National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

Inhibition of cellular proliferation by retinoids and transforming growth factor-betas in bovine mammary cells correlates with increased connexin43 expression

by Terry L. Woodward

Department of Animal Science,

McGill University, Montreal

March, 1996

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of

DOCTORATE OF PHILOSOPHY

©Terry L. Woodward, 1996

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-612-12511-4

Canada

To my fiancée Carolyn

TABLE OF CONTENTS

ABSTRACT

Bovine fibroblasts and epithelial cells were isolated from surgically biopsied mammary tissue. Characterization of population doubling time, cytoskeletal intermediate filaments, cryopreservation survival, and viability were performed on all fibroblast and epithelial cells. Several clonal fibroblast cell lines were cotransfected with a plasmid bearing the SV-40 Large-T-antigen, and the pSV-2 neo plasmid. Transfected cells were subsequently selected with G418 sulfate and cloned.

MAC-T cells and non-clonal primary bovine mammary epithelial cells proliferated in response to IGF-I, insulin, serum and serum albumin. MAC-T cells did not proliferate when cultured in EGF, estrogen, progesterone, estrogen+progesterone, growth hormone, prolactin, and only modest proliferation was obtained after TGF-α treatment. Subsequent experiments used serum, insulin or IGF-I (and its analogues) to stimulate cellular proliferation. Serum albumin was not added to serum-free media preparations since it stimulated cellular proliferation.

TGF-β receptors were characterized in MAC-T cells and normal fibroblasts. Affinity labelling studies revealed that MAC-T and MF-2 cells contained type I, II, and III autoregulatable receptors. Fibroblast proliferation, was inhibited 50% by TGF-β. TGF-β inhibited MAC-T cellular proliferation at concentrations among the lowest ever reported, ED_{50} = 4 pm. TGF-β was not cytotoxic at concentrations 1000-fold higher.

Retinoic acid (RA) also inhibited proliferation of MAC-T cells. Inhibition of proliferation did not occur when cells were growth stimulated by IGF-I analogues that do not bind IGFBPs. Unlike TGF-β, RA treatment increased IGFBP-2 and decreased IGFBP-3 protein expression by cells into media and on the cell's membrane. RA was cytotoxic at concentrations 10-fold higher than ED_{100}.

Fibroblasts and epithelial cells expressed the gap junction (GJ) protein, connexin43, with transformed fibroblasts expressing significantly less connexin43. Perinuclear and cell surface connexin43 was immunodetected in epithelial and fibroblasts cells. TGF-β, RA or cAMP, increased connexin43 protein expression, especially phosphorylated species. Only cAMP noticeably altered immunolocalization patterns of connexin43, causing a shift from perinuclear pools to the cell surface. None of the growth inhibitors affected GJ communication as measured by dye transfer. Therefore, mammary epithelial cells are growth inhibited by TGF-β and RA by distinct mechanisms and both growth inhibitors significantly enhance the gap junction protein, connexin43, without increasing GJ communication.

Résumé

Les fibroblastes mammaires bovins et les cellules épithéliales ont été isolés de tissus provenant de biopsies chirurgicales. La caractérisation du temps de doublement de population, des filaments intermédiaires cytosquelettiques, de survie à la congélation et de viabilité a été faite sur tous les fibroblastes et cellules épithéliales. Plusieurs lignées cellulaires de fibroblastes ont été cotransfectées avec le large Antigène-T de SV-40 et le plasmide pSV-2néo, sélectionnées et clonées.

Les cellules MAC-T et les cellules épithéliales mammaires bovines primaires non-clonées prolifèrent en réponse à l'IGF-I, l'insuline, le sérum et l'albumine sérique. Les cellules MAC-T ne prolifèrent pas en culture en présence d'EGF, d'estrogène, de progestérone, d'estrogène+ progestérone, d'hormone de croissance, de prolactine. Une croissance modérée est obtenue suite au traitement avec TGF-alpha. Subséquemment, le sérum, l'insuline ou l'IGF-I (et ses analogues) ont été utilisés pour stimuler la prolifération cellulaire. L'albumine sérique n'a pas été ajoutée aux préparations de milieux sans sérum puisqu'elle stimule la prolifération cellulaire.

Les récepteurs de TGF-ß ont été caractérisés dans les cellules MAC-T et fibroblastes normaux. Les études de marquage d'affinité révèlent que les cellules MAC-T et MF-2 contiennent les récepteurs autorégulés de types I, II et III. La prolifération des fibroblastes est inhibée de 50% par le TGF-ß. Ce dernier inhibe la prolifération des

cellules MAC-T à des concentrations parmi les plus basses rapportées (ED50=4pM) alors qu'il ne présente aucun effet toxique à des concentrations 1000 fois supérieures.

L'acide rétinoïque (AR) inhibe aussi la prolifération des cellules MAC-T. L'inhibition n'apparaît pas quand la croissance cellulaire est stimulée par des analogues de IGF-I ne se fixant pas aux protéines liantes de l'IGF (IGFBP). Contrairement au TGF-ß, le traitement au AR augmente l'expression des protéines IGFBP-2 alors qu'elle diminue les IGFBP-3 dans le milieu et à la surface cellulaire. L'acide rétinoïque est cytotoxique à des concentrations 10 fois supérieure à ED100.

Les fibroblastes et cellules épithéliales expriment la protéine de jonction (GJ), connexin43, les fibroblastes transformés en exprimant beaucoup moins. La connexin43 périnucléaire et de surface est immunodétectée dans les cellules épithéliales mais les fibroblastes présentent moins de connexin43 périnucléaire. Le TGF-ß, le AR et l'AMPc augmentent l'expression de la connexin43, spécifiquement les espèces phosphorylées. Seul l'AMPc altère l'immunolocalisation, causant un déplacement à la suface des groupes périnucléaires. Aucun inhibiteur de croissance n'affecte la communication GJ mesurée par transfert de colorant. Conséquemment, les cellules épithéliales sont inhibées par TGF-ß et AR suivant des mécanismes distincts et tous deux augmentent de façon sigificative la connexin43 sans altérer significativement la communication GJ.

ACKNOWLEDGMENTS

I would like to extend my gratitude to several people for their assistance throughout my doctoral studies. First, I would like to thank Dr. Jeffrey Turner, who enticed me to come to Canada and pursue my studies, despite the lack of good college football. His enthusiasm, confidence, and persistence in scientific and nonscientific endeavors have inspired and hopefully 'rubbed off' on me. Next, I would to thank Dr. Dale Laird. From our first meeting, Dale's pursuit of excellence has been apparent. Dale is one of few scientists who has demonstrated complete dedication to all aspects (and excels in all) of science from administration to bench work to teaching. Dale and Jeff are well on their way to shaking up the scientific community in their respective disciplines.

I was also lucky enough to continue to work with Dr. Mike Akers, my Master's supervisor, perhaps the best mammary physiologist in the field. Special thanks to Dr. Xin Zhao, who I have spoke with at length about all aspects of science. Like Dale, Xin is a tireless scientist, devoted to excellence in all aspects of science. I have learned a great deal about science from Jeff, Dale, Mike and Xin, developed strong friendships with them and hope to continue collaborations with all these scientists, regardless of my future career interests.

I would like to extend my gratitude to several professors who have helped expand my scientific knowledge or have personally helped with research in this thesis, including: Dr.

Anie Philip, Dr. Stan Kubow, Dr. Ashraff Ismail, Dr. Hans Zingg, Dr. Maureen O'Connor-McCourt, Dr. Bruce Downey, Dr. Ng Kwai-Hang, Dr. Elliot Block, Dr. Tony Capuco, Dr. Tom McFadden and Dr. Pangala Bhat.

My research would not have been possible without the technical expertise of Manon Legare, who I shared the laboratory, lunches and friendship with when I first arrived. Elaine DeHeuvel provided enormous support by way of her laboratory skills and constant support and friendship. Josee Plamondon made life much more comfortable with her comradery, and as one of the best qualified technicians I have met. My thanks are also extended to Josee for the french translation of this thesis' abstract. Though BRI will never be a McGill. I would also like to thank two other excellent technicians: Leny and Susan (ask Susan she'll know) Smith. Jan Pikas' skills were invaluable to myself and the department in surgery, photography, and fixing or making nearly anything.

The department of Animal Science at McGill is fortunate to have (or had) Joanne Ten Eyck, Sharon O'Toole, Joyce Read, Barb Stewart and especially Lisa Gloutney. Unlike many departments in universities, these individuals were always great to be around at and outside work, worked together superbly and still did an excellent job. They made all the technicalities of my Ph.D. from travel to pay to formatting a paper far easier.

I can not thank enough the support of the many graduate students who have helped with all aspects of my research and 'off-campus' life, especially: Paul Feldman, Mike Sia,

Rochelle Chodock, Annick Delaquis, Costas Karatzsas, Zhiying Zhang, Elizabeth MacDonald, Wei Wang, and Nancy DuMont.

I was also lucky to supervise two hard working dedicated intelligent undergraduate students, Lisa Antonitti and Sigrid Gertram, who I am sure will set the world on fire with their aspirations

Thanks also to my parents, Luther and Hazel, my brothers, Gary and Mark, and friends for their support. Finally, and most importantly, I would like to thank a girl I met during my interview for this Ph.D. position, starting dating soon after my arrival and will soon marry, Carolyn, who has improved every aspect of my life, not necessarily by way of her constant brainic veterinarian advice.

PREFACE

Chapters 2 and 3 are published manuscripts, and Chapter 4 and 5 have been submitted for publication. All these chapters have contributions from multiple authors. These contributions are detailed below.

Chapter 2. All experiments and data analyses were performed by Terry Woodward. Drs. Michael Akers and Jeffrey Turner assisted Terry Woodward in experimental design and review of the manuscript, written by Terry Woodward.

Chapter 3. Dr. Anie Philip and Nancy DuMont performed all labelling studies. Creation of cell lines, transfections, thymidine and DNA assays, cytotoxicity assays and data analyses were performed by Terry Woodward. Dr. Maureen O'Connor-McCourt assisted Dr. Philip and Ms. DuMont in labelling study design. Dr. Turner assisted Terry Woodward in experimental design. All authors reviewed the manuscript, written by Terry Woodward.

Chapter 4. Dr. Hung Huynh was responsible for running the Western blots in Fig. 7 and 8, though these samples were prepared and Western blots were analyzed by Terry Woodward. All other experiments and data analyses were performed by Terry Woodward. Drs. Jeffrey Turner and Xin Zhao assisted Terry Woodward in experimental design. All authors reviewed the manuscript, written by Terry Woodward.

Chapter 5. Dr. Dale Laird assisted Terry Woodward in experimental design. Dr. Laird also assisted in refining several images. Drs. Jeffrey Turner and Dale Laird reviewed the manuscript, written by Terry Woodward. All experiments and data analyses were performed by Terry Woodward.

In accordance with McGill thesis submission guidelines the following paragraphs have been reproduced to inform external reviewers of Faculty regulations:

INTRODUCTION AND REVIEW OF LITERATURE

I. INTRODUCTION

This monograph will detail the regulation of cellular proliferation and its effect on cell to cell communication in bovine mammary cells. The major current areas of mammary gland research, outlined below, are intimately concerned with either cellular proliferation and its modulation and/or cellular communication. In vivo and in vitro, classical and contemporary methods of measuring mammary cell proliferation will be compared and contrasted. This literature review will describe major growth inhibitors of mammary cell proliferation in all species and their relevance to bovine mammary gland biology. Finally, gap junctional communication and its role in cellular homeostasis and interactions with growth inhibitors will be reviewed.

II. MAMMARY GLAND BIOLOGY

The mammary gland has intrigued researchers from many disciplines for decades. The magnitude and diversity of research generated by a single gland only found in mammals can basically be attributed to 4 disparate subdisciplines.

A. Breast Cancer Research

Research regarding breast cancer has been and is currently an area of intense study, as breast cancer incidences have increased over the past 20 years, especially in North America. In fact, greater than 1 in 9 women in the United States and Canada will be afflicted with breast cancer (Klijn et al., 1993). Recently, the identification and cloning of BRCA1, the first gene linked to heritable breast cancer, has spurred many researchers to study this gene and its protein product, as well as, search for other genes involved in breast cancer (Easton et al., 1993). However, the majority of breast cancer cases are not familial. In fact, BRCA1 represents only 5-10% of all breast cancer (Claus et al., 1991). The intense search for the breast cancer gene has, however, reminded scientists that breast cancer is not a single disease. Instead breast cancer is many diseases resulting from abnormalities in endocrine/paracrine/autocrine function, receptors, cell communication/attachment, secondary messengers, protooncogenes, extracellular matrices, and metalloproteinases. However, one common theme in breast cancer is a loss of control of normal cellular proliferation.

B. Improved Milk Production

Success in improving milk production has far exceeded expectations. In the early nineteenth century, average annual milk yields of dairy cattle were approximately 2000 liters/cow, by 1965 this figure had risen to 3600 liters and today North American standards are approximately 6500 liters/year, with the Pacific regions reporting an 8500 liters/year average (U.S. department of agriculture, dairy situation and outlook reports,

1965 and 1990; Cowie et al., 1980). However, farmers are still far from realizing maximal milk production as peak yields of over 25,000 liters/year have been reported (Cowie et al., 1980). Nutritional, genetic, managerial and physiological techniques have been employed to achieve these increases. The recent approval by the Food and Drug Administration (U.S.) of recombinant bovine somatotropin should increase herd milk yields that use bST by 10-15%, over previous figures (Burton et al., 1994). Although many techniques have been used to enhance milk production, all techniques are dependent upon 3 criteria: a) the length of lactation, b) the number of epithelial cells present in the mammary gland, and c) the individual capacity of each epithelial cell to secrete milk proteins, carbohydrates and lipids.

C. Mastitis

Mastitis is the most costly disease of dairy cows. In fact, losses, primarily from lost milk production, have been estimated at between 1 to 2 billion dollars/year in North America (Lightner et al., 1988; DeGraves and Fetrow, 1993). Mastitis results when bacteria (especially: *Staphylococcus aureus*, *Streptococcus uberis* and *Streptococcus agalactiae*) and other pathogens (including mycoplasma) enter the teat and infect the udder (Tyler et al., 1993) by breaking tight junctions and entering between epithelial cells (MacDonald et al., 1994, Lin et al., 1995) or by destroying epithelial cells (Sordillo et al., 1989 a,b). Research involved in controlling mastitis is quite diverse including immunotherapy, management, decreasing response to pathogens (limiting resultant oxidative stress, swelling), and enhancing tight junction function (DeGraves and

Fetrow, 1993; Erskine, 1993; Miller et al., 1993; Shearer and Harmon, 1993; Tyler et al., 1993; Harmon, 1994; and Kirk et al., 1994). Mastitis research probably has the least relevance to cellular proliferation and its control, however, cell-cell interactions are important in preventing mastitis, impeding its progression and improving mastitis treatment.

D. Altering Milk Composition

Advances in targeted expression of transgenes has resulted in the appearance or livelihood of numerous biotechnology companies, government and university laboratories attempting to convert the mammary gland into a massive bioreactor. The mammary gland has been targeted as an alternative to prokaryotic in vitro bioreactors, since mammary epithelial cells can properly process proteins post-transcriptionally (i.e. glycosylation, phosphorylation, cleavage, and folding) that are needed as human pharmaceuticals (Wilmut et al., 1991; Henninghausen, 1992). Additionally, the mammary gland, especially in high producing dairy cattle, has the capacity to produce very large quantities of proteins which can readily be harvested without harm to the animal. Furthermore, others have reported milk is a poor source of proteolytic enzymes, which can potentially damage the protein of interest (Houdebine, 1993).

In addition to targeting the bovine mammary gland for the expression of human pharmaceutical proteins, researchers are also interested in modifying the composition of milk for various purposes: increasing or altering the protein content, making a natural

substitute for 'formula' or human breast milk, and eliminating lactose. Although many ideas have been postulated to introduce foreign genes or alter endogenous genes that are specifically expressed within the mammary gland, production of transgenic animals are and have been the most widely studied (Houdebine, 1993). Many hurdles exist in creating a predictable repeatable method for efficient expression of foreign proteins in the bovine mammary gland (Houdebine, 1993; Janne, 1994). A comprehensive understanding of the physiology of the normal and transgenic mammary gland is critical to eventually overcoming these obstacles (Henninghausen, 1992).

III. ACCESSING MAMMOGENIC AND MAMMOSTATIC AGENTS: IN VIVO & IN VITRO METHODS OF MEASURING MAMMARY CELL PROLIFERATION

Discernment of direct acting primary mitogens and primary growth inhibitors from secondary or tertiary regulators of mammary growth is inherently complex when data are obtained solely from whole animal studies. In contrast, studies involving culture of mammary tissue explants, eliminates interference of circulatory mitogens. However, explant cultures have an unknown composition of cell types; including, epithelium, fibroblasts, myoepithelium, endothelium, adipocytes, mast cells, plasma cells and cells from the circulation. Explant cultures are often static in growth and suffer from cellular hypoxia (Banerjee et al., 1976; McGrath, 1987). A more defined method for measuring cellular response to mitogens is primary cell culture. Primary cells are isolated from

tissue and separated into different cell types. However, primary cell culture is labor intensive and plagued by heterotypic cell contamination. Other difficulties with primary cell culture include: early senescence, static growth, frequent surgical biopsies and subsequent typing and characterization of cells (McGrath, 1987).

The in vitro method of choice by scientists studying breast cancer has been utilization of clonal cell lines. The isolation, cloning and characterization of the major cell types within the mammary gland have circumvented many of the difficulties inherent with in vivo, explant and primary cell culture. However, maximal utilization of cell lines are only possible when the cells are able to be cultured for extended periods of time or have been 'immortalized'. Cell lines offer researchers the ability to study individual cell types or specific cell combinations. Additionally, individual extracellular matrices, combinations of purified extracellular matrices, or extracellular matrices isolated from the mammary gland (Wicha et al., 1979; Wicha et al., 1982) can readily be used; thus, delineating how each cell type or extracellular matrix contributes to a particular growth factor's or growth inhibitors' actions.

IV. ESTABLISHED MAMMARY CELL LINES

Numerous breast tumor cell lines exist and display an array of different characteristics (for review, Taylor-Papadimitriou et al., 1993; Stampfer and Yawsen, 1993; Dickson et

al., 1994; Rochefort, 1994; Sobek et al., 1994). These include, breast tumor lines with or without estrogen, progesterone and epidermal growth factor (EGF) receptors. Breast cancer cell lines that do or do not respond to other growth factors and vitamins, cells from benign, tumorigenic, metastatic or aggressive metastatic tumors of the breast have also been reported. Far fewer normal breast cell lines exist and fewer lines still retain differentiation properties in culture (Huynh et al., 1995). The most commonly used normal breast epithelial cell line, the HBL-100 line, was originally reported to synthesize and secrete caseins, and thus show some degree of functional differentiation (Gaffney, 1982). However, substantial drift has occurred in this cell line and a more recent study has demonstrated that the HBL-100 cell line lacks estrogen and prolactin receptors and does not secrete caseins (Laherty et al., 1990). A myriad of epithelial and fibroblast cell populations and lines have been described in recent years, but few have been well characterized. In fact, only the murine mammary epithelial cell line, Comma-1D, has been reported to synthesize appreciable amounts of casein (Danielson et al., 1984). Unfortunately, subcloning of this line revealed several cell types, and prolonged passage of these cells led to increased oncogenic potential (Medina et al., 1986).

Regardless of the suitability of these cells for modeling lactation, researchers from human or bovine fields studying normal mammary gland biology have, however, benefitted from two mammary epithelial cell lines of murine origin, the Comma-1D and NMuMg cell lines. Both cell lines are reportedly spontaneously immortal non-transformed murine mammary epithelial cell lines (Sizemore, 1979; Danielson et al., 1984). In addition to

questionable functionality (NMuMg) and oncogenic state (Comma-1D), using these murine cell lines to extrapolate data from one species to another can often be errorenous. In fact, substantial species variation exists between mammary gland development, growth, hormonal responsiveness, remodeling, carcinogenesis, cellular composition and anatomy between rodent, human and ruminant mammary glands (Akers, 1985; Akers, 1990; Politis et al., 1989, 1990; Woodward et al., 1993).

In 1992, at the beginning of my research project, there existed only two bovine mammary epithelial cell lines. Both of these lines were epithelial, the MAC-T cell line and the PS-BME line (Huynh et al., 1991; Gibson et al., 1991, 1992). Additionally, both lines have been reported to morphologically and functionally differentiate in culture when plated on the appropriate extracellular matrix and in the presence of lactogenic hormones. The MAC-T cell line was transfected with a plasmid bearing the simian virus-40 Large-T-antigen, the expression of which has allowed continuous culture. The PS-BME, like the NMuMG and Comma-1D murine cell lines was reportedly spontaneously immortal. Unfortunately, the PS-BME line was later determined to be murine in origin and most likely originated from the Comma-1D or NMuMG cell lines which were also used in the laboratory of origin (Craig Baumrucker, personal communication). Thus, only a single bovine mammary cell line remained. No myoepithelial, fibroblast or adipocyte lines from the bovine mammary were reported and/or available for use. In July 1995, another bovine mammary epithelial cell line was reported (Huynh et al., 1995), that has not yet been distributed or well characterized.

Most researchers who use cell lines to better understand how the cell type studied normally functions within an organ or a gland realize that multiple cell lines must be used to accurately represent the heterogeneity of cells within a gland. As can easily be recognized from the multitude of heterogenous breast cancer cell lines, a single cell line taken from a single mammary gland is not representative. Thus, the availability of a single epithelial cell line has limited in vitro modelling of the bovine mammary gland. Also, the lack of stromal cell lines is particularly limiting as many hormones and growth factors in the mammary gland are not able to directly effect the mammary epithelium, but instead function by endocrine or paracrine mechanisms (see below, Mammary Cell Proliferation) (Haslam, 1986; Haslam, 1988; Woodward et al., 1993; Burton et al., 1994).

V. MAMMARY CELL PROLIFERATION

A. Mitogens (Mammogens)

Bovine mammogenesis in vivo occurs in response to estrogen, progesterone, and growth hormone. However, human, murine and bovine studies have demonstrated that none of these hormones directly affect mammary epithelial cell proliferation. Growth hormone (somatotropin) exerts its mitogenic effects on mammary epithelium through insulin-like growth factor-I (IGF-I or somatomedin-C), most of which is synthesized in the liver

(Burton et al., 1994). However, the seemingly simple growth hormone/IGF-I axis is complicated by: 1) A family of at least 7 IGF binding proteins (IGFBPs) that can inhibit or potentiate IGF-Is' effects (Jones and Clemmons, 1995; Swisshelm et al., 1995); 2) the expression of growth hormone receptor mRNA in the mammary gland (Glimm et al., 1990); 3) local production of autocrine/paracrine IGFs that have very low affinity for or do not bind IGFBPs within the mammary gland (Jones and Clemmons, 1995); 4) alterations in partitioning of nutrients, increased cell growth, increased synthetic capacity of individual cells, changes in blood flow, and whole body metabolic changes accompanying GH treatment (Burton et al., 1994). These factors combined with hypothalamic and pancreatic regulators of growth hormone secretion add to the complexity involved in increased milk synthesis/secretion elicited by treatment of dairy cattle with exogenous bST. Regardless, an "unprecedented number of technical papers, abstracts, and short communications have been published in the past decade regarding the effects of exogenous bovine growth hormone on milk production" (Burton et al., 1994), and bovine growth hormone remains the best understood and well studied hormone or growth factor in the bovine animal.

Comparatively, little is known about the mechanisms of action of ovarian steroids on bovine mammary tissue. Estrogen and progesterone are known to elicit mammary growth and development in vivo, but do not have direct effects on bovine mammary epithelium (Woodward et al., 1993) and may function by a paracrine pathway (Woodward et al., 1992). The direct effects of many more growth factors/inhibitors,

hormones, vitamins, and fatty acids are not known because of the lack of in vitro bovine mammary cell models.

B. Inhibitors of Mammary Cell Proliferation

Although bovine mammary mitogens have been rigorously investigated for most of the century, regulators or inhibitors of cellular proliferation have not received this degree of scrutiny. Only in breast cancer research has significant effort been directed toward understanding the control of cellular proliferation as well as mitogenesis. In the murine and bovine mammary glands, growth hormone, IGF-I, IGF-II, EGF, TGF-α, thyroxine, triiodothyronine, corticosteroids, acidic and basic FGF, insulin, estrogens, progesterones, fatty acids, albumin and other cytokines have all been identified as mammary cell mitogens (Tucker, 1981; Akers, 1985, 1991; Oka et al., 1991). In sharp contrast, only a single growth inhibitor has been well characterized in the bovine mammary gland, aptly named mammary derived growth inhibitor (MDGI) (Grosse et al., 1992). Two major growth inhibitors have been well characterized in mouse mammary epithelium: MDGI and the TGF-βs (Yang et al., 1994; Daniel and Robinson, 1992). The void of rigorous examination of growth regulators in normal mammary gland biology is particularly perplexing since excessive growth inhibition would limit mammary gland growth, development and subsequent lactation potential, important to the dairy industry. While, absence of mammogenic regulators could result in uncontrolled cellular proliferation and tumor formation, important for clinical oncology.

VI. SPECIFIC GROWTH INHIBITORS OF MAMMARY CELLS

Control and regression of breast tumor proliferation is a goal of all researchers studying breast cancer. As a result of this, researchers have identified many growth inhibitors of mammary tumor cells. Surprisingly, this work has infrequently been extended to include normal breast epithelial cells, which would invariably be effected by in vivo treatments aimed at the tumor.

This thesis has concentrated only on reversible physiological receptor-mediated growth inhibitors. Four major physiological growth inhibitors have been described 1) transforming growth factor-betas (TGF-βs), 2) retinoids and/or carotenoids, 3) vitamin D, and 4) mammary derived growth inhibitors (MDGI). Interleukins and interferons have also been ascribed to this class of growth inhibitors, although these agents often induce programmed cell death, and results vary considerably from study to study (Solary et al., 1991; Bajaj et al., 1993, Coradini et al., 1994; Otto, 1994). Likewise, the diminutive growth inhibitory effects of vitamin D are inconsistent and vitamin D has also been demonstrated to induce apoptosis (Vandewalle et al., 1995). More research is needed to understand the roles of these interleukins, interferons and vitamin D and their control of proliferation in the mammary gland. Additionally, vitamin D, retinoids and carotenoid research has focused on tumorigenic mammary cells. Only TGF-β and MDGI have been intensely investigated in non-transformed mammary epithelial cells, and

as previously stated only MDGI has been characterized in the bovine mammary gland.

A. Mammary-Derived Growth Inhibitor (MDGI)

MDGI was originally purified from the bovine mammary gland (Grosse et al., 1992). Consequently, much research has been conducted in the bovine mammary gland or using bovine mammary tissue or cells in vitro. MDGI is a member of the fatty acid binding protein (FABP) family. Synthesis of MDGI by bovine mammary epithelium has been demonstrated by in vivo and in vitro trials (Erdman and Breter, 1993; Huynh and Pollak, 1995). Additionally, MDGI has been reported to be regulated by lactogenic hormones, maximally expressed at peak differentiation and to inhibit proliferation of mammary epithelial cells (Grosse et al., 1992; Huynh and Pollak, 1995). Interestingly, growth inhibition did not occur in MAC-T cells unless these cells were previously serum starved and synchronized in G_0 (Zavizion et al., 1993). The same study reported that the inhibition of cellular proliferation by MDGI was transient and proliferation was not different than control cells after 4 d of MDGI treatment. In a murine study, researchers have determined that MDGI specifically inhibits mammary epithelial cell proliferation, while stromal cell proliferation was not inhibited (Yang et al., 1994).

In addition to a detailed examination of effects of MDGI on growth and differentiation in the bovine mammary, others have cloned and characterized the cDNA for MDGI and determined that an 11-amino acid sequence in the COOH terminus of MDGI is responsible for its growth inhibitory actions (Treuner et al., 1994; Yang et al., 1994).

Another group has recently reported that MDGI is a potent breast tumor suppressor, despite only being a modest inhibitor of normal breast cell proliferation (Huynh et al., 1995). MDGI, reportedly, exerts its tumor suppressor effects by inducing a proliferating cell to enter a differentiated state when also cultured with lactogenic hormones (Huynh et al., 1995). Research regarding MDGI and growth inhibition in the mammary has encompassed: the gene encoding MDGI, the MDGI protein and its regulatory sequences, the distribution and expression patterns in vivo, as well as the ability of MDGI to induce differentiation, suppress cellular proliferation, and suppress tumor progression. Thus, MDGI remains the only well understood inhibitor of bovine mammary cell proliferation.

B. Transforming Growth Factor-Beta (TGF-β)

TGF-β is a family of 5 polypeptide growth factors, designated TGF-β_1, TGF-β_2, TGF-β_3, TGF-β_4, and TGF-β_5. However, only TGF-β_1, -β_2, and -β_3 are expressed in mammals (reviewed in Roberts and Sporn, 1990). TGF-βs have a high sequence conservation between species and are expressed in nearly all mammalian cells (Roberts and Sporn, 1990). The major actions of TGF-β include: growth inhibition and growth stimulation, enhancing extracellular matrix synthesis/secretion, inhibiting metalloproteinases and enhancing tissue inhibitors of metalloproteinases (TIMPs), and potent local immunosuppression. TGF-β isoforms generally do not differ substantially in eliciting a physiological response, though isoform variability in degree of response has often been reported. Much of the variability in response can be attributed to differences in TGF-β isoform's 1) affinity for receptors and 2) developmentally regulated patterns of expression

(Massague, 1990).

All TGF-βs bind to three major TGF-β receptors, designated type I, type II and type III. The cDNAs for these receptors have been cloned (Roberts and Sporn, 1990). The type III (betaglycan) receptor is a transmembrane proteoglycan with a short cytoplasmic domain that has been postulated to enhance binding of TGF-β to the signaling receptors (Lopez-Casillas et al., 1991; Wang et al., 1991). Type I and II receptors are members of a small family of transmembrane serine/threonine kinase signaling receptors (Lin et al., 1992; Franzen et al., 1993).

Although MDGI is the best documented inhibitor of cellular proliferation in the bovine mammary gland, TGF-β has generated recent interest regarding its role in controlling normal mammary cell proliferation and mammary gland development in the mouse (Silberstein and Daniel, 1987; Daniel et al., 1992; Jhappan et al., 1993; Robinson et al., 1993). In the murine mammary gland, TGF-β reversibly inhibits ductal growth, while alveolar morphogenesis is not inhibited (Silberstein and Daniel, 1987; Daniel et al., 1989). Additionally, TGF-β is present in a latent form (acid/protease activated), in the extracellular matrix surrounding ducts and ductules of the murine mammary gland (Robinson et al., 1991; Daniel and Robinson, 1992). Ductal side branching appears to occur exclusively in areas devoid of TGF-β activity. Thus, TGF-β appears to play a major role in controlling growth, morphogenesis and development in the murine mammary gland.

TGF-β has also been reported to control differentiation of murine mammary epithelial cells. Robinson and coworkers (1993) found that explants from pregnant mice have reduced β-casein expression following exogenous TGF-β treatment. Jhappan and colleagues (1993) created a series of transgenic mice overexpressing TGF-β_1. These mice not only showed impaired alveolar development and function, but exhibited greatly diminished lactation functionality as expressed as a unit of alveolar cells present. Thus, in addition to the marked growth and development inhibitory properties of TGF-β in the murine mammary gland, several laboratories have demonstrated that TGF-β also impairs functional differentiation of mammary epithelium. Similarly, another laboratory has reported that when normal murine mammary epithelial cells (NMuMg cells) are cultured in TGF-β containing media, morphological and functional transdifferentiation is induced (Miettinen et al., 1994). NMuMg cells became fibroblastic after TGF-β treatment in this study as measured by 1) morphology, 2) cytoskeletal proteins, 3) production of extracellular matrices, and 3) loss of epithelial specific cadherins. The epithelial to mesenchyme transition supports the role of TGF-β in inhibiting mammary epithelial cell function/differentiation by inducing a change in cellular phenotype.

Knowledge of TGF-β action in the bovine mammary is very limited. TGF-β_1 and TGF-β_2 have been identified in bovine milk and colostrum (Cox and Burke, 1991; Jin et al., 1991; Tokuyama and Tokuyama, 1993). All three mammalian TGF-β isoforms are expressed in both the non-lactating and lactating bovine mammary gland (Maier et al.,

1991). Despite the potent effects exhibited on normal murine mammary epithelial proliferation and development, and studies that report TGF-β inhibits milk protein synthesis, there have been no reports on the action of TGF-β nor have TGF-β receptors been characterized in bovine mammary tissue or cells. If TGF-β behaves similarly in the bovine mammary gland as it does in the murine, TGF-β may prove to be especially interesting to many dairy scientists, who are concerned with maximizing mammary development, proliferation and lactation.

C. Retinoids

i. Enzymatic Processing, Binding Proteins and Receptors

Animals are not capable of *de novo* synthesis of vitamin A-active substances, neither retinol nor the carotenoid precursor forms. For example, carotenoids are synthesized exclusively by photosynthetic microorganisms and by plants, where they assist in plant metabolism (Underwood, 1984). On average, one sixth of β-carotene absorbed by the small intestine is cleaved by carotene 15,15'-dioxygenase, presumably in the cytosol of enterocytes, to yield two all trans-retinals (Ross, 1993). Subsequently, retinal can be converted to either retinol or retinoic acid. Alternatively, retinyl esters can be obtained directly from a meat, milk or egg diet. Retinyl esters from the diet can be hydrolyzed directly to retinol in the intestine. Retinol is the primary form of vitamin A in circulation. Retinol and retinyl esters, are transported in the circulation by retinol binding protein (RBP) and retinol is transported interstitially by a distinct RBP, appropriately termed interstitial RBP. Retinol is esterified with palmitic or stearic acid

in the liver (Olson, 1989, Ong, 1993). Stored retinyl esters can be hydrolyzed to retinol to maintain the relatively narrow range of plasma retinol concentrations (Ross, 1993).

When the retinol-RBP complex reaches target tissues, RBP is released and the retinol freely transverses the cell membrane (Creek et al., 1993). Retinol is transported intracellularly by cellular retinol binding proteins (CRBPs). Here it can be enzymatically oxidized into retinoic acid. Retinoic acid, is now recognized as the major physiologically active metabolite of retinol (Ross et al., 1993). Retinoic acid is transported intracellularly by its own set of cellular retinoic acid binding proteins (CRABPs). Interestingly, CRABPs and CRBPs belong to a larger family of fatty acid-binding proteins that include MDGI. Retinoic acid is released from CRABPs and is 'transported' into the nucleus. In the nucleus, all-trans-RA binds to retinoic acid receptors (RARα, RARβ, RARγ), and this complex binds to specific sites within promoters termed retinoic acid response elements (RARE) and induces gene transcription (Soprano et al., 1993). RARE have been identified in the promoter of many genes important to mammary gland function including: lactoferrin, tissue plasminogen activator, oxytocin, growth hormone, laminin β, as well as in their own RARs and CRBPs (DeLuca, 1991; Richard and Zingg, 1991; Smith et al., 1991; Bulens et al., 1995; Lee et al., 1995). Additionally, 3 more nuclear receptors, RXRα, RXRβ, RXRγ, bind 9-cis RA, and can bind their own RARE or facilitate RARs binding to RARE as a heterodimer (Zhang and Phahl, 1993). Interestingly, 13-cis RA does not bind RARs nor RXRs (Wolf, 1993) or binds these receptors with very low affinity (Wu et al., 1994), and may

function independently of nuclear steroid receptor binding (Wolf, 1993).

Regulation of retinoic acid is not limited to the intestinal and hepatic enzymes, the 2 RBP, the 5 CRBPs or CRABPs and the 6 RAR or RXRs. RARs, RXRs, CRBPs and CRABPs have been shown to have RARE in their own promoters and can be up or downregulated accordingly (Napoli, 1993; Wolf, 1993; Zhang and Phahl, 1993). Moreover, a number of ligand-less nuclear 'orphan' receptors have been cloned, and several of these have recently been reported to bind either retinoids or retinoid receptors (Perlmann and Jansson, 1995; Seol et al., 1995), further adding to the complexity of regulation of RA induced gene transcription.

ii. Retinoids and Mammary Gland Function

Retinoids inhibit the proliferation of many mammary carcinoma cell lines and may also inhibit breast tumor promotion and progression (Lacroix and Lippman, 1980; Fontana et al., 1991; Adamo et al., 1992; Decensi et al., 1993; Halter, 1993; and Shemer et al., 1993). Toxic effects of retinoids have limited their therapeutic usage (David et al., 1988). Excess retinoids cause: 1) mucocutaneous side effects, 2) abnormal lipid profiles in sera, 3) liver toxicity, 4) teratogenic effects (all but eliminating use by pregnant and nursing mothers), and 5) permanent idiopathic hyperostosis skeletal abnormalities (for review see, David et al., 1988). However, now more than 3000 naturally-occurring and synthetic vitamin A analogues have been described, many of which have little or no

major side effects in vivo (Lippman et al., 1987; DiGiovanna, 1992; Arnold et al., 1994). With the advent of these new retinoids has came a surge in retinoid interest in controlling tumor mammary cell proliferation and several in vivo human trials are underway.

In contrast to significant investigation with breast tumors, only two studies exist regarding RA's action on normal mammary epithelial cell proliferation. The first, by Lotan (1979) found no significant change in growth of a nonmalignant myoepithelial line and minimal inhibition (13%) in the breast epithelial line, HBL-100 following RA treatment. Although the HBL-100 cell line was originally reported to be 'normal', more recent evidence has demonstrated HBL-100 cells cause tumors when transplanted into nude mice (Ohaliwal et al., 1990). Additionally, as previously stated, this line lacks estrogen and prolactin receptors and does not secrete casein (Laherty et al., 1990). Sixteen years after Lotan's study (1979), Lee and colleagues (1995), have shown non-clonal primary mammary epithelial enriched cells from the rat are considerably growth inhibited and possibly growth arrested following culture in RA containing media for 5 d - 25 d. This recent study, which cultured these cells in an undefined basement membrane from the Englebreth-Holm-Swarm sarcoma in a growth factor and hormone enriched, BSA containing media, is the only examination of the role of RA on non-transformed mammary cell proliferation. No parallel studies have been performed with non-transformed bovine, ovine, murine or human mammary cells.

Although many studies have been designed to examine how vitamin A or β-carotene affects mammary development, mammogenesis and lactogenesis in vivo, results are inconsistent. Studies that have limited vitamin A or β-carotene intake, have encountered other endocrinological problems; while mammary development and growth has been reported to be not effected or severely repressed (Sankaran and Topper, 1982; Chew et al., 1985, respectively). Researchers have supplemented the diet of dairy cattle with β-carotene and demonstrated both increases in milk production and no changes in milk production (Swanson et al., 1968; Oldham et al., 1991). Thus, despite considerable in vivo efforts, no clear outcome has prevailed.

VII. GAP JUNCTIONAL COMMUNICATION AND REGULATION OF CELLULAR PROLIFERATION

A. Cellular Communication and Gap Junctions

Communication between cells within the mammary gland is important in development and function. Cellular communication occurs at many levels: autocrine/paracrine signals, extracellular matrix signals from homotypic or heterotypic cells, gap junctions and various contact mediated reponses from: desmosomes, calcium dependent (cadherins) and independent (CAMs) cell adhesion molecules, and zonula occuldens.

Gap junctions are channels that allow for the direct passage of small molecules (Ca^{2+}, amino acids, second messengers) from one cell to another. A gap junction channel is

composed of a hemichannel, connexon, from one cell pairing with a connexon from another cell. Each connexon is formed by six polypeptides (connexins) arranged in a hexamer, forming a central pore (Beyer et al., 1993). 'Closed' connexons are transported to the cell surface and aggregate into clusters termed 'plaques' as junctions are beginning to form between aggregating cells (Laird and Saez, in press). The connexins are a family of gap junctional proteins, which are differentially expressed in virtually all cell types (Casico et al., 1995). Twelve different connexins have been cloned in the rat (Laird and Saez, in press). To add to the complexity, several studies have identified that different connexins can occur in the same gap junctional plaque and they can form heteromeric hemichannels (Traub et al., 1989; Stauffer, 1995, respectively).

B. Regulation of Gap Junctional Communication

Regulation of gap junctional communication is multifactorial, dependent upon: synthesis, assembly, transport, insertion of connexins/connexons, formation of gap junctional plaques with opposing cells, gating of the junctions, and turnover of the junctions. Opening and closing of individual gap junctions have been reported to be regulated by phosphorylation, calcium, pH, hydrogen, voltage, acidification and second messengers (Liu et al., 1993; Suchyna et al., 1993). Although gating represents a very quick mechanism of up or downregulating intracellular communication, connexins themselves are dynamic proteins with a reported half life of 1-3 h in vitro (Laird et al., 1991; Laird et al., 1995) and 5 h in vivo (Fallon and Goodenough, 1981). Thus, turnover of gap

junctions also represents a process by which gap junctional communication can quickly be up or downregulated.

Connexin trafficking may be particularly important in gap junction regulation, because of their rapid turnover. Additionally others have shown that pools of cytoplasmic connexin may be responsible for rapid increases in gap junction plaques (Larsen et al., 1991), since increases in connexin transcripts are often not sufficient to explain the quantity of plaque increases (Beyer, 1993). For example, at the onset of labor in mammals, Cx43 protein increases 50 to 100-fold in the uterus, whereas Cx43 mRNA is only amplified 5-fold (Beyer, 1993). Interestingly, the regulation of connexin gene expression in the liver, carcinogenic states, during tissue injury, and during development is an area of considerable research effort. However, other tissue or organs which have been examined for gap junction proteins have been largely ignored when studying connexin gene expression (Beyer, 1993; Wolburg and Rohlmann, 1995). Therefore, control of gap junctions remains a complex, but dynamic system with connexins having a short half-life, and an even more rapid gating system.

C. Gap Junctional Communication: General Function and Mammary Gland Function

Gap junctions permit homologous cell communication, via trans-membrane pores. The purposes of this type of communication are extensive. Gap junctions can magnify cellular responses to external and internal stimuli by transmitting second messengers, ions

and metabolites from one cell to another. In addition to amplifying hormone/growth factor responses, gap junctions allow groups of cells to respond similarly by averaging and transmitting the response. For example, muscle and nerve cells function as a syncytium, by transmitting electrical impulses through gap junctions. Alternatively, waste products and xenobiotics can be diluted and their harmful effects minimized (Holder et al., 1993). Additionally, gap junctions can close very rapidly during cell death, cell injury and cell division to prevent transmitting toxins or inappropriate messengers and maintain a barrier between the extracellular environment and the cell.

It has been demonstrated that adhering junctions, especially cadherins are responsible for pairing homologous cell types (Steinberg and Takeichi, 1994). Researchers have acknowledged that in the absence of cell adhesion molecules or when cell adhesion molecule binding is blocked, gap junctions do not form or their formation is substantially inhibited (Musil et al., 1990; Jongen et al., 1991; Meyer et al., 1992). Alternatively, gap junctions can form when cadherins are introduced into a cell type lacking gap junctions (Musil et al., 1990; Jongen et al., 1991). Moreover, gap junctions have been reported between homologous cell types in nearly every tissue and cell type examined in multicellular organisms (Holder et al., 1993). Data concerning heterologous gap junctional communication are not as clear, as several researchers have reported heterologous GJIC while others found no GJIC in the same cell types, i.e. epithelium and fibroblasts (Tomasetto et al., 1993; Pitts and Kam, 1985, respectively). Lack of heterologous GJIC is important in allowing the transfer of molecular and hormonal

signals between homologous cells and preventing other cells to receive these messages. It is easy to recognize the importance of separating intercellular signalling in heterologous cells so external stimuli affect only one group of cells. For example, muscle and nerve cells transmit intercellular signals by GJIC. Lack of GJIC communication barriers, could cause unplanned stimulated of other nerves, muscles or cells by the signals meant for a single group of cells, say caridac muscle.

In mammary parenchymal tissue, gap junctions are believed to be important in 1) transmitting oxytocin signals from one myoepithelial cell to adjoining myoepithelial cells to cause a synchronized contraction enabling efficient milk let down, and 2) synchronizing epithelial cells to responses of mammogenic and lactogenic hormones enabling magnification and averaging of hormones/growth regulators/lactogens and even extracellular matrix signals (Monaghan et al., 1994; Pozzi et al., 1995). Several reports have identified that stromal cells may modulate epithelial cell proliferation or differentiation by transmitting paracrine growth factors after blood borne hormones/growth factors bind the stromal cell. Therefore, gap junctional communication is equally important in these cells, not only for their own growth/differentiation, but also for their subsequent influence on epithelial cells by paracrine mechanisms.

Few reports have described gap junctional communication or connexin expression in the normal mammary gland. Two independent studies have examined the expression of

connexins in mice and rat mammary glands. Monaghan and colleagues (1994) reported that Cx32, Cx40 and Cx43 were not expressed in the mouse mammary gland. Cx26 was developmentally expressed in the epithelium: it was absent in the virgin, and expression increased during pregnancy, was highest during lactation, and dramatically fell at involution. Perezamendariz and coworkers (1995), however, reported that in murine mammary glands, Cx26, Cx32 and Cx43 were expressed. Cx43 was expressed in mammary tissue from virgin and pregnant mice and increased during lactation. Whereas, Cx26 and Cx32 had low expressional levels in mammary tissue from virgin mice, rising slightly during pregnancy and peaking during lactation. Additionally, this study confirmed that Cx26 and Cx32 were present in epithelial cells, while Cx43 was present in epithelium, fibroblasts, myoepithelium and adipocytes. A separate study determined that Cx43 was expressed in normal human breast tissue (Wilgenbus et al., 1992). Yet another laboratory found the expression of Cx26 and Cx43 in normal human mammary epithelial cells, but these cells lacked Cx31.1, Cx32, Cx33, Cx37, and Cx40 (Lee et al., 1992). This study also determined by Northern blot analysis and immunocytochemistry that Cx26 and Cx43 were both downregulated in tumor cells. Gap junctional communication has not been examined in the bovine mammary gland by in vivo or in vitro methods.

D. Cellular Proliferation and Gap Junctional Communication

Loss of cellular homeostasis leads to cancer and disease states. This has been well documented by several proto-oncogenes, p53, p105RB and pp60c-src, that function to

maintain normal cellular proliferation and cellular homeostasis (Chiarugi et al., 1994; Shalloway et al., 1992; Ozbun and Butel, 1995; Weinberg, 1995). Likewise, gap junctions are critical to the maintenance of cellular homeostasis in many cells. Many independent studies have demonstrated that gap junctions regulate cellular proliferation, while loss of gap junctional communication causes cellular dysfunction and may lead to tumorigenesis (Lowenstein, 1967; Eghbali et al., 1991; Zhu et al., 1992; Ruch, 1994; Mesnil et al., 1995). Several key observations have provided compelling evidence that gap junctions are involved in the regulation of cellular proliferation: 1) Tumor promoters, such as TPA have been shown to block gap junctional communication (Oh et al., 1991). Additionally, many tumor cell lines, such as the rat C6 glioma cells, human SKHep 1 hepatoma cells and human mammary 21MT-2 cells, lack gap junctional communication, (Eghbali et al., 1991; Naus et al., 1991; Lee et al., 1992) 2) Transfection of communication-deficient tumor cell lines with plasmids bearing connexin26, connexin32 or connexin43 results in both a decreased rate of cellular proliferation and a reversal of the neoplastic phenotype as shown by in vivo an in vitro studies (Eghbali et al., 1991; Zhu et al., 1993; Ruch, 1994; Mesnil et al., 1995) 3) subtractive hybridization for mRNAs expressed in normal breast epithelial cells, but not in breast tumor cells led to the identification of connexin26 (Lee et al., 1992).

Another important link to gap junctions and control of cellular proliferation involves connexin trafficking during the cell cycle. Gap junctions are subject to defined modulations during normal cell cycle progression. For example, Xie and colleagues (D.

Laird, personal communication) demonstrated that during mitosis, a novel form of connexin is present that is near or at the cell membrane. This novel mitosis-specific connexin, however, is not functional. Thus, during mitosis, gap junctions are reportedly disrupted and therefore cellular communication via these junctions is also disabled. Additionally, recent evidence from our laboratory has demonstrated that when cells enter quiescence, connexin43 protein content is decreased by 95%, as measured by Western blotting (Sia et al., 1995). However, gap junctional communication is **not** dramatically effected. These cell cycle specific modulations in connexin and gap junctional communication support the idea that gap junctions play a role in regulating cellular proliferation.

We propose that gap junctional communication potentiates and sustains the effect of growth inhibitors across mammary epithelium by allowing transfer of their second messengers and metabolites. Reciprocally, growth inhibitors that do not cause apoptosis or cellular toxicity, upregulate gap junctional communication. Conversely, toxins should inhibit gap junctional communication, to prevent intercellular spread of these potentially lethal toxins. The increase in gap junctional communication following reversible growth inhibition should function to: 1) increase mitogenic responsiveness to cells in a quiescent state that may be lacking appropriate nutrients/growth factors to proliferate, 2) maintain a syncytium of homeostatic cells, or 3) maximize effects of lactogenic hormones on cellular differentiation.

VIII. SUMMARY AND RESEARCH PROPOSAL

This thesis examines how direct regulators of mammary epithelial cell proliferation function, and whether their function alters gap junctional communication. Growth inhibition studies will concentrate on TGF-βs and retinoids, since they have been largely ignored in the bovine mammary gland. The thesis is divided into 5 paper-formatted chapters. The first chapter, details the development of mammary epithelial and fibroblast cell lines and their isolation, cloning and characterization. Additionally, this chapter describes transfection of these cell lines with an 'immortalizing' oncogene. The cell lines described in chapter 1 are utilized as in vitro models throughout the remaining chapters.

Chapter 2 characterizes the growth responsiveness of mammary epithelial cells to growth factors and hormones that have been reported as mitogenic to bovine mammary epithelium in vivo. Several important differences among bovine, human and murine mammary epithelial cell proliferation were observed and discussed. Additionally, endocrine and paracrine hormone action is implicated for many previously described in vivo 'mammogens' that do not alter cellular proliferation of mammary epithelial cells directly.

Chapter 3, characterizes, for the first time in the bovine, the potent growth inhibition by TGF-β1 and TGF-β2. The effect of these growth inhibitors on mammary fibroblasts and epithelium are extensively characterized and contrasted, including the effect of

immortalization by expression of the Large-T-antigen on growth inhibition. This is particularly important to TGF-β, since other laboratories have shown that the expression of several oncogenes including the Large-T-antigen may interfere with TGF-β action. Additionally, Chapter 3 details the receptors involved in TGF-β binding and auto-regulation of these receptors.

In Chapter 4, retinoic acid and retinol's effectiveness as a potent growth inhibitor of non-transformed mammary epithelium is described for the first time in any species. Additionally, a novel proposal for the mechanism of inhibition of cellular proliferation by retinoic acid is described.

In Chaper 5, connexin protein type and localization are characterized in bovine mammary fibroblasts and epithelium. Gap junctional communication and connexins have not previously been examined in the bovine mammary gland. Next, function of the identified gap junctions in normal and SV-40 large-T-antigen transfected fibroblasts and epithelial cells are discussed. Lastly, this chapter compares the effect of three growth inhibitors, that act by independent pathways, on the protein expression of Cx43 by immunohistochemical and biochemical analysis. We also propose a relationship between a class of physiological growth inhibitors, that are not cytotoxic or apoptotic, and gap junctional communication. These studies, for the first time, outline the potent growth inhibitory effects of TGF-β and RA and relate this growth inhibiton to gap junctional communication.

LITERATURE CITED

Adamo, M.L., Z. Shao, F. Lanau, J.C. Chen, D.R. Clemmons, C.T. Roberts, D. LeRoith, and J.A. Fontana. 1992. Insulin-like growth factor-I (IGF-I) and retinoic acid modulation of IGF-binding proteins (IGFBPs): IGFBP-2, -3, -4 gene expression and protein secretion in a breast cancer cell line. Endocrinology, 131:1858-1866.

Akers, R.M. 1985. Lactogenic hormones: binding sites, mammary growth, secretory cell differentiation, and milk biosynthesis in ruminants. J. Dairy Sci., 68:501-519.

Akers, R.M. 1990. Lactation physiology: a ruminant animal perspective. Protoplasma, 159:96-111.

Arnold, A., B. Kowaleski, R. Tozer, and H. Hirte. 1994. The arotinoids: early clinical experience and discussion of future development. Leukemia, 8:S42-S49.

Banerjee, M.R. 1976. Response of mammary cells to hormones. Int. Rev. Cytol., 47:1-23.

Bajaj, P., J. Lawry, G. Shenton, and R.C. Rees. 1993. Interleukin-6 and tumor necrosis factor alpha synergistically block S-phase cell cycle and upregulate intercellular adhesion molecule-1 expression on MCF7 breast carcinoma cells. Cancer Let., 71:143-149.

Beyer, E.C. 1993. Gap junctions. Int. Rev. Cytol., 137C:1-37.

Bulens, F., I. Ibanez-Tallon, P. VanAcker, A. DeVriese, L.Nelles, A. Belayew, and D. Collen. 1995. Retinoic acid induction of human tissue-type plasminogen activator gene expression via a direct repeat (DR5) located at -7 kilobases. J. Cell Biol., 270:7167-7175.

Burton, J.L., B.W. McBride, E. Block, D.R. Glimm, and J.J. Kennelly. 1994. A review of bovine growth hormone. Can. J Anim. Sci., 74:167-201.

Casico, M., N.M. Kumar, R. Safarik, and N.B. Gilula. 1995. Physical characterization of gap junction membrane connexons (hemi-channels) isolated from rat liver. J. Biol. Chem., 270:18643-18648.

Chew, B.P., C.S. Zamora, and L.O. Luedecke. 1985. Effect of vitamin A deficiency on mammary gland development and susceptibility to mastitis through intramammary infusion with *Staphylocoocus aureus* in mice. Am. J. Vet. Res., 46:287-293.

Chiarugi, V., L. Magnelli, M. Cinelli, and G. Basi. 1994. Apoptosis and the cell cycle. Cell. Mol. Biol. Res., 40:603-612.

Claus, E.B., N. Risch, and W.D. Thompson. 1991. Genetic analysis of breast cancer in the cancer and steroid hormone study. Am. J. Human Genetics, 48:232-241.

Coradini, D., A. Biffi, E. Pirronello, and G. Di Fronzo. 1994. The effects of alpha-, beta-, and gamma- interferon on the growth of breast cancer cell lines. AntiCancer Res., 14:1779-1784.

Cowie, A.T., I.A. Forsyth, and I.C. Hart. 1980. Hormonal control of lactation. F. Gross, M.M. Grumbach, A. Labhart, M.B. Lipsett, T. Mann, L.T. Samuels, and J. Zanders. Eds, Springer-Verlag. New York. Vol. 15. pp 1-145.

Cox, D.A., and R.R. Burk. 1991. Isolation and characterization of milk growth factor, a transforming-growth-factor-β2-related polypeptide, from bovine milk. Eur. J. Biochem., 197:353-358.

Daniel, C.W., G.B. Silberstein, K. Van Horn, P. Strickland, and S. Robinson. 1989. TGF-β1-induced inhibition of mouse mammary ductal growth: development specificity and characterization. Dev. Biol., 135:20-30.

Daniel , C.W., and S.D. Robinson. 1992. Regulation of mammary growth and function by TGF-β. Mol. Reprod. Dev., 32:145-151.

Danielson, K.G., C.J. Oborn, E.M. Durban, J.S. Butel, and D. Medina. 1984. Epithelial mouse mammary cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. Cell Biol., 81:3756-3760.

David, M., E. Hodak, and N.J. Lowe. 1988. Adverse effects of retinoids. Med. Toxic. Adverse Drug Exp., 3:273-288.

Decensi, A., F. Formelli, R. Torrisi, and A. Costa. 1993. Breast cancer chemoprevention: studies with 4-HPR alone and in combination with tamoxifen using circulating growth factors as potential surrogate endpoints. J. Biol. Chem., 17G:226-233.

DeGraves, F.J. and J. Fetrow. 1993. Economics of mastitis and mastitis control. Vet Clinics N. Am., 9:421-434.

DeLuca, L.M. 1991. Retinoids and their receptors in differentiation, embryogenesis and neoplasia. FASEB J., 5:2924-2933.

Dickson, R.B., Y.E. Shi, and M.D. Johnson. 1994. Matrix-degrading proteases n hormone-dependent breast cancer. Breast Cancer Res. Trt., 31:167-173.

DiGiovanna, J.J. 1992. Retinoids for the future: oncology. J. Am. Acad. Derm., 27:534-537.

Easton, D., F. Deborah, and J. Peto. 1993. Inherited susceptibility to breast cancer. Cancer Surveys, 18:95-113.

Eghbali, B., J.A. Kessler, L.M. Reid, C. Roy, and D.C. Spray. 1991. Involvement of gap junctions in tumorigenesis: transfection of tumor cells with Cx32 cDNA retards growth in vivo. Proc. Natl. Acad. Sci., 88:10701-10705.

Erdmann, B., and H. Breter. 1993. Irregular distribution of mammary-derived growth inhibitor in the bovine mammary epithelium. Cell & Tissue Res., 272:383-389.

Erskine, R.J. 1993. Nutrition and mastitis. Vet Clinics N. Am., 9:551-561.

Fallon, J.F., and D.A. Goodenough. 1981. Five hour half-life of mouse liver gap-junctional protein. J. Cell Biol., 90:521-526.

Fontana, J.A., A. Burrows-Mezu, D.R. Clemmons, and D. LeRoith. 1991. Retinoid modulation of insulin-like growth factor-binding proteins and inhibition of breast carcinoma proliferation. Endocrinology, 128:1115-1122.

Franzen, P., P. ten Dijke, H. Ichijo, H. Yamashita, P. Schultz, C.H. Heldin, and K. Miyazono. 1993. Cloning of the TGF-β type I receptor that forms a heteromeric complex with the type II TGF-β receptor. Cell, 75:681-692.

Gaffney, E.V. 1992. A cell line (HBL-100) established from human breast milk. Cell & Tissue Res., 227:563-568.

Gibson, C.A., J.R. Vega, C.R. Baumrucker, C.S. Oakley, and C.W. Welsch. 1991. Establishment and characterization of bovine mammary epithelial cell lines. In Vitro, 27:585-594.

Gibson, C.A., D.E. Kerr, and C.R. Baumrucker. 1992. Growth responsiveness of bovine mammary epithelial cells (PSBME). J. Dairy Sci., 75(Suppl. 1):225.

Glimm, D.R., V.E. Baracos, and J.J. Kennelly. 1990. Molecular evidence for the presence of growth hormone receptors in the bovine mammary gland. J. Endocr., 126:R5-R8.

Grosse, R., F.D. Bohmer, B. Binas, A. Kurtz, E. Spitzer, T. Muller, and W. Zschiesche. 1992. Mammary-derived growth inhibitor (MDGI). Cancer Trt. Res., 61:69-96.

Halter, S.A., A.R. Winnier, and C.L. Arteaga. 1993. Pretreatment with vitamin A inhibits transforming growth factor-α stimulation of human mammary carcinoma cells. J. Cell. Physiol., 156:80-87.

Harmon, R.J. 1994. Physiology of mastitis and factors affecting somatic cell counts. J. Dairy Sci., 77:2103-2112.

Haslam, S.Z. 1986. Mammary fibroblast influence on normal mouse mammary epithelial cell responses to estrogen in vitro. Cancer Res., 46:310-316.

Haslam, S.Z. 1988. Local versus systemically mediated effects of estrogen on normal mammary epithelial cell deoxyribonucleic acid synthesis. Endocrinology, 122:860-867.

Hennighausen, L. 1992. The prospects for domesticating milk protein genes. J. Cell. Biochem., 49:325-332.

Holder, J.W., E. Elmore, and J.C. Barrett. 1993. Gap junction function and cancer. Cancer Res., 53:3475-3485.

Houdebine, L.M. 1993. Expression de proteines recombinants dans le lait d'animaux transgeniques. Revue Francais de Transfusion et d'Hemobiologie, 36:49-72.

Huynh, H.T., G. Robitaille, and J.D. Turner. 1991. Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. Exp. Cell Res., 197:191-199.

Huynh, H.T., and M. Pollak. 1995. HH2A, an immortalized bovine mammary epithelial cell line, expresses the gene encoding mammary derived growth inhibitor (MDGI). In Vitro Cell. Dev. Biol., 31:25-29.

Huynh, H.T., C. Larsson, S. Narod, and M. Pollak. 1995. Tumor suppressor activity of the gene encoding mammary-derived growth inhibitor. Cancer Res., 55:2225-2231.

Janne, J., J.M. Hyttinen, T. Peura, M. Tolvanen, L. Alhonen, R. Sinervirta, and M. Halmekyto. 1994. Transgenic bioreactors. Int. J. Biochem., 26:859-870.

Jhappan, C., A.G. Geiser, E.C. Kordon, D. Bagheri, L. Henninghausen, A.B. Roberts, G.H. Smith, and G. Merlino. 1993. Targeting expression of a transforming growth factor-β1 transgene to the pregnant mammary gland inhibits alveolar development and lactation. EMBO J., 12:1835-1845.

Jin, Y., D.A. Cox, R. Knecht, F. Raschdorf, and N. Cerletti. 1991. Separation, purification, and sequence identification of TGF-β1 and TGF-β2 from bovine milk. J. Prot. Chem., 10:565-575.

Jongen, W.M., D.J. Fitzgerald, M. Asamoto, C. Piccoli, T.J. Slaga, D. Gros, M. Takeichi, and H. Yamasaki. 1991. Regulation of connexin43-mediated gap junctional intercellular communication in mouse epidermal cells is controlled by E-cadherin. J. Cell Biol., 114:545-555.

Jones, J.I., and D.R. Clemmons. 1995. Insulin-like growth factors and their binding proteins: biological actions. Endocr. Rev., 16:3-34.

Kirk, J.H., F. DeGraves, J. Tyler. 1994. Recent progress in treatment and control of mastitis in cattle. J. Am. Vet. Med. Assoc., 204:1152-1158.

Klijn, J.G.M., E.M.J.J. Berns and J. A. Foekens. 1993. Prognostic factors and response to therapy in breast cancer. Cancer surveys, 18:165-256.

Laherty, R.F., W.X. Balcavage, C. Goff, T. Alvager, S. Ghosh, R.W. Geib, M. King, and D. Personett. 1990. HBL-100 cells do not secrete casein and lack prolactin and estradiol receptors. In Vitro Cell. Devel. Biol., 26:933-935.

Laird, D.W., K.L. Puranam, and J.P. Revel. 1991. Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes. Biochem. J., 273:67-72.

Laird, D.W., and J.C. Saez. 1995. Posttranscriptional events in the expression of gap junctions. In: Advances in Molecular Cell Biology, Vol. 18. In Press.

Laird, D.W., M. Castilo, and L. Kasprzak. 1995. Gap junction turnover, intracellular trafficking and phosphorylation of connexin43 in brefeldin A-treated rat mammary tumor cells. J. Cell Biol., In Press.

Larsen, W.J., L. Chen, S. Wert, P.T. Russell, and E.M. Hendrix. 1991. Reproductive implications of responses of the cumulus to the ovulatory surge. Bul. L'Assoc. Anatomistes., 75:73-76.

Lee, M.O., Y. Liu, and X.K. Zhang. 1995. A retinoic acid response element that overlaps an estrogen response element mediates multihormonal sensitivity in transcriptional activation of the lactoferrin gene. Molec. Cell. Biol., 15:4194-4207.

Lee, P.P., M.T. Lee, K.M. Darcy, K. Shudo, and M.M. Ip. 1995. Modulation of normal mammary epithelial cell proliferation, morphogenesis, and functional differentiation by retinoids: a comparison of the retinobenzoic acid derivative RE80 with retinoic acid. Endocrinology, 136:1707-1717.

Lee, S.W., C. Tomasetto, D. Paul, K. Keyomarsi, and R. Sager. 1992. Transcriptional downregulation of gap junction proteins blocks junctional communication in human mammary tumor cell lines. J. Cell Biol., 118:1213-1221.

Lehrman, S. 1994. BST enters battle for consumers. Nature, 367:585.

Lightner, J.K., G.Y. Miller, W.D. Hueston, and D.R. Dorn. 1988. Estimation of the costs of mastitis using National Animal Health Monitoring System and milk somatic cell count data. J. Am. Vet. Med. Assoc., 192:1410-1419.

Lin, H.Y., Z.F. Wang, E. Ng-Eaton, R.A. Weinberg, and H.F. Lodish. 1992. Expression cloning of the TGF-β type II receptor, a functional transmembrane serine/threonine kinase. Cell, 68:775-785.

Lin Y., L. Xia, J.D. Turner, and X. Zhao. 1995. Morphological observation of neutrophil diapedesis across bovine mammary gland epithelium in vitro. Am. J. Veter. Res., 56:203-207.

Lippman, S.M., J.F. Kessler, and F.L. Meyskens Jr. 1987. Retinoids as preventative and therapeutic anticancer agents. Cancer Trtmt. Reports, 71:391-405, 493-515.

Liu, S., S. Taffet, L. Stoner, H. Delmar, M.L. Vallano, and J. Jalife. 1993. A structural basis for the unequal sensitivity of the major cardiac and liver gap junctions to intracellular acidifications: the carboxyl tail length. Biophys. J., 64:1422-1433.

Lopez-Casillas, F., S. Cheifetz, J. Doody, J. Andres, W.S. Lane, and J. Massague. 1991. Structure and expression of the membrane proteoglycan betaglycan, a compenent of the TGF-β receptor system. Cell, 67:785-795.

Lotan, R. 1979. Different susceptibilities of human melanoma and breast carcinoma cell lines to retinoic acid-induced growth inhibition. Cancer Res., 39:1014-1019.

Loewenstein, W.R. 1967. Intercellular communication and tissue growth. J. Cell Biol., 33:225-234.

Macdonald, E.A., L. Xia, H. Monardes, and J.D. Turner. 1994. Neutrophil function in vitro: diapedesis and phagocytosis. J. Dairy Sci., 77:628-638.

Maier, R., P. Schmid, D. Cox, G. Bilbe, and G.K. McMaster. 1991. Localization of transforming growth factor-β1, -β2 and -β3 gene expression in the bovine mammary gland. Molec. Cell. Endo., 82:191-198.

Massague, J. 1990. The transforming growth factor-β family. Ann. Rev. Cell Biol., 6:597-641.

McGrath, M.F. 1987. A novel system for mammary epithelial cell culture. J. Dairy Sci., 70:1967-1980.

Medina, D., C.J. Oborn, F.S. Kitrell, and R.L. Ullrich. 1986. Properties of mouse mammary epithelial cell lines characterized by in vivo transplantation and in vitro immunocytochemical methods. J. Natl. Cancer Inst., 76:1143-1156.

Mesnil, M., V. Krutovskikh, C. Piccoli, C. Elfgang, O. Traub, K. Willecke, and H. Yamaski. 1995. Negative growth control of HeLa cells by connexin genes: connexin species specificity. Cancer Res., 55:629-639.

Meyer, R.A., D.W. Laird, J.P. Revel, and R.G. Johnson. 1992. Inhibition of gap junction and adherens junction assembly by connexin and A-CAM antibodies. J. Cell Biol., 119:179-189.

Miettinen, P.J., R. Ebner, A.R. Lopez, and R. Derynck. 1994. TGF-β induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. J. Cell Biol., 127:2021-2036.

Miller, J.K., E. Brzezinska-Slebodzinska, and F.C. Madsen. 1993. Oxidative stress, antioxidants, and animal function. J. Dairy Sci., 76:2812-2823.

Monaghan, P., N. Perusinghe, G. Carlile, and W.H. Evans. 1994. Rapid modulation of gap junction expression in murine mammary gland during pregnacy, lactation and involution. J. Histoch. Cytoch., 42:931-938.

Musil, L.S., B.A. Cunningham, G.M. Edelman, and D.A. Goodenough. 1990. Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. J. Cell Biol., 111:2077-2088.

Naus, C.C.G., J.F. Bechberger, S. Caveney, and J.X. Wilson. 1991. Expression of gap junction genes in astrocytes and C6 glioma cells. Neurosci. Lett., 126:33-36.

Oh, S.Y., C.G. Grupen, and W.C. Murray. 1991. Phorbol ester induces phosphorylation and down-regulation of connexin43 in WB cells. Bioch. Biophys. Acta, 1094:243-245.

Ohaliwal, M.K., B.C. Giovanella, and S. Pathak. 1990. Cytogenetic characterization of 2 human milk derived cell line (HBL-100) passages differing in tumorigenicity. AntiCancer Res., 10:113-117.

Oka, T., M. Yoshimura, S. Lavandero, and K. Wada. 1991. Control of growth and differentiation of the mammary gland by growth factors. J. Dairy Sci., 74:2788-2800.

Oldham, E.R., R.J. Eberhart, and L.D. Muller. 1991. Effects of supplemental vitamin A or β-carotene during the dry period and early lactation on udder health. J. Dairy Sci., 74:3775-3781.

Olson, J.A. 1989. Provitamin A function of carotenoids: the conversion of β-carotene into vitamin A. J. Nutr., 119:105-108.

Ong, D.E. 1993. Retinoid metabolism during intestinal absorption. J. Nutr., 123:351-355.

Ozbun, M.A., and J.S. Butel. 1995. Tumor suppressor p53 mutations and breast cancer: a critical analysis. Adv. Cancer Res., 66:71-141.

Perezamendariz, E.M., J. Luna, C. Aceves, and D.T. Tapia. 1995. Connexins 26, 32 and 43 are expressed in virgin, pregnant and lactating mammary glands. Develop. Growth Diff., 37:421-431.

Perlmann, T., and L. Jansson. 1995. A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURRI. Genes Devel., 9:769-782.

Pitts, J.D., and E. Kam. 1985. Communication compartments in mixed cell cultures. Exp. Cell Res., 156:439-449.

Politis, I., E. Lachance, E. Block, and J.D. Turner. 1989. Plasmin and plasminogen in bovine milk: a relationship with involution? J. Dairy Sci., 72:900-906.

Politis, I., E. Block, and J.D. Turner. 1990. Effect of somatotropin on the plasminogen and plasmin system in the mammary gland: proposed mechanism of action for somatotropin on the mammary gland. J. Dairy Sci., 73:1494-1499.

Pozzi, A., B. Risek, D.T. Kiang, N.B. Gilula, and N.M. Kumar. 1995. Analysis of multiple gap junction gene products in the rodent and human mammary gland. Exp. Cell Res., 220:212-219.

Richard, S., and H.H. Zingg. 1991. Identification of a retinoic acid response element in the human oxytocin promoter. J. Biol. Chem., 266:21428-21433.

Roberts, A.B., and M.B. Sporn. 1990. The transforming growth factor-betas. In: Peptide Growth Factors and Their Receptors. M.B. Sporn and A.B. Roberts, eds. Springer-Verlag, Heidelberg, Germany, pp. 421-472.

Robinson, S.D., G.B. Silberstein, A.B. Roberts, K.C. Flanders, and C.W. Daniel. 1991. Regulated expression and growth inhibitory effects of transforming growth factor-β isoforms in mouse mammary gland development. Development, 113:867-878.

Robinson, S.D., A.B. Roberts, and C.W. Daniel. 1993. TGF-β suppresses casein synthesis in mouse mammary explants and may play a role in controlling milk levels during pregnancy. J. Cell Biol., 120:245-251.

Rochefort, H. 1994. Oestrogens, proteases and breast cancer. From cell lines to clinical applications. Eur. J. Cancer, 30A:1583-1586.

Ross, C.A. 1993. Overview of retinoid metabolism. J. Nutr., 123:346-350.

Ruch, R.J. 1994. The role of gap junctional intracellular communication in neoplasia. Annals Clin. Lab. Sci., 24:216-231.

Sankaran, L., and Y.J. Topper. 1982. Effect of vitamin A deprivation on maintenance of rat mammary tissue and on the potential of the epithelium for hormone-dependent milk protein synthesis. Endocrinology, 111:1061-1067.

Seol, W., H.S. Choi, and D.D. Moore. 1995. Isolation of proteins that interact specifically with the retinoid-X receptor: 2 novel orphan receptors. Mol. Endo., 9:72-85.

Shalloway, D., S. Bagrodia, I. Chackalaparampil, S. Shenoy, P.H. Lin, and S.J. Taylor. 1992. c-Src and mitosis. Ciba Found. Symp., 170:248-265.

Shearer, J.K., and R.J. Harmon. 1993. Mastitis in heifers. Vet. Clinics N. Am., 9:583-595.

Shemer, J., A. Yaron, H. Werner, Z.M. Shao, M.S. Sheikh, J.A. Fontana, D. LeRoith, and C.T. Roberts. 1993. Regulation of insulin-like growth factor (IGF) binding protein-5 in the T47D human breast carcinoma line by IGF-I and retinoic acid. J. Clinic. Endocr. Metab., 77:1246-1250.

Sia, M.A., T.L. Wooward, J.D. Turner, and D.W. Laird. 1995. The mechanism of gap junction removal in live normal rat kidney cells. Am. Soc. Cell Biol., Washington D.C. December, 1995.

Silberstein, G.B., and C.W. Daniel. 1987. Reversible inhibition of mammary gland growth by transforming growth factor-β. Science, 237:291-293.

Sizemore, S.R. 1979. Hormonal responsiveness of NMuMG, a mammary epithelial cell line. J. Dairy Sci., 41:28. Abstract.

Smith, W.C., H. Nakshatri, P. Leroy, J. Rees, and P. Chambon. 1991. A retinoic acid response element is present in the murine cellular retinol binding protein-I (mCRBP-I) promoter. EMBO J., 10:2223-22230.

Sobek, L., D. Di Lorenzo, M. Oettel, and G. Kaufmann. 1994. Normal and stable transfected cancer cell lines: tools for a screening of progestogenic, antiprogestogenic and antiglucocorticoid substances. Meth. & Findings Exp. Clin. Pharm., 16:545-551.

Soprano, D. R., D.C. Harnish, K.J. Soprano, D.M. Kochhar, and H. Jiang. 1993. Correlations of RAR isoforms and cellular retinoid-binding proteins mRNA levels with retinoid-induced teratogenesis. J. Nutr., 123:367-371.

Sordillo, L.M.. S.C. Nickerson, and R.M. Akers. 1989a. Pathology of Staphylococcus aureus mastitis during lactogenesis: relationships with bovine mammary structure and function. J. Dairy Sci., 72:228-235.

Sordillo, L.M., M.Z. Doymaz, and S.P. Oliver. 1989b. Morphological study of chronic Staphylococcus aureus mastitis in the lactating bovine mammary gland. Res. Vet. Sci., 47:247-253.

Stampfer, M.R., and P. Yaswen. 1993. Culture systems for study of human mammary epithelial cell proliferation, differentiation and transformation. Cancer Surv., 18:7-34.

Stauffer, K.A. 1995. The gap junction proteins β_1-connexin (connexin-32) and β_2-connexin (connexin-26) can from heteromeric hemichannels. J. Biol. Chem., 270:6768-6772.

Steinberg, M.S., and M. Takeichi. 1994. Experimental specification of cell sorting, tissue spreading, and specific spatial patterning by quantitative difference in cadherin expression. Proc. Natl. Acad. Sci. USA, 91:206-209.

Suchyna, T.M., L.X. Xu, F. Gao, C.R. Fourtuer, and B.J. Nicholson. 1993. Identification of a proline residue as a transduction element involved in voltage gating of gap junctions. Nature, 365:847-849.

Swanson, E.W., G.G. Martin, F.E. Pardue, and G.M. German. 1968. Milk production of cows fed diets deficient in vitamin A. J. Anim. Sci., 27:541-548.

Swisshelm, K., K. Ryan, K. Tsuchiya, and R. Sager. 1995. Enhanced expression of an insulin growth factor-like binding protein (mac25) in senescent human mammary epithelial cells and induced expression with retinoic acid. Proc. Natl. Acad. Sci., 92:4472-4476.

Taylor-Papadimitriou, J., F. Berdichevsky, B. D'Souza, and J. Burchell. 1993. Human models of breast cancer. Cancer Surv., 16:59-78.

Tokuyama, Y., and H. Tokuyama. 1993. Purification and identification of TGF-β2-related growth factor from bovine colostrum. J. Dairy Res., 60:99-109.

Tomasetto, C., M.J. Neveu, J. Daley, P.K. Horan, and R. Sager. 1993. Specificity of gap junction communication among human mammary cells and connexin transfectants in culture. J. Cell Biol., 122:157-167.

Traub, O., J. Look, R. Dermietzel, F. Brunner, D. Husler, and K. Willecke. 1989. Comparative characterization of the 21-kD and 26-kD gap junction proteins in murine liver and cultured hepatocytes. J. Cell Biol., 108:1039-1051.

Treneuner, M., C.A. Kozak, D. Gallahan, R. Grosse, and T. Muller. 1994. Cloning and characterization of the mouse gene encoding mammary-derived growth inhibitor/heart-fatty acid-binding protein. Gene, 147:237-242.

Tucker, H.A. 1981. Physiological control of mammary growth, lactogenesis, and lactation. J. Dairy Sci., 64:1403-1421.

Tyler, J.W., J.S. Cullor, and D.C. Ruffin. 1993. Immunization and immunotherapy for mastitis. Vet. Clinics N. Am., 9:537-549.

Underwood, B.A. 1984. Natural sources of vitamin A. In: The retinoids, Vol. 2, A.B. Roberts and M.B. Sporn, eds. Academic Press, Inc., Raven, NY. pp. 284-300.

Vandewalle, B., L. Hornez, N. Wattez, F. Revillion, and J. Lefebvre. 1995. Vitamin-D3 derivatives and breast-tumor cell growth: effect on intracellular calcium and apoptosis. Int. J. Cancer, 61:806-811.

Wang, Z.F., H.Y. Lin, E. Ng-Eaton, J. Downward, H.F. Lodish, and R.A. Weinberg. 1991. Expression cloning and characterization of the TGF-β type III receptor. Cell, 67:797-805.

Weinberg, R.A. 1995. The retinoblastoma protein and cell cycle control. Cell, 81:323-330.

Wicha, M.S., L.A. Liotta, S. Garbisa, and W.R. Kidwell. 1979. Basement membrane collagen requirements for attachment and growth of mammary epithelium. Exp. Cell Res., 124:181-191.

Wicha, M.S., G. Lourie, E. Kohn, P. Bagavandos, and T. Mahn. 1982. Extracellular matrix promotes mamamry epithelial growth and differentiation in vitro. Proc. Natl. Acad. Sci. USA, 79:3213-3218.

Wilgenbus, K.K., C.J. Kirkpatrick, R. Knuechel, K. Willecke, and O. Traub. 1992. Expression of Cx26, Cx32 and Cx43 gap junction proteins in normal and neoplastic human tissues. Int. J. Cancer, 51:522-529.

Wilmut, I., A.L. Archibald, M. McClenaghan, J.P. Simons, C.B. Whitelaw, and A.J. Clark. 1991. Production of pharmaceutical proteins in milk. Experentia, 47:905-912.

Wolburg, H., and A. Rohlmann. 1995. Structure-function relationships in gap junctions. Int. Rev. Cytol., 157:315-373.

Wolf, G., and D. Phil. 1991. The intracellular vitamin A-binding proteins: an overview of their functions. Nutr. Rev., 49:1-12.

Wolf, G. 1993. The newly discovered retinoic acid-X receptors (RXRs). Nutr. Rev., 51:81-84.

Woodward, T.L., R.M. Akers, T.B. McFadden, H.T. Huynh, and J.D. Turner. 1992. Estrogen mediated bovine mammary epithelial cell proliferation: evidence of indirect action. J. Dairy Sci., 75:P444. (Abstract)

Woodward, T.L., W.E. Beal, and R.M. Akers. 1993. Cell interactions in initiation of mammary epithelial proliferation by oestradiol and progesterone in prepubertal heifers. J. Endocr., 136:149-157.

Wu, Y.N., M. Gadina, J.H. Tao-Cheng, and R.J. Youle. 1994. Retinoic acid disrupts the Golgi apparatus and increases the cytosolic routing of specific protein toxins. J. Cell Biol., 125:743-753.

Xie, H., D.W. Laird, and V.W. Hu. 1995. A novel mitosis-specific modification of a gap junction protein in human endothelial cells: biochemical characterization, localization and functional analysis. (In revision, J. Cell Biol.)

Yang, Y., E. Spritzer, N. Kenney, W. Zschiesche, M. Li, A. Kromminga, T. Muller, F. Spener, A. Lezius, and J.H. Veerkamp. 1994. Members of the fatty acid binding protein family are differentiation factors for the mammary gland. J. Cell Biol., 127:1097-1109.

Zavizion, B., I. Politis, and R.C. Gorewit. 1992a. Bovine mammary myoepithelial cells. 1. Isolation, culture, and characterization. J. Dairy Sci., 75:3367-3380.

Zavizion, B., I. Politis, and R.C. Gorewit. 1992b. Bovine mammary myoepithelial cells. 2. Interactions with epithelial cells in vitro. J. Dairy Sci., 75:3381-3393.

Zavizion, B., I. Politis, R.C. Gorewit, J.D. Turner, E. Spritzer, and R. Grosse. 1993. Effect of mammary-derived growth inhibitor on proliferation of MAC-T bovine mammary epithelial cells. J. Dairy Sci., 76:3721-3726.

Zhang, X.K., and M. Phafl. 1993. Retinoic acid receptors (RARs) and thyroid receptors (TRs) require heterodimer formation with retinoic acid-X-receptors (RXRs) for efficient DNA binding and transcriptional activation. Receptors, 3:183-191.

Zhu, D., G.M. Kidder, S. Caveney, and C.C. Naus. 1992. Growth retardation in glioma cells cocultured with cells overexpressing a gap junction protein. Proc. Natl. Acad. Sci., 89:10218-10221.

CHAPTER 1

Isolation, cloning, immortalization and characterization of bovine mammary epithelial and fibroblasts cells.

INTRODUCTION

Research regarding hormonal control of mammogenesis and lactogenesis in the bovine animal has been dominated by in vivo studies, and explant cultures (Tucker, 1981; Akers, 1985; McGrath, 1987; Akers, 1991). Consequently, direct action of mitogens and differentiation agents on individual cell types is largely unknown. These studies have identified many mammary specific mitogens including: estrogen, progesterone, growth hormone, insulin, insulin-like growth factors, thyroxine, and corticosteroids. Additionally, data from murine and human studies have demonstrated that epidermal growth factor, IGF-I, IGF-II, insulin, fibroblast growth factors, transforming growth factor-α, etc. are direct mitogens to mammary epithelial cells (Tonelli and Sorof, 1980; Imai et al., 1981; Taketani and Oka, 1983; Vonderhaar, 1987; Sakthivel et al., 1993). Limited data has been obtained from primary cell culture. However, results have often been inconsistent because of limitations in primary cell culture steming from: 1) a non-clonal population of cells, often containing stromal, endothelial, myoepithelial and blood

cells as well as the extracellular matrices they secrete, 2) a lack of characterization of these cell with regard to cytoskeletal cell-typing markers, growth responsiveness, and differentiation capabilities, and 3) early senescence and a lack of cellular responsiveness.

Although many human and murine mammary cell lines exist, including normal and transformed; only a single clonal bovine mammary epithelial cell line has been well characterized. This cell line, MAC-T, is a mammary epithelial cell line isolated from a lactating Holstein cow (Huynh et al., 1991). Unfortunately, because only a single cell line exists, mitogens or mitostatic agents identified using the MAC-T cell line can only be confirmed with biopsied primary cell populations. Additionally, no other cell lines representing mammary stromal cells have been established.

The mammary gland is composed of many cell types which potentially affect mammary epithelial cell growth and differentiation (Haslam, 1988), with epithelial cells, myoepithelial cells, adipocytes and fibroblasts comprising the major cellular components of the gland. Stromal tissue in the rodent mammary gland is mostly adipose with close physical association of adipocytes and epithelial cells (Cowie, 1974; Woodward et al., 1993). In contrast in the bovine mammary gland, adipocytes are not in close apposition to epithelial cells (Akers, 1990; Woodward et al., 1993). Instead, epithelial cells are separated from the adipose tissue by a 100 μM or larger expanse of stromal cells, the vast majority of which are fibroblasts (Woodward et al., 1993). Therefore, we have chosen to isolate the major cellular components of the mammary gland parenchyma

(epithelial cells) and stroma (fibroblast cells). This chapter will describe the isolation, cloning, 'immortalization' and characterization of bovine mammary epithelial and fibroblast cells.

MATERIALS AND METHODS

Hormones, Enzymes and Culture Materials

All culture reagents, media, serum, and enzymes were obtained from GIBCO BRL (Burlington, Ontario, Canada) and Becton Dickinson (Ville St. Laurent, Quebec, Canada), unless otherwise noted. The collagenases, hyaluronidase, and pronase used in tissue dissociation were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cell Isolation

Mammary biopsies were taken from one nulliparous pubertal Holstein heifer and two pregnant Holstein heifers. Hair was shaved from the area selected for incision and the skin was scrubbed with iodine. The animals were given a general anaesthetic of Xylazine (100 mg/ml; Rompum®, Mobay Corporation, Shawnee, KA, USA) at 0.2 ml i.m. (caudal to the scapula) and 0.1 ml i.v. (via the jugular).

An electroscalpel was used to cut the skin and cauterize small blood vessels. An incision of approximately 4 cm was made immediately dorsal to the teat on the lateral side of the udder. A mass of fascia and attached parenchymal tissue was removed using sterile forceps and immediately placed in sterile complete medium (Medium 199/DMEM

mixture containing 10% FBS, 100 IU/ml penicillin G sodium, 10 μg/ml streptomycin sulfate, 25 μg/ml amphotericin B, 50 μg/ml gentamicin, 10 μg/ml bovine insulin, 1 μg/ml hydrocortisone, 1.85 mg/ml sodium bicarbonate and 15 mM HEPES). The vial containing tissue was stored on ice for transport to the laboratory, approximately 10 min.

Tissue was next minced with two scalpel blades and transferred to 75 ml dissociation solution (HBSS with 30 mM HEPES, 12.2 mM glucose, 100 IU/ml collagenase type XI, 100 IU/ml collagenase type IA, 25 IU/ml hyaluronidase, 0.41 IU/ml pronase, 1 mg/ml serum albumin and antibiotics as per complete media). The 75 mls of dissociation solution with 1-5 g minced tissue was placed in a 300 ml trypsinizing flask. Next, the tissue was incubated for 90 min at 150 rpm in a 37° C gyratory water bath. Dissociation solution was centrifuged at 100 X g for 3 min and supernatant transferred to a new vial. The pellet consisting of undigested tissue clumps was digested again in fresh dissociation solution for 60 min further. Supernatant was centrifuged at 700 X g for 5 min, supernatant discarded and the pellet resuspended in 30 mls complete medium. This centrifugation and resuspension step was repeated, to remove tissue debri, adipocytes and erythrocytes, and then passed through a sterile nitex 65 μM pore size mesh filter (Technico, Montreal, Quebec, Canada). The filtered cell suspension consisting of single cells and small cell clusters were plated at 40,000 cells/cm^2 on tissue culture dishes. Clumps of cells not passing through the filter were plated in separate dishes. All cells were plated in complete medium and incubated in a 5% CO_2 humidified incubator at 37° C.

Cell Cloning and Typing

Following 1-3 passages, plates were enriched for fibroblast or epithelial cells by differential trypsinization (Schumann et al., 1988). Enriched cultures were next cloned by limiting dilution into 96-well tissue culture plates. Wells that contained one cell were marked and colonies resulting from these wells progressively transferred to 24 well plates, 35 mm dishes and 100 mm dishes when cells were approaching confluency.

Cell typing (as epithelial or fibroblast) was confirmed by immunohistochemical analysis after incubation with monoclonal antibodies that react with vimentin (Boehringer-Mannheim, Indianapolis, IN) and cytokeratin (pan-specific, Boehringer-Mannheim) as described previously (Huynh et al., 1991).

Transfections

Fibroblasts and epithelial cell clones were co-transfected with the simian virus-40 Large-T-antigen directed from the plasmid, pBAPSV40TtsA58 (Otsuka et al., 1979), and the pSV-2 neo plasmid which provided a dominant selectable marker (Southern and Berg, 1982). Transfections and subsequent selection were the same as that previously reported (Huynh et al., 1991), except that 45 μl lipofectin reagent (GIBCO BRL, Burlington, Ontario, Canada) and 55 μl HBSS containing 5 μg pBAPSV40TtsA58 plasmid and 1.1 μg pSV2 plasmid were incubated for 5 h per 100 mm plate. Lipofectin transfections resulted in a dramatic increase in the percentage of cells stably transfected/μg DNA over calcium phosphate transfections (Woodward et al., 1995).

Transfected cells were transferred to 6 well multiplates 24 h following transfection at a density of 5000 cells/cm^2 in DMEM containing 10% FBS and 200 μg/ml G418 sulfate (GIBCO). This concentration of G418 sulfate was previously determined to cause 100% cell death of non-transfected epithelial cells after 7 d culture. Selection media were changed every 3 days. Following 14 d of selection, surviving colonies were isolated with cloning rings as previously described (Huynh et al., 1991). Some epithelial and fibroblast transfectants were recloned by limiting dilution, described above. All transfected cells were recharacterized with regard to cytoskeleton and population doubling times as per Ethier (1985) in 10% FBS.

Transformed Phenotype Assessment

Cells were analyzed for a transformed phenotype, based on two criteria: loss of topo-inhibition and loss of adherence to substratum. Contact inhibition was assessed histologically and by Giemsa staining of 10 d confluent plates as previously described (Bertram, 1979). Foci formation on plastic identified by Giemsa staining was representative of loss of contact inhibition. Substratum adherence was measured by time of trypsinization (0.25%) and ability to grow and form colonies in 0.1% soft agar as previously described (Leone et al., 1991). Quick trypsinization times or removal of cells without trypsin are indicative of a transformed phenotype. Likewise, growth in soft agar demonstrates a lack of need for a substrata and is representative of a transformed phenotype.

RESULTS

Cell Cloning and Selections:

# Cell Populations Viable after Cloning, Transfection and Cryopreservation		
	Epithelial cells	**Fibroblast cells**
# cloned	99	763
# not senencing after cloning and passage	2	16
Nontransfected cells surviving cryopreservation	1	3
# clonal cells transfected	12	16
# surviving selection	3	10
# surviving transfection & cryopreservation	0	4

Only 2 epithelial cell lines, the FbE (AKA MEB-E) and the PrE, survived cloning, though neither proliferated following Large-T-antigen transfection. The FbE line was the only epithelial line to survive cryopreservation and thawing.

Similarly, 16 fibroblast cell populations survived cloning and only 3 of these did not enter crisis after passage and cryopreservation/thawing: 3hUnfil (AKA MFB-3hUnfl), MF2 (MFB-Pr), FibC (MFB-3). Of the 16 fibroblast cell populations transfected, 4 fibroblast cell lines were viable and survived selection (MF-T2 (Fib D), MF-T3 (Fib 00),

Fib A, and Fib B. However, only MF-T2 and MF-T3 cells did not enter crisis after passage and cryopreservation/thawing.

Characterization of Morphology, Cytoskeletal Proteins, Growth and Phenotype

All fibroblast lines were positive for vimentin and negative for cytokeratin and had a fibroblastic morphology. A phase contrast micrograph of MF2 cells is representative of the nontransfected fibroblast morphology (Figure 1). An immunofluorescent micrograph of MAC-T cells cocultured with a FibC cell is shown in Figure 2a, the punctate staining between MAC-T cells is connexin43. Figure 2b is the same cells showing positive vimentin staining of FibC and negative vimentin staining MAC-T cells. Figure 2c is an overlay of 2a and 2b, vimentin staining is green. The epithelial morphology of the FbE cell line is captured in a phase contrast micrograph (Figure 3).

CELLULAR CHARACTERIZATION (population doubling time = PDT; transformed = Tfm; Not Tfm = NEG)					
Cell Type	*Giemsa*	*Foci form*	*Soft agar*	*Trypsin time*	*PDT*
EPITHELIAL					
EbE	NEG	NEG	NEG	15-20 min	19 h
MAC-T	NEG	NEG	NEG	20-30 min	17 h
FIBROBLAST					
EbC	NEG	NEG	NEG	10 min	28 h
MF2	NEG	NEG	NEG	4 min	26 h
ShUnpl	NEG	NEG	NEG	6 min	25 h
Large-T Fibroblast					
EbA	Tfm	Tfm	Tfm	0 min	16 h
EbB	Tfm	Tfm	NEG	5 min	34 h
MF2	Tfm	Tfm	NEG	2 min	32 h
MF3	Tfm	Tfm	Tfm	< 1 min	21 h

Therefore all of the Large-T-antigen expressing fibroblasts show signs of transformation, though MAC-T cells (which also have been transfected with the simian virus-40 Large-T-

antigen) do not exhibit a transformed phenotype. Giemsa staining of petri dishes (Figure 4) can quickly quantify foci formation. The positive stained plate, FibA, has extensive foci formation, whereas the FibC have no foci. A phase contrast micrograph of FibA cells in Figure 5 shows large foci and the inset is a foci being trypsinized. Figure 6 represents the morphology of the MF-2 cell line, with the transformed phenotype of the MF-T2 cell line in the inset.

DISCUSSION

Bovine mammary fibroblasts grew more vigorously than epithelial cells in primary culture (previous to cloning) and allowed for extensive cloning. Three clonal non-transfected fibroblast lines (3hUnfil, MF2, and FibC) and one epithelial line (FbE) display normal morphology and normal growth after at least 25 passages and could also be cryostored and thawed without significant cell death or cell crisis. None of these lines have a transformed phenotype. Several epithelial and fibroblast lines were successfully transfected. Oddly, all of the fibroblasts and none of the epithelial cell lines displayed a transformed phenotype. All parameters of epithelial transformation could not be assessed, since all epithelial transfectants entered crisis. Cell crisis was monitored by 1) increased population doubling times, 2) decreased substratum adherence, 3) appearance of numerous giant cells, 4) appearance of numerous mutlinucleated cells, 5) cytoplasmic vacuolation and 6) increased cellular cytoxicity as previously defined by others (Freshney, 1994).

Upon transfecting fibroblast cells with the Large-T-antigen, all fibroblasts tested showed variable degrees of a transformed phenotype, including the more aggressive FibA line. The MAC-T bovine mammary epithelial cell line has also been transfected with the SV-40 Large-T-antigen (Huynh et al., 1991). The MAC-T cell line is not transformed as the cells 1) do not form foci on plastic or in soft agar, 2) are substratum dependent and contact inhibited, 3) do not form tumors in nude mice (Huynh et al., 1991).

Researchers studying breast cancer have benefitted from numerous tumor cell lines for more than 25 years (Turkington, 1969; Dickson et al., 1989). Additionally, a few non-transformed lines representative of the human and murine mammary gland are available (Sizemore, 1979; Danielson et al., 1984; Gaffney, 1992). Although, no immortalized mammary fibroblast cell lines have been reported, several fibroblast cell populations from the human breast have been described (Cullen et al., 1991; Adam et al., 1994; Brouty-Boye et al., 1994). In this study, we introduce for the first time: 1) normal bovine fibroblast lines, 2) SV-40 Large-T-antigen transfected bovine fibroblast cell lines displaying a transformed phenotype, 3) an epithelial cell line FbE that has the potential for extended passage in culture (passages exist as high as 40), without displaying any signs of crisis. The FbE line may have become spontaneously immortalized, since nontransfected epithelial cells typically enter crisis after 5-25 passages (Huynh et al., 1991; Freshney, 1994). However, as demonstrated, none of the nontransfected lines have a transformed phenotype.

The MAC-T cell line itself, is a good in vitro model for bovine lactation and mammogenesis. However, expression of the Large-T-antigen has been shown to effect proliferation and differentiation of many cell types. For example independent studies by several laboratories have shown that the expression of the Large-T-antigen results in a loss of growth inhibition to TGF-β (Hosobuchi and Stampfer, 1989; Laiho et al., 1990; Pietenpol et al., 1990; Missero et al., 1991). The Large-T-antigen probably exerts this effect and its ability to permit long term culture or 'immortalization' of cells by binding p53 protein and hypophosphorylated retinoblastoma protein (pRB) (Weinberg, 1991). The hypophosphorylated state of pRB protein and wild-type p53 protein prevents progression of the cell cycle by holding cells in a middle or late G_1 phase (Weinberg, 1995). TGF-β and other growth inhibitors may function by retaining pRB in an underphosphorylated state (Laiho et al., 1990). Others have found wild-type p53 and hypophosphorylated pRB are important in normal cellular differentiation (Moles et al., 1991; Weinberg, 1995) and mammary epithelial cell differentiation (Geradts et al., 1994). Large-T-antigen expressing fibroblast (FibA, Fib B, MFT2, MFT3) and epithelial cells (MAC-T) and their nontransfected counterparts, fibroblast (3hUnfil, FibC and MF2) and epithelial (FbE) can now be easily compared and contrasted. Thus, Large-T-antigen modulation of growth or differentiation in MAC-T cells can be directly assessed with the cell lines described within.

In addition to cloning a nontransfected bovine mammary epithelial cell line, this report describes the establishment of 3 nontransfected and 4 SV-40 Large-T-antigen transfected

fibroblast cell lines. Fibroblasts and adipocytes represent the major cell types present in bovine mammary stroma. Additionally, fibroblasts, unlike adipocytes, lie in close apposition to stromal cells. Murine and human mammary studies have documented that many hormones, growth factors and growth inhibitors may exert their effects through fibroblast interactions (Sheffield, 1988; Haslam, 1988; Haslam and Counterman, 1991; Silberstein, 1992; Schrey et al., 1992; Cunha, 1994; Vollmer et al., 1995). Additionally, we have previously shown that estrogen does not directly effect bovine mammary epithelial cell (MAC-T) proliferation in vitro (Woodward et al., 1992). However, fibroblasts adjacent to epithelium in vivo proliferate in response to exogenous estrogen in prepubertal heifers (Woodward et al., 1993). Moreover, when bovine primary mammary fibroblast cells are treated with estrogen, their conditioned media stimulates the proliferation of MAC-T cells more than conditioned media from untreated fibroblasts (Woodward et al., 1992).

These bovine mammary fibroblast cell lines may prove useful in analyzing paracrine interactions in the mammary. Additionally, the establishment of both normal and SV-40 Large-T-antigen transfected lines of epithelial and fibroblast origin enable researchers to perform experiments with the immortalized lines and determine whether Large-T-antigen expression alters hormonal responsiveness. Most importantly, the effects of hormones/growth factors on bovine mammary epithelium can now be efficiently analyzed: 1) directly, 2) with or without exogenous extracellular matrices, 3) and in epithelial/fibroblast coculture models.

LITERATURE CITED

Adam L., M. Crepin, J. LeLong, E. Spanakis, and L. Israel. 1994. Selective interactions between mammary epithelial cells and fibroblasts in co-culture. Int. J. Cancer, 59:262-268.

Akers, R.M. 1985. Lactogenic hormones: binding sites, mammary growth, secretory cell differentiation, and milk biosynthesis in ruminants. J. Dairy Sci., 68:501-519.

Akers, R.M. 1990. Lactation physiology: a ruminant animal perspective. Protoplasma, 159:96-111.

Bertram, J.S. 1979. Modulation of cellular interactions between C3H/10T1/2 cells and their transformed counterparts by phosphodiesterase inhibitors. Cancer Res., 39:3502-3508.

Brouty-Boye, D., C. Mainguene, V. Magnien, L. Israel, and R. Baupain. 1994. Fibroblast-mediated differentiation in human breast carcinoma cells (MCF-7) grown as nodules in vitro. Int. J. Cancer, 56:731-735.

Cowie, A.T. 1974. Overview of the mammary gland. J. Invest. Dermat., 63:2-9.

Cullen, K.J., H.S. Smith, S. Hill, N. Rosen, and M.E. Lippman. 1991. Growth factor messenger RNA expression by human breast fibroblasts from benign and malignant lesions. Cancer Res., 51:4978-4985.

Cunha, G.R. 1994. Role of mesenchymal-epithelial interactions in normal and abnormal development of the mammary gland and prostate. Cancer, 74:1030-1044.

Danielson, K.G., C.J. Oborn, E.M. Durban, J.S. Butel, and D. Medina. 1984. Epithelial mouse mammary cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. Cell Biol., 81:3756-3760.

Dickson, R.B., E.W. Thompson, and M.E. Lippman. 1989. Hormones and breast cancer in vitro. Human Cell, 2:219-230.

Ethier, S.P. 1985. Primary culture and serial passage of normal and carcinogen treated rat mammary epithelial cells in vitro. J. Natl. Cancer Inst., 74:1307-1318.

Freshney, R.I. 1994. Specialized techniques. In: Culture of Animal Cells: A Manual of Basic Technique. R.I. Freshney, ed. Wiley-Liss Inc., New York, Vol. 3, pp. 319-444.

Gaffney, E.V. 1992. A cell line (HBL-100) established from human breast milk. Cell & Tissue Res., 227:563-568.

Geradts, J., S.X. Hu, C.E. Lincoln, W.F. Benedict, and H.J. Xu. 1994. Aberrant retinoblastoma gene expression in routinely processed, archival tumor tissues determined by three different anti-RB antibodies. Int. J. Cancer, 58:161-167.

Haslam, S.Z. 1988. Cell to cell interactions and normal mammary gland function. J. Dairy Sci., 71:2843-2854.

Haslam, S.Z. and L.J. Counterman. 1991. Mammary stroma modulates hormonal responsiveness of mammary epithelium in vivo in the mouse. Endocrinology, 129:2017-2023.

Hosobuchi, M., and M.R. Stampfer. 1989. Effects of transforming growth factor-β on growth of human mammary epithelial cells in culture. In Vitro Cell. Dev. Biol., 25:705-713.

Huynh, H.T., G. Robitaille, and J.D. Turner. 1991. Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. Exp. Cell Res., 197:191-199.

Imai, Y., C.K.H. Leung, H.G. Friessen, and R.P.C. Shiu. 1982. Epidermal growth factor receptors and effect of epidermal growth factor on growth of human breast cancer cells in long-term tissue culture. Cancer Res., 42:4394-4398.

Laiho, M., J.A. DeCaprio, J.W. Ludlow, D.M. Livingston, and J. Massague. 1990. Growth inhibition by TGF-β linked to suppression of retinoblastoma protein phosphorylation. Cell, 62:175-185.

Leone, A., U. Flatow, C.R. King, M.A. Sandeen, I.M. Margulies, L.A. Liotta, P.S. Steeg. 1991. Reduced tumor incidence, metastatic potential and cytokine responsiveness of nm23-transfected melanoma cells. Cell, 65:25-35.

McGrath, M.F. 1987. A novel system for mammary epithelial cell culture. J. Dairy Sci., 70:1967-1980.

Missero, C., E. Filvaroff, and G. Paolo Dotto. 1991. Induction of transforming growth factor β1 resistance by the E1A oncogene requires binding to a specific set of cellular proteins. Biochemistry, 88:3489-3493.

Moles, J.P., J.T. Schiller, A. Tesniere, I.M. Leigh, J.J. Guilhou, and N. Basset-Seguin. 1994. Analysis of HPV16 E6 and mutant p53-transfected keratinocytes in reconstituted epidermis suggests that wild-type p53 inhibits cytokeratin 19 expression. J. Cell Sci., 107:435-441.

Pietenpol, J.A., J.T. Holt, R.W. Stein, and H.L. Moses. 1990. Transforming growth factor-β1 suppression of c-myc gene transcription: role in inhibition of keratinocyte proliferation. Proc. Natl. Acad. Sci., 87:3758-3762.

Sakthivel, R., M. Hamdan, J. Yang, R.C. Guzman, and S. Nandi. 1993. Effect of TGF-alpha on growth of normal human breast epithelial cells in serum-free primary culture using 3-dimensional collagen gels. Cell Biol. Int., 17:387-397.

Schrey, M.P., K.V. Patel, and N. Tezapsisis. 1992. Bombesin and glucocorticoids stimulate human breast cancer cells to produce endothelin, a paracrine mitogen for breast stromal cells. Cancer Res., 52:1786-1790.

Sheffield, L.G. 1988. Organization and growth of mammary epithelia in the mammary gland fat pad. J. Dairy Sci., 71:2855-2874.

Silberstein, G.B., K.C. Flanders, A.B. Roberts, and C.W. Daniel. 1992. Regulation of mammary morphogenesis: evidence for extracellular matrix-mediating inhibition of ductal budding by transforming growth factor-beta-1. Devel. Biol., 152:354-362.

Sizemore, S.R. 1979. Hormonal responsiveness of NMuMG, a mammary epithelial cell line. Dairy Sci., 41:28.

Taketani, Y., and T. Oka. 1983. Epidermal growth factor stimulates cell proliferation and inhibits functional differentiation of mouse mammary epithelial cells in culture. Endocrinology, 113:871-876.

Tonelli, Q.J., and S. Sorof. 1980. Epidermal growth factor requirement for development of cultured mammary gland. Nature, 285:250-252.

Tucker, H.A. 1981. Physiological control of mammary growth, lactogenesis, and lactation. J. Dairy Sci., 64:1403-1421.

Turkington, R.W. 1969. Hormonal regulation of cell proliferation in breast cancer cells in vitro. New York St. J. Med., 69:2649-2655.

Vollmer, G., N. Ellerbrake, A. Hopert, R. Knauthe, W. Wunsche, and R. Knuppen. 1995. Extracellular matrix induces hormonal responsiveness and differentiation in RUCA-I rat endometrial adenocarcinoma cells. J. Steroid Bioch., 52:259-269.

Vonderhaar, B.K. 1987. Local effects of EGF, α-TGF, and EGF-like growth factors on lobuloalveolar development of the mouse mammary gland in vivo. J. Cell. Physiol., 132:581-584.

Weinberg, R.A. 1991. Tumor suppressor genes. Science, 254:1138-1146.

Weinberg, R.A. 1995. The retinoblastoma protein and cell cycle control. Cell, 81:323-330.

Woodward, T.L., R.M. Akers, T.B. McFadden, H.T. Huynh, and J.D. Turner. 1992. Estrogen mediated bovine mammary epithelial cell proliferation: evidence of indirect action. J. Dairy Sci., 75:P242. (Abstract).

Woodward, T.L., W.E. Beal, and R.M. Akers. 1993. Cell interactions in initiation of mammary epithelial proliferation by oestradiol and progesterone in prepubertal heifers. J. Endocr., 136:149-157.

Woodward, T.L., N. DuMont, M. O'Connor-McCourt, J.D. Turner, and A. Philip. 1995. Characterization of transforming growth factor-β growth regulatory effects and receptors on bovine mammary cells. J. Cell. Phys., 165:339-348.

Figure 1: A phase contrast micrograph of MF2 normal fibroblast cells cultured in 10% FBS. Cells are confluent and have formed the typical swirl pattern characteristic of normal fibroblasts.

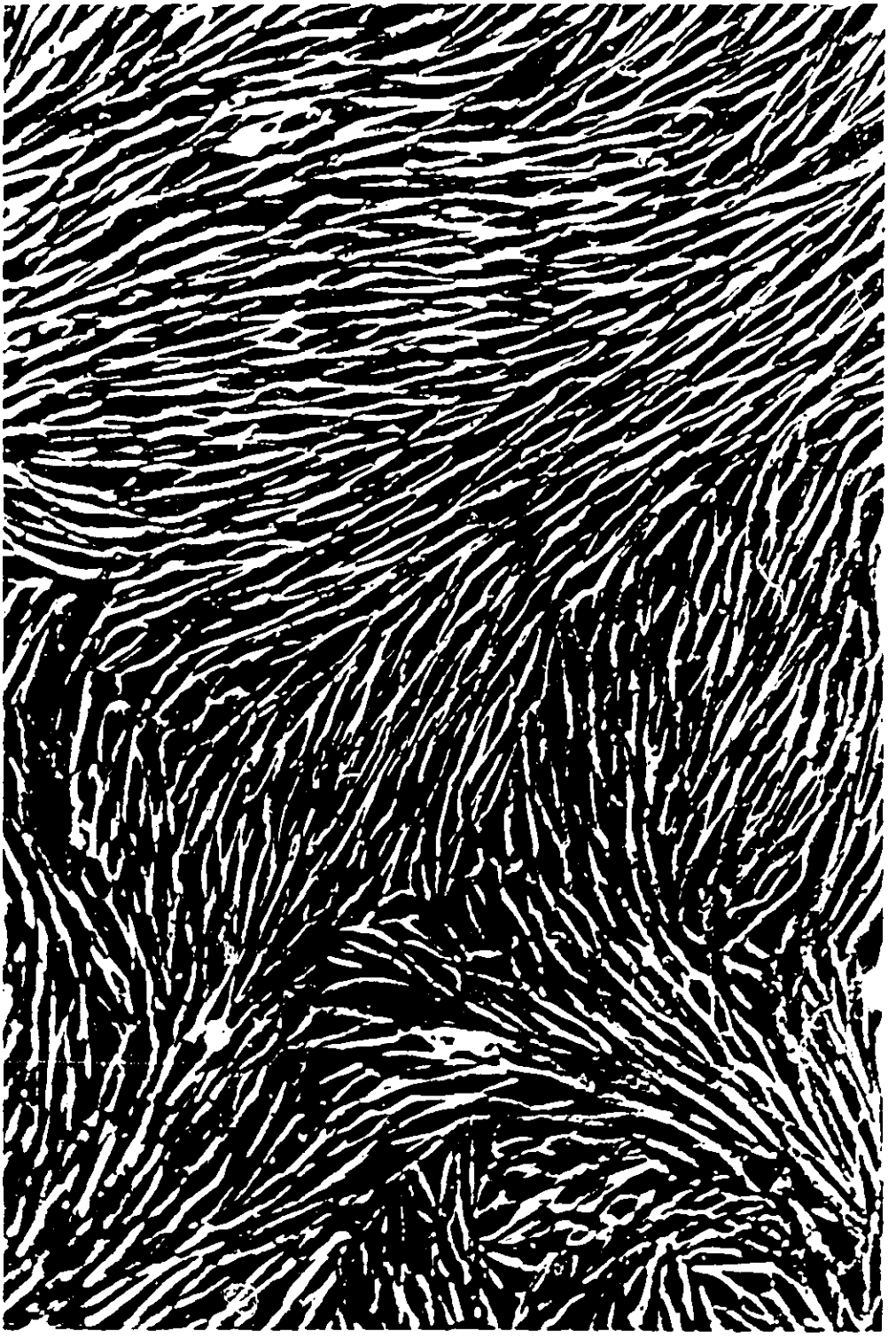


Figure 2: Immunoflorescent micrographs of FibC fibroblast cells cocultured with MAC-T epithelial cells. Panel A is a micrograph of all 3 cells: 2 MAC-T cells and 1 FibC cell (Note: punctate staining between MAC-T cells is Connexin43 protein). Panel B is stained for vimentin. The FibC cell stained positive, whereas no staining was observed in the MAC-T cells. Panel C is an overlay of A and B.

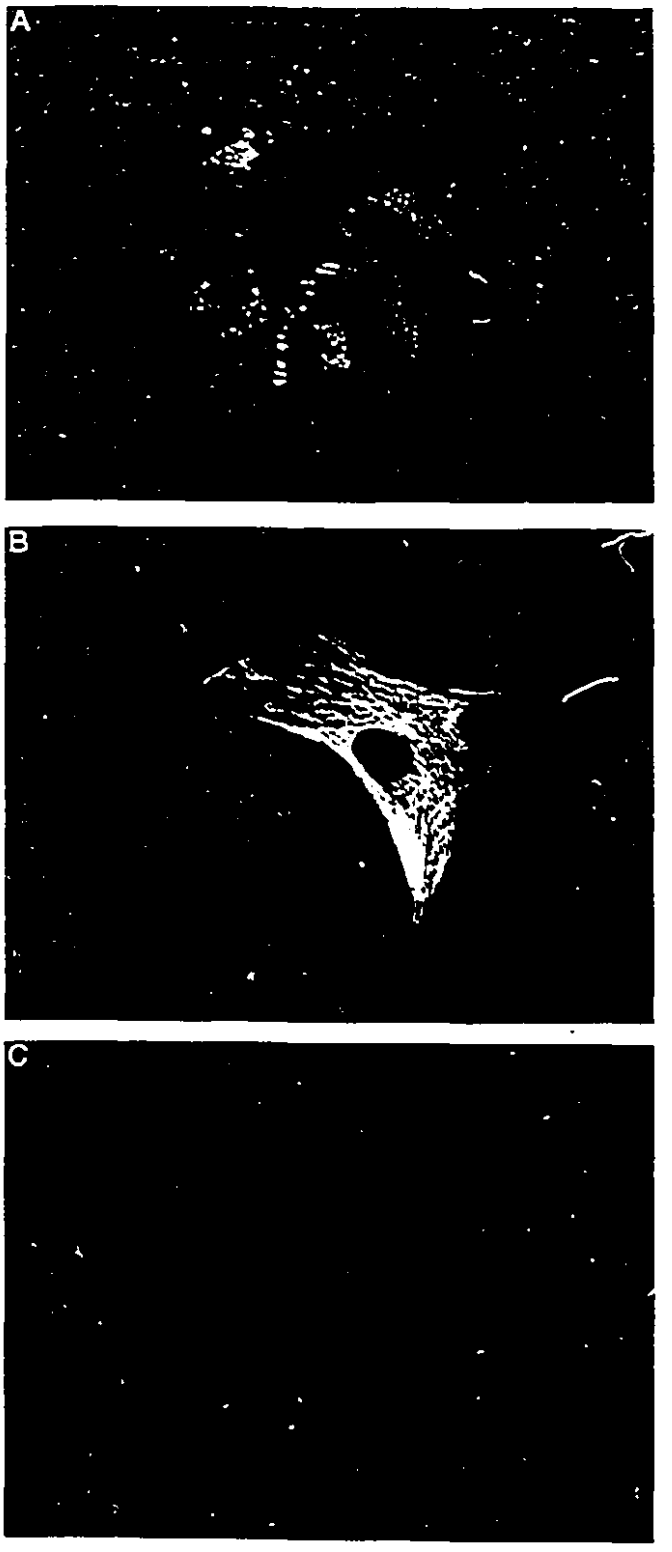

A
B
C

Figure 3: Phase contrast micrograph of FbE epithelial cells. Micrograph was taken at the edge of a cluster. Note homogenous cuboidal morphology, typical of mammary epithelium.

Figure 4: A photograph of Giemsa stained confluent cultures of 2 cell types: FibC (top dish) and FibA (bottom dish). Fib A cells were transfected with the SV-40 Large-T-antigen and display a transformed phenotype. Dark spots on the plate represent formation of foci. Note: nontransfected FibC cells have no foci stained, while FibA cells have extensive foci formation.

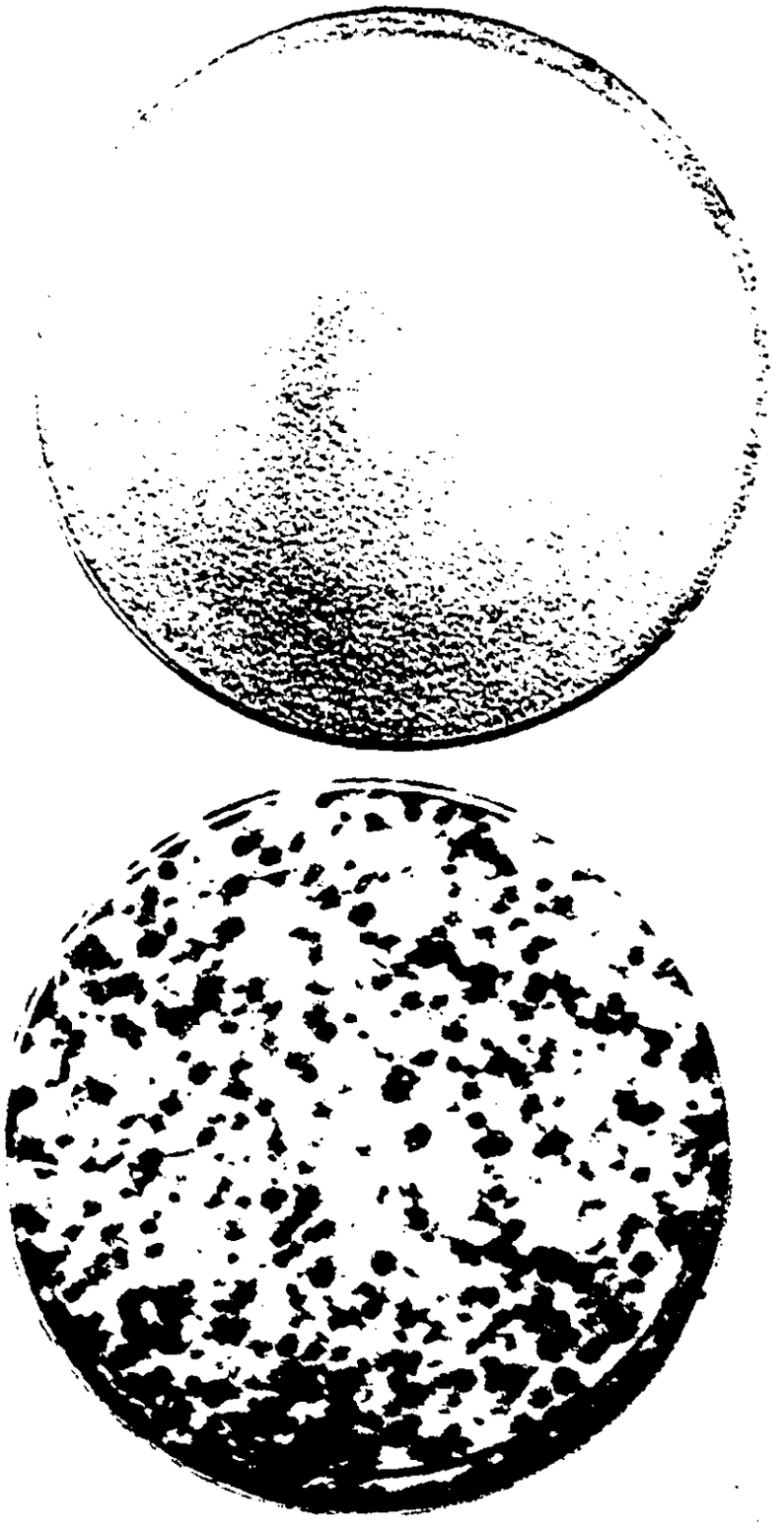


Figure 5: Phase contrast micrographs of foci formed by FibA cells. Note the lack of cells outside the foci area and the arrangement of fibroblasts at the periphery of the foci. The inset is a micrograph of the same culture of FibA cells being trypsinized. This demonstrates that these clusters are foci, composed of layers of fibroblasts typical of transformed cells.

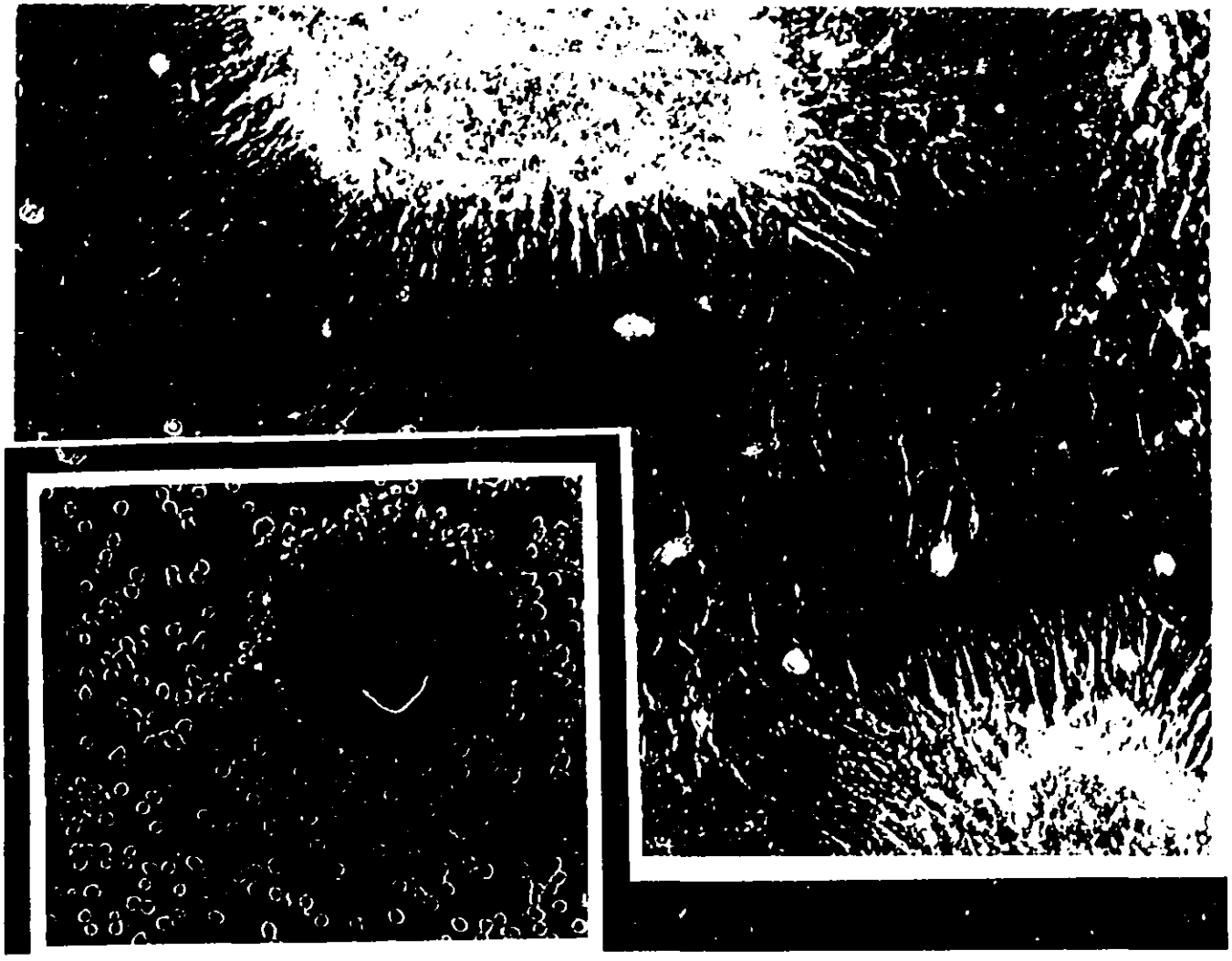


Figure 6: Phase contrast micrograph of MF-T2 transformed fibroblasts. These fibroblasts are MF-2 cells transfected with the SV-40 Large-T-antigen. Compare to Figure 1, MF-2 cells. MF-T2 cells lack contact inhibition and cells overlap one another. Swirl patterns formed by confluent MF-2 fibroblasts are lost in MF-T2 fibroblasts.

SUMMARY 1

The major objective, described in this first chapter, was the creation of a series of bovine mammary cell lines. Realization of this objective allows for extensive in vitro examination of bovine mammary cell proliferation, its control, and interactions among homologous and heterologous cells. This chapter has described the isolation, cloning, characterization and transfection of bovine mammary fibroblasts and epithelial cells. Previous to this study, only a single cell line from the bovine mammary existed, the MAC-T epithelial cell line.

A cell line isolated from a single animal and immortalized by constitutive expression of the SV-40 Large-T-antigen, leaves many questions unanswered when modeling the bovine mammary gland. 1) Does this line accurately represent mammary epithelum of the bovine mammary gland?, 2) Do cells isolated from animals in different reproductive states in the mammary vary in response?, 3) Does expression of the SV-40 Large-T-antigen alter responsiveness of these cells to hormones and growth factors?, 4) Are other major cell types affected by the hormone/growth factor being examined? By creating several epithelial and fibroblast cell lines from three different animals representing two reproductive states (three if the MAC-T cell line is included), by creating fibroblast lines that have not been transfected with the SV-40 Large-T-antigen and others that do express the Large-T-antigen, this chapter will allow these questions to be addressed in subsequent chapters. Finally, this chapter has demonstrated for the first time that

mammary fibroblasts are easily transformed by expression of the SV-40 Large-T-antigen, but mammary epithelial cells appear to be resistant to this transformation. Cells, including the FbE, FibC, MF2, PrE, 3hUnfil, MFT2 and MFT3, created in this chapter are used in studies throughout this thesis.

CHAPTER 2

Lack of mitogenic response to EGF, pituitary and ovarian hormones in bovine mammary epithelial cells.

Endocrine (1994), 2, 529–535

© Macmillan Press Ltd. 1994

Lack of mitogenic response to EGF, pituitary and ovarian hormones in bovine mammary epithelial cells

T.L. Woodward[1], R.M. Akers[2] & J.D. Turner[1]

[1]*Department of Animal Science, McGill University, Montreal, Canada H9X 3V9;* [2]*Department of Dairy Science, Virginia Polytechnic Institute & State University, Blacksburg, Virginia 24061, USA*

The direct hormonal regulation of bovine mammary epithelial cell proliferation was examined using the MAC-T cell line as an *in vitro* model. Unlike human and murine mammary epithelial cells, MAC-T cells show little or no growth response to epidermal growth factor (murine (mEGF) or human (hEGF)) and only modest proliferation after treatment with TGF-α. Similarly, primary mammary epithelial cells isolated from a pregnant non-lactating Holstein cow showed no response to mEGF or hEGF ($P > 0.1$). The vast differences between species were realized when MAC-T cells and a murine cell line (NMuMG) were cultured concurrently under identical conditions. The murine cell lines' proliferative responses were 22 times greater than MAC-T cells when cultured in hEGF or mEGF spiked media. Additionally, several hormones (bGH, bPRL, E, P or E + P) which can elicit dramatic mammogenic activity *in vivo*, were completely inactive. Similar to murine and human mammary trials, IGF-I was a potent mitogen (maximal stimulation at 10 ng/ml, $P < 0.01$), while insulin was most effective at higher concentrations (1000 ng/ml or greater, $P < 0.05$). The diminutive response to EGF may represent a fundamental difference between the endocrine regulation of mammogenesis in the bovine when compared to the mouse or human.

Introduction

Milk production is the summation of the secretory activity of mammary epithelial cells over time. Total milk production is thus affected by (1) the length of lactation and the non-lactating period (2) the combined secretory capacity of each mammary epithelial cell, and (3) the total number of actively secreting epithelial cells. Efforts to increase milk production by increasing cell number depends on understanding the response of epithelial cells to mitogens. These mitogens affect mammary epithelial cells directly or through interactions with other cells to induce mammary epithelial cell proliferation.

Mammary epithelial cell lines of murine or human origin, both normal and neoplastic have provided valuable insight into the specific effects of many hormones, growth factors and inhibitors (Soule *et al.*, 1973; Sizemore, 1979; Danielson *et al.*, 1984). However, this wealth of information must be prudently applied to ruminant dairy animals as substantial differences exist between the ruminant and rodent or human mammary gland. Previous reports have identified differences in the development of these glands, hormones involved in: mammaogenesis, lactation and its induction, and the events of involution (Cowie *et al.*, 1980; Wiens *et al.*, 1992), substantial variations in milk composition (Jenness, 1985, 1988; Mepham, 1987) as well as marked differences in gland gross morphology and cellular composition (Schmidt, 1971). We have previously reported species variations in response to estrogen and TGF-β on mammary growth and development (Woodward *et al.*, 1992, 1993a,b) as well as cellular and gross anatomical differences (Akers, 1990).

Research regarding hormonal control of mammogenesis and lactogenesis in dairy cattle has been dominated by *in vivo* studies, explant and primary cell cultures (Tucker, 1981; Dils & Forsyth, 1981; McGrath, 1987; Akers, 1990). Only recently has a functional bovine mammary epithelial cell line become available as an *in vitro* model of lactation. The establishment of bovine mammary epithelial cell lines by our laboratory, denoted MAC-T (Mammary Alveolar Cell-large T antigen), has provided an appropriate experimental model (Huynh *et al.*, 1991). These clonal cells grow well in culture and are responsive to extracellular matrix signals and prolactin for the induction of differentiation. In this state, MAC-T cells synthesize and secrete α and β casein, lactose and lipid (Huynh *et al.*, 1991). With regard to regulation of cell growth, MAC-T cells are reported to have high affinity receptors for IGF-I and marked mitogenic responses to IGF-I (Zhao *et al.*, 1992) and that certain whey proteins, namely α-lactalbumin and lactoferrin, are known to inhibit cell proliferation (Rejman *et al.*, 1992).

The MAC-T cell line represents the most reductionist biological model for the evaluation of direct hormonal effects on the bovine mammary gland; a single clonal cell line, in defined media into which hormones are added. In contrast, explant culture retains the tissue architecture and cellular diversity of the mammary gland, and therefore presents a broader repertoire of hormonal responsiveness, for example paracrine interactions. In this report we evaluated the direct effects of hormones or growth factors previously determined to be mammogenic or lactogenic in the bovine *in vivo* or in murine and human studies. By first describing the direct hormonal effects on bovine mammary epithelial cells, we have a foundation from which we can begin to unravel the more complex regulation of bovine mammogenesis at the tissue and organismal level.

Correspondence: J.D. Turner, Ph.D.
Received 13 January 1994; accepted 2 March 1994

Results

Clonal bovine mammary epithelial cells responded directly to physiological concentrations of hormones/growth factors including: insulin, IGF-I, IGF-II and preparations of bovine serum albumin (BSA). Most hormones known to be mammogenic *in vivo*, including: growth hormone, prolactin, EGF, estrogen and progesterone, did not exert a direct biological effect on the proliferation of bovine mammary epithelial cells.

Neither mEGF nor hEGF was mitogenic for bovine mammary epithelial cells at concentrations up to 250 ng/ml (Figure 1 (DNA), Table 1 (3H-thymidine incorporation)). As a positive control, 5% FBS amplified tritiated thymidine incorporation by nearly 2000% (Table 1). Both EGFs often gave rise to minimal growth increases, though, results were never significantly different than control in any of eight trials with as many as 18 replicates per treatment. Further, mEGF in combination with FBS and/or hIGF-I did not affect MAC-T cell proliferation whether measured by DNA accumulation (Figure 1B) or thymidine incorporation (Table 2). To determine whether the clonal line (MAC-T) differed because of its adaptation to culture or since it was isolated from a lactating animal, primary bovine mammary epithelial cells from pregnant non-lactating Holstein cows were tested. These cells also failed to elicit significant proliferative responses when cultured in DMEM containing hEGF or mEGF (Figure 2). To confirm that our EGF preparations were active, MAC-T cells and NMuMG cells (a murine mammary epithelial cell line known to respond to EGF) were directly compared. MAC-T cell proliferation showed minimal non-significant increases (12%) while NMuMG cells increased thymidine incorporation more than 250% (Figure 3, $P<0.01$). Note that both MAC-T cells and NMuMG cells were very responsive to even low levels of FBS (0.75%) as well as higher levels (5%).

Insulin-like growth factor -I and -II were found to be potent mitogens for MAC-T cells. DNA accumulation was stimulated by 0.1 ng/ml hIGF-I in serum free media and 10 ng/ml hIGF-I in the presence of FBS (Figure 4A, $P<0.05$). [^{3}H]thymidine incorporation results exhibited maximal mitogenic activity for IGF-I and IGF-II at 10 ng/ml. Des (1-3) IGF-I and Long R^3 IGF-I were slightly more effective than IGF-I at lower concentrations ($\leqslant$20 ng/ml), but were equally mitogenic at higher concentrations ($\geqslant$40 ng/ml; data not shown). Insulin at 10 ng/ml was also uniformly mitogenic in both the DNA accumulation and thymidine incorporation assays (Figure 4B, Table 2, $P<0.05$). The addition of 1% FBS did not alter the effective dose of insulin, 10 ng/ml, but it did increase the magnitude of DNA accumulation at this concentration of insulin (Figure 4B).

Bovine growth hormone (bGH) and bovine prolactin (bPRL) were not mitogenic to bovine mammary epithelial cells when tested at concentrations ranging from 10 to 1000 ng/ml. The presence of 1% FBS was not permissive for either bGH or bPRL. Furthermore, the addition of FBS at levels of 2, 4, or 8% to media containing GH or PRL did not cause a proliferative response above that caused by the serum alone (data not shown). No synergistic action between insulin and bGH or bPRL was found concerning thymidine incorporation (Table 2).

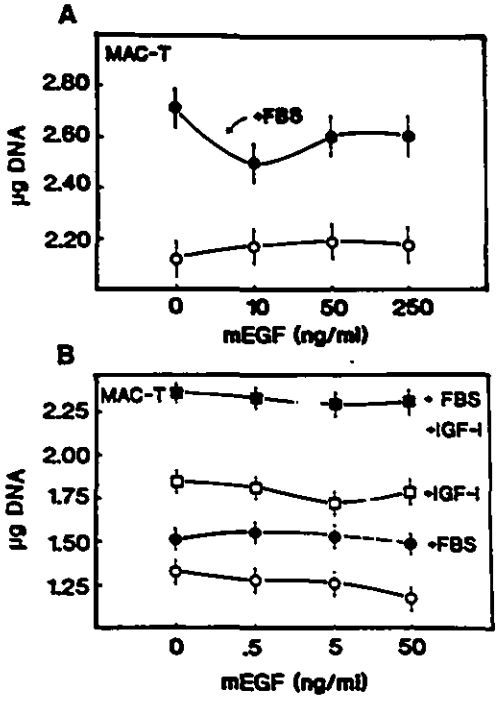


Figure 1 Effect of mEGF, alone or in combination with FBS and/or IGF-I on the growth of MAC-T cells. The effect of mEGF alone (○), in combination with 1% FBS (●); 10 ng/ml hIGF-I (□) or 1% FBS and 10 ng/ml IGF-I (■)

Table 1 Effect of concentration of hEGF and mEGF on tritiated thymidine incorporation into serum starved MAC-T cells

Conc (ng/ml)	*hEGF*	*mEGF*
Control (DMEM)	3.2±0.5	3.2±0.5
0.1	2.5±0.1	3.4±0.1
0.5	3.2±0.1	3.4±0.2
1.0	2.6±0.1	3.2±0.1
5.0	3.2±0.3	4.0±0.4
10.0	2.6±0.1	3.0±0.2
50.0	2.7±0.1	3.6±0.1
100.0	2.7±0.2	2.7±0.1
250.0	3.5±0.2	3.6±0.3
FBS 5.0%	62.4±4.9*	62.4±4.9*

Data presented as mean±SE ($\times 10^{-3}$ c.p.m. $n=9$).
*Indicates value is greater than control ($P\leqslant 0.05$)

Table 2 Effects of hIGF-I, mEGF, bovine insulin, bGH and bPRL on tritiated thymidine incorporation into serum starved MAC-T cells*

Factor(s)	*Conc**	*c.p.m./well*
Control (DMEM)	–	84±10
bI	10 ng	147±16†
mEGF	10 ng	83±8
hIGF-I	10 ng	227±9†
hIGF-I + mEGF	10 + 10 ng	226±13†
bGH	1 µg	73±8
bPRl	1 µg	64±16
bI + hIGF-I	10 + 10 ng	231±11†
bI + mEGF	10 + 10 ng	157±12†
bI + hIGF-I + mEGF	10 + 10 + 10 ng	219±3†
bI + bPRl	10 ng + 1 µg	134±17†
bI + bGH	10 ng + 1 µg	120±10†

*All concentrations per ml, data given as mean±SD $\times 10^{-3}$. ($n=4$).
†Indicates value is greater than control ($P\leqslant 0.05$)

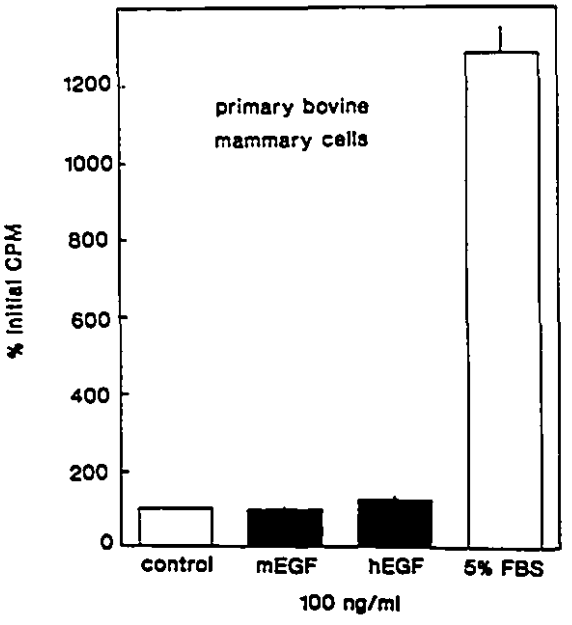


Figure 2 The figure shows the effect of mEGF and hEGF on primary bovine mammary epithelium from pregnant non-lactating cows. CPM were standardized to initial CPM and % initial CPM is displayed

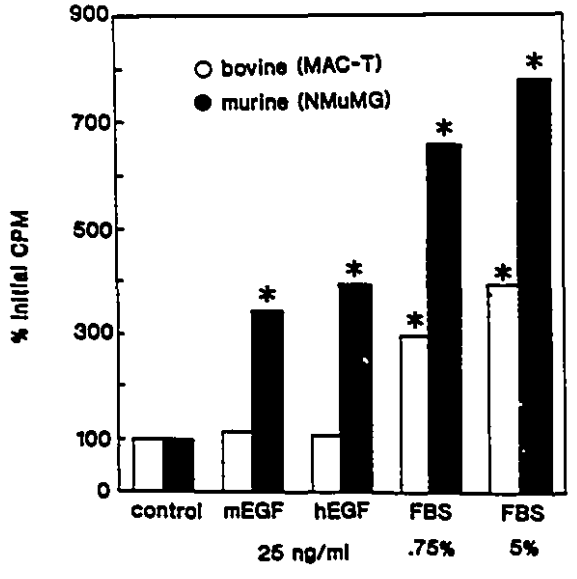


Figure 3 Comparison between a murine line (NMuMG) and a bovine line (MAC-T) to EGF standardized to the same control are shown. (*) Indicates a significant difference ($P<0.05$) from the control

Estradiol 17-β concentrations from 100 pg/ml to 100 ng/ml or progesterone concentrations from 1 ng/ml to 1000 ng/ml did not affect MAC-T cell proliferation, either in serum-free media or in the presence of 1% FBS. When estradiol 17-β and progesterone were added together over several logs of concentrations, they were also without effect on MAC-T cell growth. Neither were these steroids able to stimualte proliferation in phenol red free media. Additionally, estradiol 17-β was unable to alter proliferative response of bovine mammary epithelial cells from pregnant non-lactating Holstein cows (data not shown).

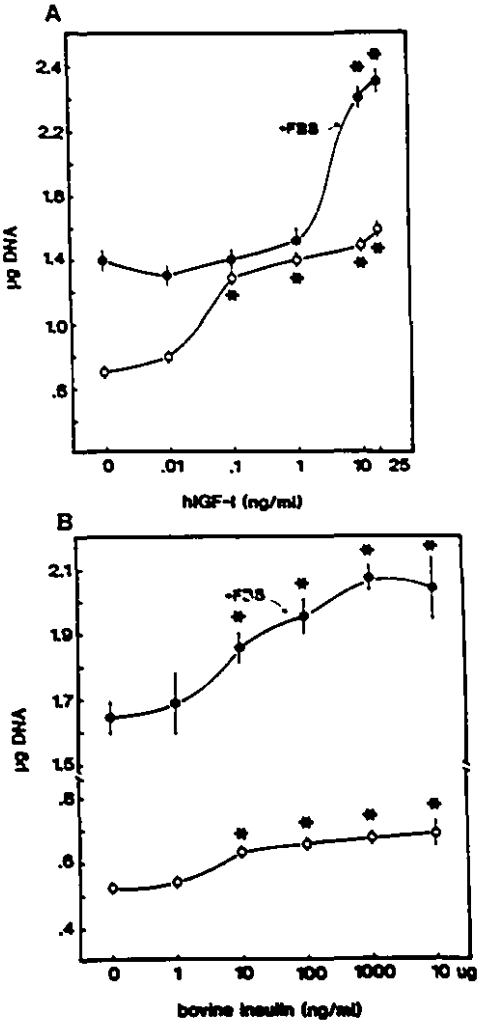


Figure 4 Effect of hIGF-I (A) and bovine insulin (B) on the growth of MAC-T cells cultured in serum free media (○) or media supplemented with 1% FBS (●). (*) Indicates a significant difference ($P<0.05$) from the control (no hormone addition)

Bovine serum albumin preparations do not induce an accumulation of DNA when added to serum free media in the range from 10 to 640 μg/ml (Figure 5A). However, further increases, from 2–16 mg/ml in BSA concentration caused large increases in DNA accumulation ($P<0.01$). When three stocks of BSA were compared, each stock increased MAC-T cell proliferation, but effective concentrations varied considerably between stocks (Figure 5B). Fatty acid-free BSA was the least effective in increasing MAC-T cell DNA accumulation ($P<0.05$).

Discussion

The direct interaction of endocrine hormones with mammary epithelial cells of domestic dairy animals is poorly understood. Considerable work with explant culture and less so with primary mammary cells has implicated many hormones, growth factors and inhibitors in the regulation of proliferation and differentiation of secretory epithelial cells (Tucker, 1981; Akers, 1985, 1990). This report defines which of the

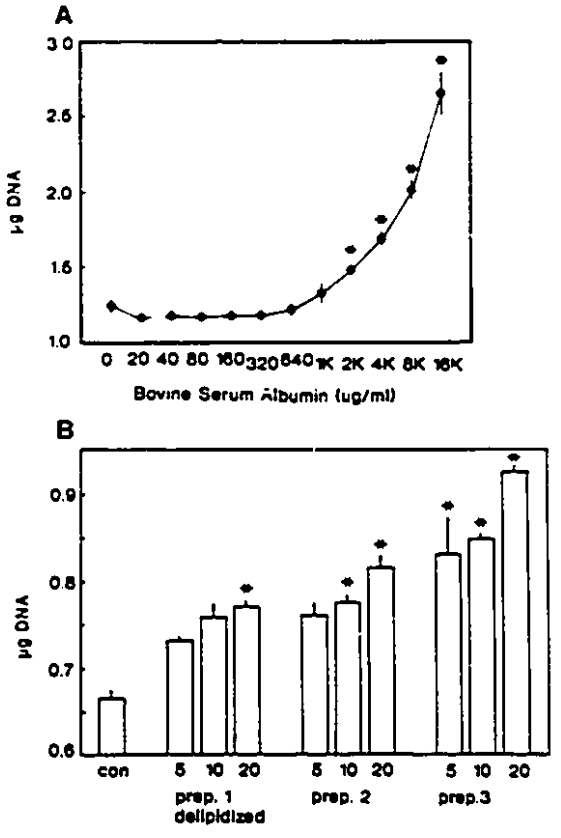


Figure 5 Bovine serum albumin (BSA) stimulation of MAC-T cell proliferation (A). Serum-free media (DMEM) was supplemented with the level of BSA indicated and DNA accumulation measured. (B) Compares different preparations of BSA including a fatty acid-free stock. (*) Indicates a significant difference ($P<0.05$) from no BSA treated cells

major mammogenic or lactogenic hormones directly affect bovine mammary epithelial cell growth. This reductionist view permits the experimental evaluation of the most basic elements of regulation, one cell-one hormone. The bovine mammary epithelial cell line MAC-T has provided the biological context *in vitro* and is not influenced by the presence of contaminating cell types nor by mammary tissue architecture. More complete regulatory motifs will emerge as other cell types are added and the effects of substratum are evaluated in this system.

Perhaps the most striking finding of these trials was the paucity or lack of EGF induced growth of bovine mammary epithelial cells. Regardless of whether the EGF was human or murine, from Sigma or UBI, tested on an immortal clonal line or a primary line, or whether the cells were from a pregnant non-lactating or lactating non-pregnant animal little to no effect was elicited. Since non-clonal early passage epithelial cells (less than five passages) confirmed results obtained with the MAC-T line, adaptation to culture conditions is unlikely to account for lack of response. To confirm our EGF preparations were active, NMuMG cells (a murine line known to respond to EGF) were compared to MAC-T cells; the murine line increased thymidine incorporation more than 260% over NMuMG cells not stimulated with EGF, some 22 times greater response than MAC-T cells (Figure 3A). Additionally, both hEGF and mEGF were comparatively effective in NMuMG cells. Data concerning EGF's effectiveness in the ruminant mammary is somewhat divided. Sheffield & Yuh (1988) found neither sialoadenectomy nor sialoadenectomy + EGF altered growth of bovine mammary tissue xenografted into cleared fat pads of athymic nude mice. Likewise, Gow & Moore (1992) found EGF decreased milk yield in lactating ewes and suggest that EGF may act as a mitotic inhibitor in the mammary gland of sheep. Several papers concerning a bovine mammary epithelial cell line (PS-BME) and its acute responsiveness to EGF must be reexamined (Gibson *et al.*, 1991, 1992), since these lines were later shown to be murine in origin (Baumrucker, personal communication) which ourselves and others have proven to be very responsive to EGF. Perhaps, most ruminant studies have found EGF to be a slight mitogen in the mammary gland, but often the criteria to achieve mitogenic responses are extraneous (Furumura *et al.*, 1990; Tou *et al.*, 1990) with modest effects.

These retarded responses in the bovine to a potent growth factor in the human and mouse should, perhaps, not be unexpected. First, in both murine and human milk, EGF is the major growth factor secreted (Shing & Klagsburn, 1984). In the ruminant no true EGF has been identified and reports of EGF-like material in the milk and colostrum vary from low to none (Shing & Klagsburn, 1984; Gow & Moore, 1992). However, recently a bovine TGF-α was identified in tissue from pregnant cows after PCR amplification (Zurfluh *et al.*, 1990). TGF-α, on the other hand, is able to induce small but significant increases in proliferation of a MAC-T-cell subclone (Akers, unpublished observation). This is in agreement that TGF-α is generally a more potent mitogen than EGF in the bovine mammary (Tou *et al.*, 1990; Zurfluh *et al.*, 1990).

One explanation for the lack of EGF response may be the relatively low conservation of sequence identity: IGF-I, TGF-α, insulin and TGF-β1, β2, β3 all share 88–100% sequence identity between bovine, human and mouse polypeptides (Altschul *et al.*, 1990; Maier *et al.*, 1991). However, EGF only shares 68% sequence identity between mouse and human with no reports of this growth factor in the bovine. In contrast, several laboratories have reported the presence of EGF/TGF-α receptors or receptor mRNA in the bovine mammary gland (Spritzer & Grosse, 1987; Glimm *et al.*, 1992). The significant (though modest) increases in MAC-T cell proliferation in response to TGF-α indicate the MAC-T cells also have TGF-α/EGF-like receptors, though binding kinetics, receptor number and regulation thereof as well as post receptor events have yet to be examined. We suggest either mEGF and hEGF are dissimilar to a potential bovine EGF such that limiting receptor binding and/or subsequent post receptor action would occur or EGF has no central role in bovine mammogenesis.

Mitogens which directly affect MAC-T cell proliferation include insulin, IGF-I and IGF-II, while BSA preparations have also been shown to enhance proliferation. The largest response of bovine insulin occurred at pharmacological levels, 1–10 µg/ml (Figure 4B). At these very high levels insulin can interact with the IGF-I receptors (Massague & Czech, 1982; Zhao *et al.*, 1992). In support of this concept is the lack of an

additive effect when insulin was added in combination with IGF-I (Table 2) as has previously been reported by other laboratories (Baumrucker & Stemberger, 1989). IGF-I in serum free media stimulated DNA accumulation at 0.1 ng/ml which is a level consistent with the IGF-I receptor binding kinetics previously reported for MAC-T cells (Zhao *et al.*, 1992). Levels of 10–25 ng/ml IGF-I appeared to have a synergistic action with other factors in the FBS. These factors were not insulin or EGF (Table 2). A separate study in our laboratory demonstrated that MAC-T cells do secrete low levels of IGFBP-3 and moderate levels of IGFBP-2 (Romagnolo *et al.*, 1994), so to determine whether these binding proteins alter IGF-I responsiveness, two analogs of IGF-I (des(1-3)IGF-I and Long R^3 IGF-I) that exhibit very low binding to IGFBPs were compared to IGF-I. The IGF-I analogs were slightly more effective at lower concentrations (1–20 ng/ml) than recombinant IGF-I, whereas concentrations of 40 ng/ml or higher were equally mitogenic, as tested by thymidine incorporation. Proliferative responses to IGF-I, -II and insulin by the MAC-T-cells are similar to results obtained in both the mouse and human mammary glands (Stockdale *et al.*, 1966; Imagawa *et al.*, 1986; Underwood *et al.*, 1986).

Reports of direct growth hormone action on bovine mammary epithelium are lacking, though GH receptor mRNA has been reported in the bovine mammary gland (Glimm *et al.*, 1990). This taken with the enormous research interest in bovine growth hormone, since substantial increases in milk production following exogenous bGH administration were demonstrated in dairy cattle (Bauman *et al.*, 1985), warranted our testing of the hormone *in vitro*. Indeed, as expected GH failed to alter cellular proliferation. *In vivo*, GH induces synthesis of IGF-I (much from hepatic stores) and release into the circulation, hence the GH/IGF-I endocrine axis is implicated in increased milk production (Davis *et al.*, 1987; Winder *et al.*, 1989). Consistent with this concept is the presence of high affinity receptors for IGF-I on mammary cells (Zhao *et al.*, 1992) and the absence of GH receptors on bovine mammary cells (Akers, 1985; Keys & Dijione, 1988; McFadden *et al.*, 1990). The absence of any direct bGH effects and the strongly mitogenic nature of IGF-I on mammary epithelial cells, supports the concept of an indirect action of GH effects *in vivo*.

Prolactin, a fundamental lactogen in mammals, is less understood when considering its effectiveness in eliciting mammary gland growth. On one hand, authors have found PRL to have mammogenic properties in both ruminants (Vandeputte-Van *et al.*, 1976) and mice (Topper & Freeman, 1980; Nagasawa *et al.*, 1986). Whereas, others have found no correlation between PRL and mammary growth in ruminants *in vivo* (Sejrsen *et al.*, 1983) and *in vitro* (Skarda *et al.*, 1977). Data from our laboratory and others have demonstrated that the MAC-T cells do respond lactogenically to PRL (Huynh *et al.*, 1991; Romagnolo & Akers, unpublished data); however, no proliferative response was evoked when MAC-T cells were cultured in PRL (over several logs of concentrations) in varying FBS concentrations. Since several reports have implicated PRL to work indirectly with other hormones and we did not test PRL's effect with GH, corticosteroids or ovarian steroids, a collaborative role of PRL directly inducing mammary epithelial cell proliferation should not be discounted.

Like GH, ovarian steroids have been known to stimulate mammary growth for some time (Franke & Rosenbloom, 1915). Indeed, plasma estrogen concentrations increase during the latter stages of pregnancy, and peak near parturition, directly coinciding with mammary growth. Estrogen alone or in combination with progesterone stimulates growth of the mammary gland and/or mammary epithelial cells in humans (Baron, 1958), mice (Haslam, 1988), goats (Cowie *et al.*, 1952) and cattle (Tucker, 1985; Woodward *et al.*, 1993a). However, *in vitro* studies with murine and human cells support an indirect effect of these steroids on mammary cells (Shyamala & Ferenczy, 1984; Richards *et al.*, 1988). The present study suggests that estrogen and progesterone also affect bovine mammary epithelial cells indirectly. MAC-T cells were not stimulated by estrogen or progesterone alone or in combination, with or without serum.

Recently, many researchers have substituted serum with BSA to eliminate unknown proteins and fatty acids when testing potential mitogens *in vitro*. BSA found in milk, ranges in concentration from 380 to 500 μg/ml (Ng-Kwai-Hang *et al.*, 1987) and has been shown not to affect MAC-T cell proliferation (Rejman *et al.*, 1992). Preliminary experiments from our laboratory revealed higher (above 5 mg/ml) BSA concentrations consistently augment proliferative response. To determine if BSA is appropriate to use as a 'safe' substitute to FBS in growth assays, four preparations of BSA were examined. At higher concentrations of BSA, 2 mg/ml (similar to 10% FBS) or more, a significant stimulation of growth of MAC-T cells (Figure 5A) occurred. Whether this stimulation is a result of BSA itself or impurities in the preparation is unknown, though substantial variation among lots suggests the latter.

It is important to note that many serum-free media used to culture mammary epithelium use BSA at concetrations from 0.5–15 mg/ml (Imagawa *et al.*, 1982; Campbell *et al.*, 1991). When BSA is used in milligram quantitites, contaminating factors found in low levels may reach biologically relevant levels (McKiernan & Bavister, 1992). Moreover, BSA is known to synergize with growth factors to cause enhanced stimulation of growth of cultured cells (Singh *et al.*, 1992). In a murine study with optimal growth in concentrations of BSA from 5–15 mg/ml, Imagawa *et al.* (1982), determined that lipids or lipid soluble substances account for much of the cellular growth. Likewise, using four different stocks of BSA we found all to retain proliferative activity at concentrations of 10 mg/ml or higher, except fatty acid-free BSA which was only effective at higher concentrations (Figure 5B). Regardless of the cause of mitogenesis, the proliferative responses to and the inconsistency of BSA preparations warrant appropriate attention from laboratories who replace sera with BSA.

This study confirms that the regulation of bovine mammary epithelial proliferation has similarities (IGF-I, IGF-II and insulin) and differences (EGF) when compared to the human or murine mammary gland. Data also indicates that many potent *in vivo* mammogens are unable to stimulate epithelial cell proliferation directly. Thus, endocrine, paracrine and autocrine

factors as well as ECM must account for much of these hormonal actions.

Materials and methods

Hormones and culture materials

All hormones, except where noted, were obtained from the Sigma Chemical Company (St. Louis, MO). Pituitary bovine prolactin was a gift from the National Hormone Pituitary Program and recombinant bovine growth hormone was from American Cyanamid Co. (Princeton, NJ, Lot 6958C-42A). The IGF-I analogs (des (1-3) IGF-I and Long R^3 IGF-I) were purchased from GroPep Pty Ltd (Adelaide, South Australia). All culture reagents, vessels, media, serum, and enzymes were obtained from Gibco (Gaithersburg, MD) unless otherwise noted. All four stocks of BSA were purchased from Sigma. BSA stock Cat no. A-7906 and A-3803 were deemed 98–99% pure; whereas the other stocks were 96–99% pure with the remainder mostly globulins. The RIA grade BSA (A-7888) was initially fractionated by cold alcohol, the other three stocks by heat shock with the two cell culture lots (A-7906 and A-3912) having a secondary charcoal/dialysis to reduce low molecular weight contaminants. The remaining preparation (A-3803) was substantially free of fatty acids (<0.003%). The RIA grade BSA was not used in comparison tests between BSA stocks. EGF (murine) was purchased from Sigma, except when human EGF and murine EGF were compared, then both EGFs were purchased from Upstate Biotechnology Inc. (UBI; Lake Placid, NY).

Cells and culture conditions

Bovine mammary epithelial cells (MAC-T) from our laboratory were used in all experiments. These cells were originally derived from mid-lactation Holstein cows and were established in culture through low level constitutive expression of the SV40 large-T antigen (Huynh *et al.*, 1991). These cells are not transformed. MAC-T cells are clonal, non-tumorigenic in nude mice, show anchorage dependency and topo-inhibition.

To confirm that our results were indicative of normal bovine mammary epithelium, primary bovine mammary epithelial cells were obtained from pregnant non-lactating Holstein cows as previously described (Huynh *et al.*, 1991). Primary cells were not clonal in origin but were substantially free of stromal cell contamination as determined by cytokeratin and vimentin staining. Cells were maintained for routine passage in DMEM with 10% FBS.

Hormones which were water soluble (insulin, IGF-I and II, prolactin, EGF and growth hormone) were added directly to the culture media from concentrated stocks. Steroid hormones were added in an ethanol vehicle to a final concentration not exceeding 0.001%. In preliminary experiments, it was found that a concentration of 0.01% ethanol or more significantly inhibited proliferation.

Quantification of cell proliferation

MAC-T-cells were plated at a density of 2×10^4 cells/well in a 24 well plate (Nunclon, Denmark) in 1 ml DMEM supplemented with 10% FBS for 24 h. The cells were then serum starved for 48 h (two media changes) by culturing the cells in DMEM with no additions. The stimulation of proliferation was then assessed during a 72 h experimental period when the cells were cultured in DMEM containing the various hormones, alone or in combination. To assure proliferation was not inhibited by contact inhibition (1) cells were visually monitored (2) a time 0 (i.e. at time of hormone addition) and a positive control (5% FBS) were collected and run with each assay. All experiments showed a significant increase in DNA or thymidine incorporation by the positive control. Each hormonal regime was also evaluated in the presence of 1% FBS. 1% FBS was capable of eliciting significant growth in both DNA and thymidine assays. Moreover, 10% FBS caused large increases in DNA accumulation, potentially masking modest proliferative responses of the hormone/growth factor being tested. In fact, even a milieu of hormones/growth factors (insulin (1 μg/ml), hydrocortisone (5 μg/ml), triiodothyronine (1 ng/ml), bovine prolactin (1 μg/ml) and IGF-I (10 ng/ml)) previously shown to be mitogenic alone were only able to modestly increase growth of MAC-T cells cultured in 10% FBS. Total DNA content per well was measured fluorometrically as per Labarca & Paigen (1980).

[^{3}H]thymidine incorporation was based on the method described by Sheffield (1988). Briefly, cells were plated as above, but serum starved 72 h (instead of 48 h) with three fresh media changes. Cells were cultured in test media for 18 h with 1 μCi/ml methyl-[^{3}H]thymidine (ICN, Irvin, CA; SA = 64 Ci/mmol) added for the last 1 h. Primary cells were only serum starved 48 h and NMuMG cells 24 h since morphological changes and decreased survival were associated with extended serum starvation. When NMuMG cells, primary epithelial cells or MAC-T cells were compared all cells were serum starved identically. The data were expressed as c.p.m./well.

Statistical analysis

The general linear models procedure (PROC GLM) of SAS (1988) was used to analyse dependent variables. Growth of MAC-T cells in hormones was analysed using Dunnett's test (Zar, 1984), comparing all hormone treatments to control (no hormone). Comparisons between treatments utilized contrasts to obtain F values. The critical F values obtained from non-orthogonal contrasts were then replaced by the Bonferroni F test (Zar, 1984). All statistical models contained treatment only.

Acknowledgements

The authors would like to thank Pat Boyle and Elaine deHeuvel for excellent technical assistance and Lisa Gloutney for preparing this manuscript for print. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC; OGF36728).

References

Akers, R.M. (1985). *J. Dairy Sci.*, 68, 501–519.

Akers, R.M. (1990). *Protoplasma*, 159, 96–111.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990). *J. Molec. Biol.*, 215, 403–410.

Baron, C. (1958). *Am. J. Obstet. Gynec.*, 75, 135–138.

Bauman, D.E., Eppard, P.J., DeGreeter, M.J. & Lanza, G.M. (1985). *J. Dairy Sci.*, 68, 1352–1362.

Baumrucker, C.R. & Stemberger, B.H. (1989). *J. Anim. Sci.*, 67, 3503–3514.

Campbell, P.G., Skaar, T.C. & Vega, J.R. (1991). *J. Endocr.*, 128, 219–228.

Cowie, A.T., Folley, S.J., Malpress, F.H. & Richardson, K.C. (1952). *J. Endocr.*, 8, 64–90.

Cowie, A.T., Forsyth, I.A. & Hart, I.C. (1980). *Hormonal Control of Lactation*. Gross, F., Grumbach, M.M., Labhart, A., Lipsett, M.B., Mann, T., Sameuls, L.T. & Zander, J. (eds.). Springer-Verlag: New York. Vol. 15. pp. 1–145.

Danielson, K.G., Oborn, C.J., Durban, E.M., Butel, J.S. & Medina, D. (1984). *Cell Biol.*, **81**, 3756–3760.
Davis, S.R., Gluckman, P.D., Hart, I.C. & Henderson, H.V. (1987). *J. Endocr.*, **114**, 17–24.
Dils, R. & Forsyth, I.A. (1981). *Meth. Enzym.*, **72**, 724–744.
Franke, R.T. & Rosenbloom, J. (1915). *Surg. Gynec. Obstet.*, **20**, 646–649.
Furumura, K., Akers, R.M. & Beal, W.E. (1990). *J. Dairy Sci.*, 73 (Suppl 1), 121.
Gibson, C.A., Kerr, D.E. & Baumrucker, C.R. (1992). *J. Dairy Sci.*, 75 (Suppl 1), 225.
Gibson, C.A., Vega, J.R., Baumrucker, C.R., Oakley, C.S. & Welsch, C.W. (1991). *In Vitro*, **27**, 585–594.
Glimm, D.R., Baracos, V.E. & Kennelly, J.J. (1990). *J. Endocr.*, **126**, R5–R8.
Glimm, D.R., Baracos, V.E. & Kennelly, J.J. (1992). *J. Dairy Sci.*, **75**, 2687–2705.
Gow, C.B. & Moore, G.P.M. (1992). *J. Endocr.*, **132**, 377–385.
Haslam, S.Z. (1988). *Endocr.*, **122**, 464–470.
Huynh, H.T., Robitaille, G. & Turner, J.D. (1991). *Exp. Cell Res.*, **197**, 191–199.
Imagawa, W., Spencer, E.M., Larson, L. & Nandi, S. (1986). *Endocr.*, **119**, 2695–2699.
Imagawa, W., Tomooka, Y. & Nandi, S. (1982). *Proc. Natl. Acad. Sci.*, **79**, 4074–4077.
Jenness, R. (1985). *Lactation*. Larson, B.L. (ed.). The Iowa State University Press: Ames, Iowa. pp. 164–197.
Jenness, R. (1988). *Fundamentals of Dairy Chemistry*. Wong, N.P., Jenness, R., Keeney, M. & Marth, E.H. (eds.). Van Nostrand Reinhold Company: New York. Vol. 3. pp. 1–38.
Keys, J. & Dijione, J. (1988). *J. Recep. Res.*, **8**, 731–740.
Labarca, C. & Paigen, K. (1980). *Anal. Biochem.*, **102**, 344–352.
Maier, R., Schmid, P., Cox, D., Bilbe, G. & McMaster, G.K. (1991). *Molec. Cell Endocr.*, **82**, 191–198.
Massague, J. & Czech, M.B. (1982). *J. Biol. Chem.*, **257**, 5038–5045.
McFadden, T.B., Daniel, T.E. & Akers, R.M. (1990). *J. Anim. Sci.*, **68**, 3180–3189.
McGrath, M.F. (1987). *J. Dairy Sci.*, **70**, 1967–1980.
McKiernan, S.H. & Bavister, B.D. (1992). *In Vitro*, **28A**, 154–156.
Mepham, T.B. (ed.) (1987). *Physiology of lactation*. Open University Press: Philadelphia, Pennsylvania. pp. 30–50.
Nagasawa, H., Ohta, K., Nakajima, K., Noguchi, Y., Miura, K., Niki, K. & Namiki, H. (1986). *Endocrinology of the Breast: Basic and Clinical Aspects*. Angeli, A., Bradlow, H.L. & Dogliotti, L. (eds.). New York Academy of Sciences: New York. pp. 301–315.
Ng-Kwai-Hang, K.F., Hayes, J.F., Moxley, J.E. & Monardes, H.G. (1987). *J. Dairy Sci.*, **70**, 563–570.
Rejman, J.J., Oliver, S.P., Muenchen, R.A. & Turner, J.D. (1992). *Cell Biol. I.*, **16**, 993–1001.
Richards, J., Imagawa, W., Balakrishnan, A., Edery, M. & Nandi, S. (1988). *Endocr.*, **123**, 1335–1340.
Romagnolo, D., Akers, R.M., Byatt, J.C., Wong, E.A. & Turner, J.D. (1994). *Endocr. J.*, (in press).
SAS Institute Inc. (1988). *SAS/STAT User's Guide*. Rel 6.03. Cary, NC.
Schmidt, G.H. (1971). *Biology of Lactation*. Salisbury, G.W. (ed.). W.H. Freeman and Company: San Francisco, California. pp. 6–35.
Sejrsen, K., Huber, J.T. & Tucker, H.A. (1983). *J. Dairy Sci.*, **66**, 845–855.
Sheffield, L.G. (1988). *J. Dairy Sci.*, **71**, 2855–2874.
Sheffield, L.G. & Yuh, I.S. (1988). *Dom. Anim. Endocr.*, **5**, 141–147.
Shing, Y.W. & Klagsburn, M. (1984). *Endocr.*, **115**, 273–282.
Shyamala, G. & Ferenczy, A. (1984). *Endocr.*, **115**, 1078–1081.
Singh, A., Blench, I., Morris, H.R., Savoy, L.A. & Reed, M.J. (1992). *Mol. Cell Endocr.*, **85**, 165–173.
Sizemore, S.R. (1979). *Dairy Sci. Abstr.*, **41**, 28.
Skarda, J., Urkanova, E. & Bilek, J. (1977). *Endocr. Exp.*, **11**, 105–115.
Soule, H.D., Vazquez, J., Long, A., Albert, S. & Brennan, M. (1973). *J. Natl. Cancer Inst.*, **51**, 1409–1413.
Spritzer, E. & Grosse, R. (1987). *Biochem. Int.*, **14**, 581–590.
Stockdale, F.E., Juergens, W.G. & Topper, Y.J. (1966). *Devel. Biol.*, **13**, 266–281.
Topper, Y.J. & Freeman, C.S. (1980). *Physiol. Rev.*, **60**, 1049–1106.
Tou, J.S., McGrath, M.F., Zupec, M.E., Byatt, J.C., Violand, B.N., Kaempfe, L.A. & Vineyard, B.D. (1990). *Biochem. Biophys. Res. Commun.*, **167**, 484–491.
Tucker, H.A. (1981). *J. Dairy Sci.*, **64**, 1403–1421.
Tucker, H.A. (1985). *Lactation*. Larson, B.L. (ed.). Iowa State University Press: Iowa. pp. 39–79.
Underwood, L.E., D'Ercole, A.J., Clemmons, D.R. & Van Wyk, J.J. (1986). *Clin. Endocr. Metab.*, **15**, 59–77.
Vandeputte-Van, M.G., Peeters, G.G. & Lauwers, H. (1976). *J. Dairy Res.*, **43**, 9–17.
Wiens, D.J., Brooks, C.L. & Hodgson, C.P. (1992). *J. Dairy Sci.*, **75**, 1857–1869.
Winder, S.J., Turvey, A. & Forsyth, I.A. (1989). *J. Endocr.*, **123**, 319–326.
Woodward, T.L., Akers, R.M., McFadden, T.B., Huynh, H.T. & Turner, J.D. (1992). *J. Dairy Sci.*, **75** (Suppl 1), 293.
Woodward, T.L., Beal, W.E. & Akers, R.M. (1993a). *J. Endocr.*, **136**, 149–157.
Woodward, T.L., Philip, A. & Turner, J.D. (1993b). *J. Dairy Sci.*, **76** (Suppl 1), 188.
Zar, J.H. (ed.). (1984). *Biostatistical Analysis*. Prentice Hall Inc: Englewood Cliffs, New Jersey. pp. 194–195.
Zhao, X., McBride, B.W., Politis, I., Huynh, H.T., Akers, R.M., Burton, J.H. & Turner, J.D. (1992). *J. Endocr.*, **134**, 307–312.
Zurfluh, L.L., Bolten, S.L., Byatt, J.C., McGrath, M.F., Tou, J.S., Kupec, M.E. & Krivi, G.G. (1990). *Grow Factor*, **3**, 257.

SUMMARY 2

Chapter 2 describes the growth responsiveness of bovine mammary epithelial cells to a host of hormones, growth factors and cellular mitogens that have previously been reported to cause mammary epithelial cell proliferation in vivo or in vitro in the bovine animal or in other species. Although substantial literature exists on the effects of mammogens on bovine mammary tissue, this is the first time the effects of these mitogens have been studied directly on isolated, clonal bovine mammary epithelial cells under defined conditions. Prior to determining how growth inhibitors affect mammary epithelium, a comprehensive study defining direct mammary epithelial mitogens was required.

This chapter utilized the MAC-T epithelial cell line and a mixture of FbE and PrE epithelial lines described in chapter 1. With these cell lines, serum, IGF-I, des(1-3) IGF-I, Long R^3 IGF-I, insulin and IGF-II were confirmed to be potent mammary epithelial mitogens. Bovine serum albumin induced substantial mammary epithelial cellular proliferation, regardless of whether the preparation of BSA was free of fatty acids. This is especially important since many mammary epithelial researchers use high concentrations of BSA in their serum-free media that would cause substantial cell proliferation.

The most potent in vivo mitogens including estrogen, progesterone, estrogen +

progesterone, growth hormone, prolactin, did not stimulate cellular proliferation when cells were treated with these hormones alone or in combination with serum. Similar results have been reported in human mammary epithelium regarding the lack of estrogen and progesterone responsiveness. However, in both murine and human mammary epithelial cells, EGF and TGF-α have been shown to be potent mitogens. In this chapter, for the first time we have shown that EGF does not stimulate bovine mammary epithelium cell proliferation, regardless of the source of EGF. Whereas in control studies, EGF enhanced murine mammary epithelial cell proliferation by nearly 300%. This is especially important since several researchers have speculated that EGF is a potent mitogen in the bovine mammary. This difference in growth response to EGF may also represent a fundamental difference between bovine mammogenesis and murine or human mammogenesis.

This chapter, in addition to enhancing our understanding of bovine mammary physiology and its complexities, has identified several key differences between murine and bovine mammogenesis.

CHAPTER 3

Characterization of transforming growth factor-β growth regulatory effects and receptors on bovine mammary cells

JOURNAL OF CELLULAR PHYSIOLOGY 165:339-348 (1995)

Characterization of Transforming Growth Factor-β Growth Regulatory Effects and Receptors on Bovine Mammary Cells

T.L. WOODWARD, N. DUMONT, M. O'CONNOR-McCOURT, J.D. TURNER, AND A. PHILIP*

Departments of Animal Science (T.L.W., J.D.T.) and Physiology and Surgery (N.D., A.P.), McGill University, Montreal, Canada, H9X 3V9; Biotechnology Research Institute, National Research Council Canada (M.O.-M.), Montreal, Canada, H4P 2R2

Transforming growth factor-β (TGF-β) has been shown to inhibit mammary morphogenesis, growth, and differentiation in murine studies. We have characterized TGF-β receptors and their autoregulation, and the growth response to TGF-β1 and TGF-β2 in cultured bovine mammary epithelium (MAC-T) and fibroblasts. Affinity labelling studies revealed that fibroblast and epithelial cells contained type I, II, and III (betaglycan) receptors, with the type III receptor being the predominant binding component. On both fibroblasts and epithelial cells, TGF-β1 and TGF-β2 had equal binding affinities for the type I and II receptors, but TGF-β2 had a higher affinity for the type III receptor. Also, preincubation of MAC-T cells with 50 pM TGF-β1 or TGF-β2 markedly downregulated TGF-β receptors. Proliferative response was measured using both total DNA and ^{3}H-thymidine incorporation. Both TGF-β isoforms were effective in inhibiting proliferation of MAC-T cells and fibroblasts. Inhibition of proliferation was not altered following immortalization of fibroblasts with SV-40 Large-T-antigen (LT), even when the cells acquired a transformed phenotype. Inhibition of proliferation was not a result of cytotoxicity, as TGF-β at concentrations 1,000-fold higher than ED_{50} levels did not increase cell death. Moreover, the inhibition was reversible as shown by return of cellular proliferation to control levels following TGF-β removal. Although growth inhibition was not transient as culture of MAC-T cells in TGF-β resulted in sustained inhibition of proliferation for at least 144 h.

© 1995 Wiley-Liss, Inc.

The profound effects of systemic hormones on mammary development, differentiation, and secretory activity are well documented. At the cell and tissue level, the action of many of these hormones is mediated by growth factors in an autocrine or paracrine manner. Of the many peptide growth factors, transforming growth factor-β (TGF-β) appears to be relatively unique in its widespread effects on numerous cell types, influencing cellular proliferation, migration, and elaboration of extracellular matrix (reviewed in Roberts and Sporn, 1990; Massague, 1990). In mammals, three isoforms of TGF-β (-β1, -β2, and -β3) have been identified, each encoded by a different gene. Although the three isoforms, in general, have similar biological activities in most in vitro assays, several studies indicate marked differences in their cellular origin, developmental expression, and binding to receptors, suggesting that the TGF-β isoforms have distinct functions in vivo (Pelton et al., 1990; Cheifetz et al., 1990; Joyce et al., 1990; Roberts and Sporn, 1992).

The actions of TGF-β are mediated by binding to highly specific cell surface receptors which are present on nearly all cell types (Wakefield et al., 1987). Affinity cross-link labelling studies with iodinated TGF-β have shown that most cells have three types of TGF-β receptors, designated type I, II, and III (Roberts and Sporn, 1990; Massague, 1990). The recent cloning of these receptors showed that type I (Franzen et al., 1993) and type II (Lin et al., 1992) receptors are transmembrane serine/threonine kinases while the type III receptor (Wang et al., 1991; Lopez-Casillas et al., 1991) is a transmembrane proteoglycan with a short cytoplasmic domain. The first two are directly involved in signal transduction while the third has been postulated to enhance binding of TGF-β to the signalling receptors.

There is an emerging interest in the role of TGF-β in mammary physiology, following the ascription of potent growth regulatory effects to mammary gland development (Silberstein and Daniel, 1987; Daniel and Robinson, 1992; Robinson et al., 1993). Using slow-release implants, in rodents, studies from Charles Daniel's laboratory have shown that TGF-β revereibly inhibits ductal growth in the murine mammary gland

Received November 18, 1994; accepted April 5, 1995.

*To whom reprint requests/correspondence should be addressed at Montreal General Hospital, 1650 Cedar Avenue, Room C9177, Montreal, Quebec, Canada H3G 1A4.

© 1995 WILEY-LISS, INC.

(Silberstein and Daniel, 1987; Daniel et al., 1989), while alveolar morphogenesis was not inhibited. An endogenous role for TGF-β in the mouse mammary gland was suggested by the observation that the mammary gland displayed developmentally regulated expression of TGF-β isoforms (Robinson et al., 1991; Daniel and Robinson, 1992). Furthermore, recent studies in mice provided evidence that TGF-β regulates functional differentiation and lactogenesis in the mammary gland. For example, TGF-β inhibited β-casein expression in mammary explants from pregnant mice (Robinson et al., 1993). Also, mice overexpressing TGF-β exhibited impaired alveolar function and deficiency in lactation (Jhappan et al., 1993).

In this report, we address the previously unexplored question as to whether TGF-β plays a similar role in bovine mammary morphogenesis and lactation through in vitro experiments. Isolation and characterization of the major cellular representatives of stromal and parenchymal tissues of the bovine mammary gland has enabled us to determine the direct effect of TGF-β on those cells. In the present study, we describe the growth regulatory effects of TGF-β isoforms and characterize the TGF-β receptors on bovine mammary epithelial cells and fibroblasts.

MATERIALS AND METHODS

Cells and culture conditions

All culture reagents, media, serum, and enzymes were obtained from GIBCO BRL (Burlington, Ontario, Canada) and Becton Dickinson (Ville St. Laurent, Quebec, Canada), unless otherwise noted. The collagenase, hyaluronidase, and pronase used in tissue dissociation were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant human IGF-I was purchased from Ingram and Bell (Lachine, Quebec, Canada).

Tissue was obtained by mammary biopsy from cycling nulliparous, non-pregnant, non-lactating Holstein heifers as previously described (Huynh et al., 1991), except for MAC-T cells which were obtained from a mid-lactational Holstein cow. The following bovine mammary cell lines were used: (1) (MAC-T) a clonal epithelial cell line immortalized by stable transfection with the SV-40 Large-T-antigen (LT) (Huynh et al., 1991); (2) (MF-2) a non-clonal primary fibroblast population; (3) (MF-T2) the MF-2 line stably transfected with the LT (5 μg plasmid); and (4) (MF-T3) a clonal fibroblast line stably transfected with LT (15 μg plasmid); unlike MF-T2 cells, they display a transformed phenotype as evidenced by loss of contact inhibition, formation of foci on plastic, and diminished anchorage dependency.

Cell typing (as epithelial or fibroblast) was confirmed by immunohistochemical analysis after incubation with monoclonal antibodies that react with vimetin (Boehringer-Mannheim, Indianapolis, IN) and cytokeratin (pan-specific, Boehringer-Mannheim) as described previously (Huynh et al., 1991). Expression of the LT was directed from the plasmid pBAPSV40TtsA58 (Otsuka et al., 1979) and the pSV2-neo plasmid provided a dominant selectable marker (Southern and Berg, 1982). Transfections and subsequent selection were the same as that previously reported (Huynh et al., 1991), except that 45 μl lipofectin reagent (Gibco BRL) and 55 μl HBSS (containing 5 μg pBAPSV40TtsA58 plasmid and 1.1 μg pSV2) were incubated for 5 h per 100 mm plate. Lipofectin transfections resulted in a dramatic increase in the percentage of cells stably transfected/μg DNA over calcium phosphate transfections (data not shown). All cells were maintained for routine culture in 10% FBS supplemented DMEM in a humidified, 37°C, 5% CO_2 incubator.

TGF-β

TGF-β1 and TGF-β2 purchased from Austral Biologicals (San Ramon, CA) were used in all experiments. All glass and plasticware were silanized (Sigmacote; Sigma) prior to TGF-β exposure. TGF-β was diluted in a 5 mM HCl/15 μM BSA solution for addition to cell cultures, unless otherwise noted. This solution did not effect cellular proliferation (data not shown). No significant difference in biological activity was observed when these growth factors were obtained from Bristol-Myers Squibb (TGF-β1) (Seattle, WA) or from Celtrix (TGF-β2) (Palo Alto, CA).

Quantification of cell proliferation

Cellular proliferation was measured by assaying both total DNA and incorporation of methyl-^{3}H-thymidine (ICN, Irvin, CA; SA = 64 Ci/mmol). Briefly, total DNA/well was measured by plating cells at a density of 2×10^4 cells/well in a 24 well plate (GIBCO, Burlington, Ontario, Canada) in 1 ml DMEM supplemented with 10% FBS for 24 h. The cells were then serum starved for 48 h with two media changes to limit exogenous mitogens/inhibitors interference. DNA was quantified following a 72 h experiment period where the cells were cultured in the presence of the various treatments (combinations of TGF-βs, IGF, FBS). Attached cells were treated with 0.25% trypsin (250 μl/well) until detachment from plastic (10 min), then 750 μl of a 1.33× concentrated (final concentration = 1×) high salt PBS (Labarca and Paigen, 1980) was added, the sample was transferred to a 4 ml borosilicate test tube, sonicated (for 5 sec/well) at intermediate setting, and assayed for total DNA using a Model TKO 100 DNA fluorometer (Hoefer Scientific, San Francisco, CA) as per Labarca and Paigen (1980). We have previously described in detail the assay procedure used for measuring thymidine incorporation (Woodward et al., 1994). Each experiment contained a minimum of 9 replicates per treatment with all experiments being repeated at least twice. Proliferation of cells was induced by addition of IGF-I (300 nM) or FBS (1 or 5%, see below) during the experiment period, following serum starvation. MAC-T cell dose response experiments have shown that near maximal DNA accumulation was obtained with 5% FBS, whereas only 1% FBS was needed to maximize thymidine incorporation (^{3}H-TdR) (Zhao et al., 1992; Woodward et al., 1994). These concentrations were used in the corresponding assays.

Cytotoxicity assays

Cytotoxicity of TGF-β was measured by culturing MAC-T cells in TGF-β2 containing media for 72 h and subsequently counting total cell number and live cell number (by Trypan Blue exclusion) on a hemocytometer, 5 replicates/treatment.

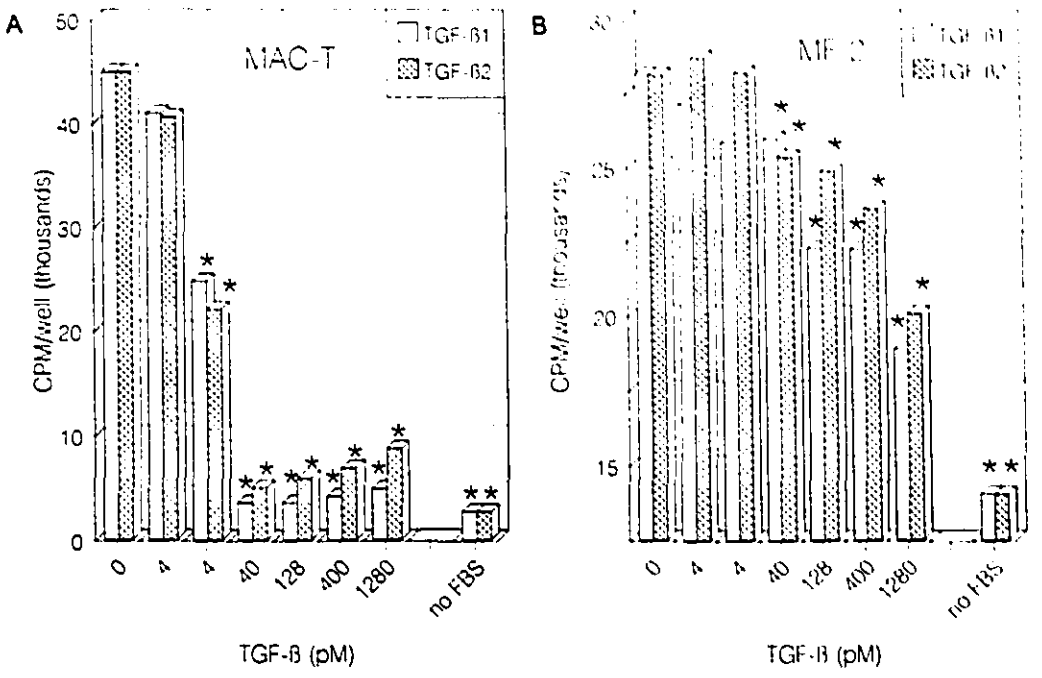


Fig. 1. A: Inhibition of thymidine incorporation in MAC-T cells by TGF-β1 or TGF-β2. Asterisks indicate significant differences in ^{3}H-TdR from control ($P \leq .05$), S.E.M. = 504. B: Inhibition of thymidine incorporation in MF-2 cells by TGF-β1 or TGF-β2, S.E.M. = 845.

Reversibility of TGF-β effect

To determine whether TGF-β growth inhibition was reversible or not, MAC-T cells were plated in 24 well dishes as previously described (Woodward et al., 1994). Subsequently, TGF-β2 (1, 10, or 100 pM) was added for 48 h in 1% FBS/DMEM. The cells were then rinsed and 1% FBS/DMEM without TGF-β was added. At 24 h intervals (up to 120 h), cells were harvested and assayed for total DNA and ^{3}H-TdR. Data are represented as CPM/μg DNA.

Iodination of TGF-β and affinity labelling of cells

Recombinant TGF-β1 (Bristol-Myers Squibb, Seattle, WA) and TGF-β2 (Austral Biologicals) were iodinated as described previously (Philip and O'Connor-McCourt, 1991). In the present study, 2–5 μg of TGF-β1 or 2 μg of TGF-β2 were iodinated and the specific activity was in the range of 1.5–3.0 μCi/pmol for both TGF-β1 and -β2. TGF-β receptors were characterized using affinity labelling and competition studies on MAC-T (epithelial) and MF-2 (fibroblast) cells. Affinity labelling was performed as previously described by Mitchell et al. (1992). Briefly, this involved the incubation of cells with ^{125}I-TGF-β1 or ^{125}I-TGF-β2 in the absence or presence of the indicated concentrations of unlabelled TGF-β1 or TGF-β2 for 3 h at 4°C. The bound ligand was cross-linked to the receptors by incubating with 1 mM BS3 and the cells were solubolized and the supernatant collected and subjected to SDS-PAGE and autoradiography.

SDS-PAGE and autoradiography

SDS-PAGE analysis was performed according to the method of Laemmli (1970) using 3–11% (w/v) linear gradient separating gel, 3% (w/v) stacking gel, and a Tris-Cl buffer system as described previously (Philip and O'Connor-McCourt, 1991). The gels were calibrated using Bio-Rad (Richmond, CA) low and high molecular weight standards. ^{14}C-methylated protein standards (Amersham, Oakville, Ontario, Canada) were used to estimate molecular weight of unknown bands. Following electrophoresis, gels were stained with Coomassie Brilliant blue, destained, dried, and exposed to Kodak Omat AR film at −80°C using Dupont Cronex Lightning (Dupont, Wilmington, DE) plus intensifying screens.

Preincubation of MAC-T cells with TGF-β before affinity labelling

MAC-T cells were plated in DMEM as described above, allowed to attach for 24 h, serum starved for 24 h, and preincubated in the absence or presence of 50 or 100 pM of unlabelled TGF-β1 or TGF-β2 for 24 h in DMEM containing 1% FBS. The cells were then washed 3 times with ice-cold DPBS for 30 min at 4°C, then affinity labelled as above. Control experiments were performed using ^{125}I-TGF-β instead of unlabelled TGF-β in the preincubation to ensure that the washing step employed before affinity labelling allowed the dissociation of any prebound TGF-β. Cell numbers were determined with a hemocytometer on identical wells prior to the solubilization step.

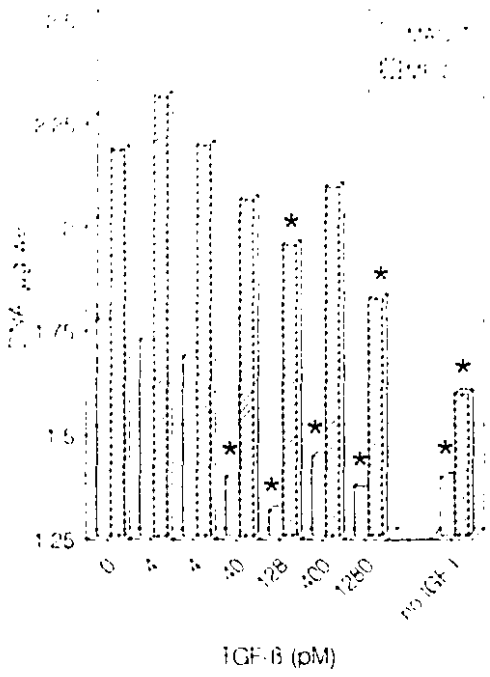


Fig. 2. Suppression of cellular proliferation of MAC-T cells and MF-2 cells cultured in serum-free, growth promoting media (IGF-I, 300 nM) by TGF-β2. Data are expressed as total DNA. Asterisks indicate a significant difference ($P \leq .05$) from control (300 nM IGF-I without TGF-β2), S.E.M. = 0.068.

Statistical analysis

The general linear models procedure (PROC GLM) of SAS (1988) was used to analyze dependent variables. Data from total DNA and ^{3}H-TdR assays were analyzed using Dunnett's test (Zar, 1984), comparing TGF-β treatments to control (no TGF-β). Comparisons between TGF-β treatments utilized non-orthogonal contrasts to obtain F values. These critical F values were then replaced by the Bonferroni F test (Zar, 1984). All statistical models contained treatment only.

RESULTS

Growth inhibition of bovine mammary epithelial cells and fibroblasts

TGF-β1 and TGF-β2 were both effective inhibitors of ^{3}H-TdR into MAC-T cells at very low concentrations (Fig. 1A). Half maximal inhibition occurred at 4 pM for both isoforms and maximal inhibition at 40 pM ($P \leq .01$). Similarly, MAC-T cells in serum-free media stimulated with 300 nM IGF-I were also maximally growth inhibited by 40 pM TGF-β2 as detected by total DNA measurement (Fig. 2). Thymidine incorporation into fibroblasts stimulated by 1% FBS was also inhibited by TGF-β1 and TGF-β2, but to a lesser degree (Fig. 1B). Fibroblasts (MF-2), stimulated by 300 nM IGF-I, were weakly inhibited by TGF-β2 as shown by a decrease in total DNA (Fig. 2).

A more detailed comparison of the maximal inhibition and the concentration at which it was observed for MAC-T cells and different fibroblast populations (MF-2, MF-T2, MF-T3) is presented in Table 1. Both the degree of maximal inhibition (as a percent of no inhibition, Table 1), and the concentration of TGF-β required to achieve maximal inhibition varied considerably between cell types: i.e., MAC-T cells were 60% growth inhibited at 40 pM TGF-β2, while MF-2 cells were only growth inhibited 7% at 1.28 nM TGF-β2. Serum starved cells showed little or no increases in total DNA and only minimal incorporation of labelled thymidine, columns 1 and 4 (Table 1). Maximal inhibition by TGF-β1 or TGF-β2 for epithelial cells (MAC-T), was similar to that observed following serum starvation whereas, for fibroblasts (MF-2, MF-T2, MF-T3), maximal inhibition by TGF-β1 or TGF-β2 was only 50% of what was observed following serum starvation. Interestingly, no change in inhibition could be demonstrated following LT transfection in fibroblasts whether they displayed a normal (MF-T2) or a transformed phenotype (MF-T3).

TGF-β induced growth inhibition is not a result of cellular toxicity and its action is reversible

Both isoforms of TGF-β inhibited MAC-T cell growth maximally at 40 pM and concentrations 100-fold higher did not decrease the number of live cells ($P \simeq .05$), as measured by Trypan Blue exclusion (Fig. 3).

Culture of MAC-T cells in TGF-β2 for 48 h and subsequent removal of the growth inhibitor revealed growth was reversibly inhibited (Fig. 4). The growth inhibitory effect of TGF-β2 (1 pM or 100 pM) was evident for 72 h after TGF-β removal ($P \leq .001$). However, the growth returned to control levels by 120 h.

MAC-T and MF-2 cells show isoform specific binding for TGF-β

Using an affinity cross-link labelling technique, we have identified three high affinity binding components (65, 85, and 250–300 kDa) on MAC-T cells (Fig. 5) and MF-2 cells (Fig. 6). Although their apparent Mr and banding pattern on SDS-PAGE were similar to those of the type I, II, and III TGF-β receptors, their isoform specificity appeared to be distinct from that normally observed for the type I, II, and III receptors. The type I and II receptors have generally been shown to exhibit a much higher affinity for TGF-β1 than for TGFβ2 while the type III receptor usually binds both isoforms with similar affinity. In contrast, the 250–300 kDa component, which is the predominant binding complex on both MAC-T and MF-2 cells, has a greater affinity for TGF-β2 than for TGF-β1 as detected by competition experiments using unlabelled TGF-β1 and TGF-β2. On MAC-T cells, 1.0 nM TGF-β1 blocks binding of either 50 pM ^{125}I-TGF-β1 or ^{125}I-TGF-β2 to the type III TGF-β receptor by approximately the same extent as 0.20 nM TGF-β2 (Figs. 5A and B). Similarly, on mammary fibroblasts (MF-2), 7.5 nM TGF-β1 blocks binding of 100 pM ^{125}I-TGF-β1 (Fig. 6A) or ^{125}I-TGF-β2 (data not shown) to the type III TGF-β receptor by approximately the same extent as 1.5 nM TGF-β2. The 65 kDa and the 85 kDa binding complexes in both mammary cell types also display isoform specificities distinct from those of classic type I and type II receptors, respectively, in that they have the same or slightly higher affinity for TGF-β2 than for TGF-β1. In previous studies using the same TGFβ1 and TGF-β2 preparations, the type I and type II receptors on other cell types

TABLE 1. Maximal inhibition (%) of ^{3}H-TdR and total DNA by TGF-β1 and TGF-β2 in MAC-T, MF-2, MF-T2, and MF-T3 cells

Cell type	^{3}H-thymidine incorporation (%)[1]			Total DNA (%)[1]		
	Serum-free	TGF-β1	TGF-β2	Serum-free	TGF-β1	TGF-β2
MAC-T[3]	84	62 (40)[2]	60 (40)	59	55 (640)	61 (640)
MF-2	64	10 (640)	7 (1,280)	44	16 (1,280)	32 (1,280)
MF-T2[4]	37	14 (400)	19 (400)	22	14 (2,560)	12 (640)
MF-T3[4]	25	18 (80)	19 (160)	39	10 (640)	21 (640)

[1]% of growth inhibition compared to serum with no TGF-β (0%).
[2]Concentration of TGF-β1 or TGF-β2 (pM) at which the maximal inhibition was obtained.
[3]Transfected with LT, not transformed.
[4]Transfected with LT and display transformed phenotype.

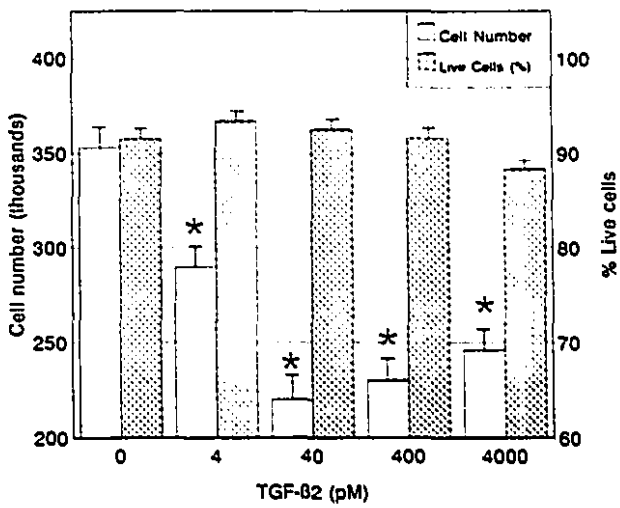


Fig. 3. Hemocytometric counts of MAC-T cells. Total cell number and percentage of live cells after treatment with physiological and supraphysiological (1,000 × ED_{50}) concentrations of TGF-β2 are presented. Asterisks indicate a significant difference ($P \leq .05$) from control.

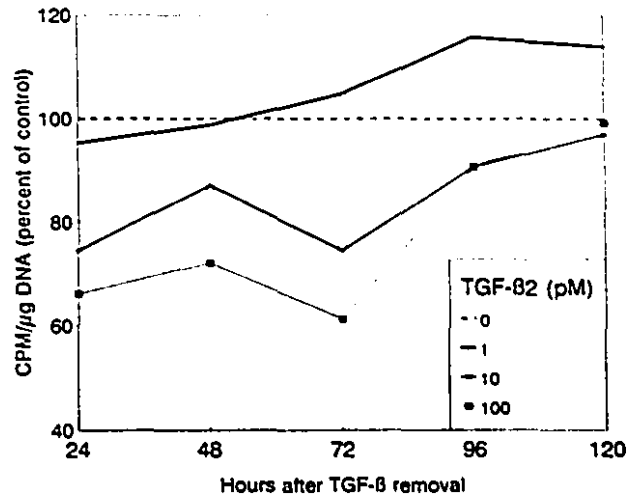


Fig. 4. Duration of MAC-T growth inhibition by TGF-β2. MAC-T cells were cultured in TGF-β2 supplemented media for 48 h, followed by removal of TGF-β2 and subsequent assay of ^{3}H-TdR and total DNA every 24 h for 120 h. Cell growth is represented as CPM/μg DNA as a percentage of control (cells cultured in 1% FBS, not exposed to TGF-β2).

displayed greater affinity for TGFβ1 than for TGFβ2 (Mitchell et al., 1992). The apparent Mr and banding pattern of the TGF-β binding components under nonreducing conditions were the same as that observed under reducing conditions for both MAC-T cells (data not shown) and MF-2 cells (Fig. 6B).

Preincubation of MAC-T cells with TGF-β results in a decrease in TGF-β receptors

A 24 h preincubation of MAC-T cells with 50 pM of TGF-β1 or -β2 induced a decrease in the affinity labelling of type III TGF-β receptors and to a lesser extent, of type I and II receptors (Fig. 7). Interestingly, TGF-β2 was at least two times more potent than TGF-β1 in inducing the down regulation of type III receptors. Under the same culture conditions, there was no decrease in cell number. This down regulation of TGF-β receptors is not due to continued occupancy of the receptors by unlabelled TGF-β, since we were able to determine, using ^{125}I-TGF-β instead of unlabelled TGF-β, that the washing step used before affinity labelling was effective in allowing the dissociation of virtually all prebound TGF-β.

A separate trial was conducted to determine if TGF-β induced growth inhibition was transient or sustained over 144 h. ^{3}H-TdR/μg DNA as a percent of control (no TGF-β) declined in TGF-β treated cells (at 10 or 100 pM) until 72 h (Fig. 8). After 72 h of culture in TGF-β, cell growth (^{3}H-TdR/μg DNA) was maintained at 5% of the rate of cells lacking TGF-β.

DISCUSSION

Several studies have documented the powerful inhibitory effects of TGF-β on the development and functional differentiation of the mouse mammary gland. It is not known whether TGF-β plays a similar role in the bovine mammary gland, where such studies may lead to avenues for increased milk production, an area of obvious interest to the dairy industry. Recent studies have reported the identification of both TGF-β1 and TGF-β2 in bovine milk (Cox and Burke, 1991; Jin et al., 1991) and TGF-β2 in bovine colostrum (Tokuyama and Tokuyama, 1993). In addition, all three TGF-β isoforms have been shown to be expressed in both the nonlactating and lactating bovine mammary gland (Maier

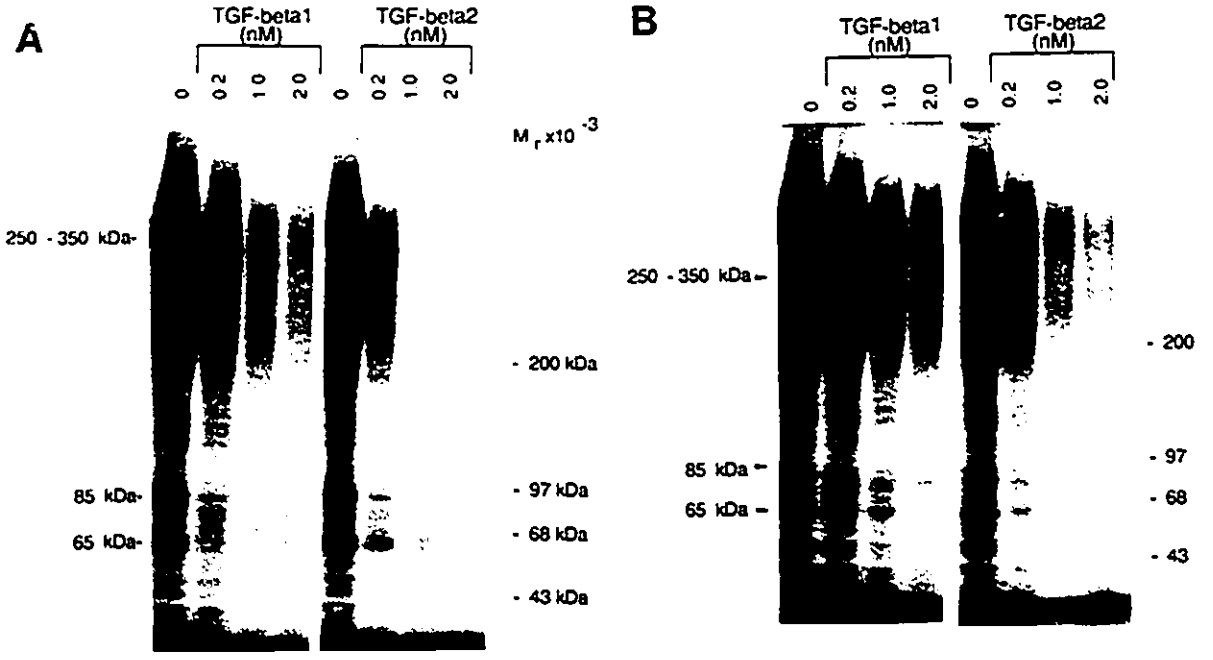


Fig. 5. TGF-β binding components on MAC-T cells. Confluent monolayers of MAC-T cells were affinity labelled with 100 pM of ^{125}I-TGF-β1 (A) or ^{125}I-TGF-β2 (B) in the presence or absence of the indicated concentrations of unlabelled TGF-β1 or -β2. Analysis on SDS PAGE was done under reducing conditions.

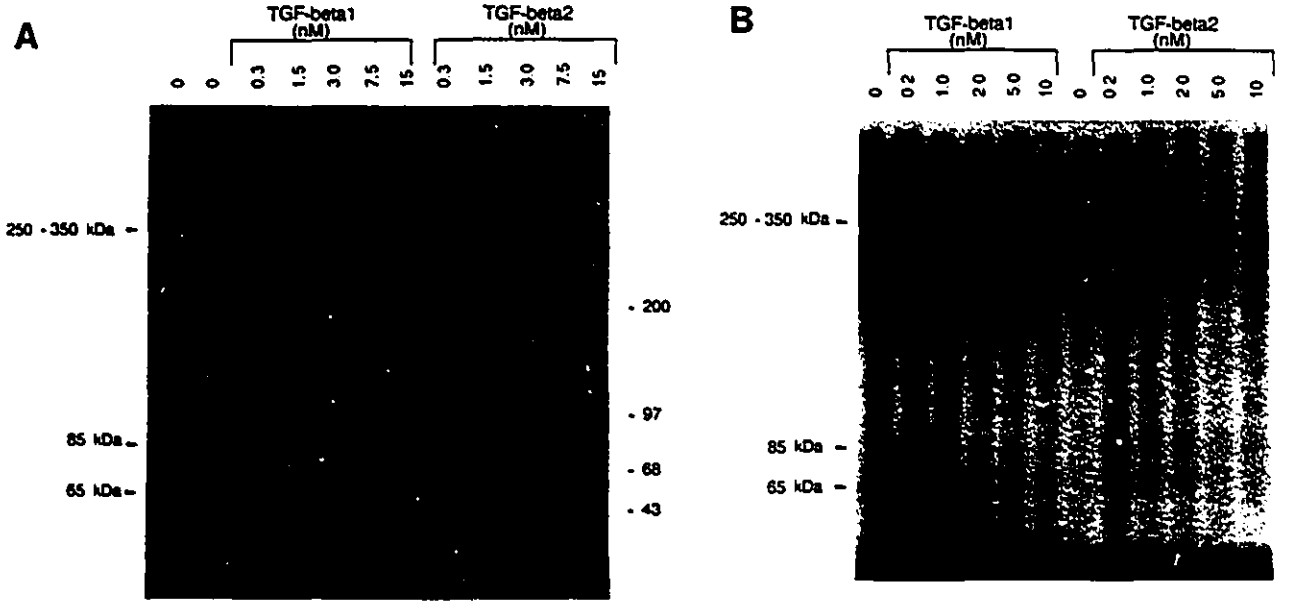


Fig. 6. TGF-β binding components on mammary fibroblasts (MF-2 cells) under reducing (A) and non-reducing (B) conditions. MF-2 cells were affinity labelled with 150 pM ^{125}I-TGF-β1 in the presence or absence of indicated concentrations of unlabelled TGF-β1 or TGF-β2.

et al., 1991). However, receptors for TGF-β have not been characterized in bovine mammary tissue and, to date, there have been no reports on the action of TGF-β in the bovine mammary gland. In the present study, we demonstrate that both TGF-β1 and TGF-β2 markedly inhibit the growth of bovine mammary epithelial cells and, to a lesser extent, mammary fibroblasts in culture. In addition, we have characterized type I, II,

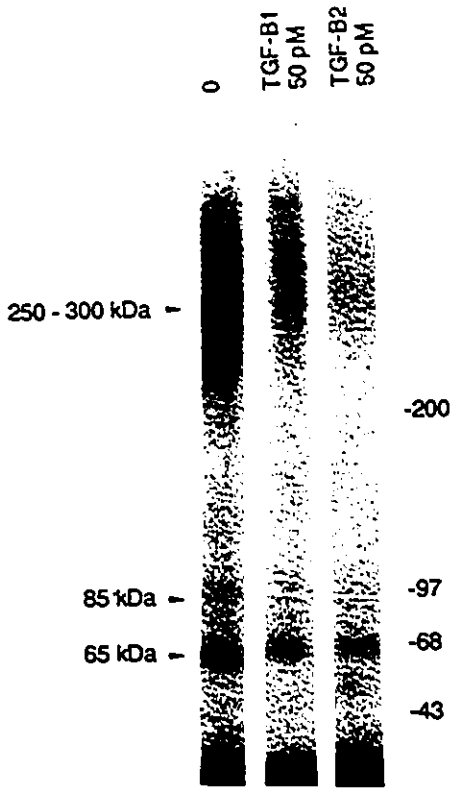


Fig. 7. TGF-β receptors on MAC-T cells are downregulated following preincubation with TGF-β1 or TGF-β2. MAC-T cells were preincubated with 50 pM TGF-β1 or TGF-β2 for 24 h and affinity labelled with 50 pM ^{125}I-TGF-β1.

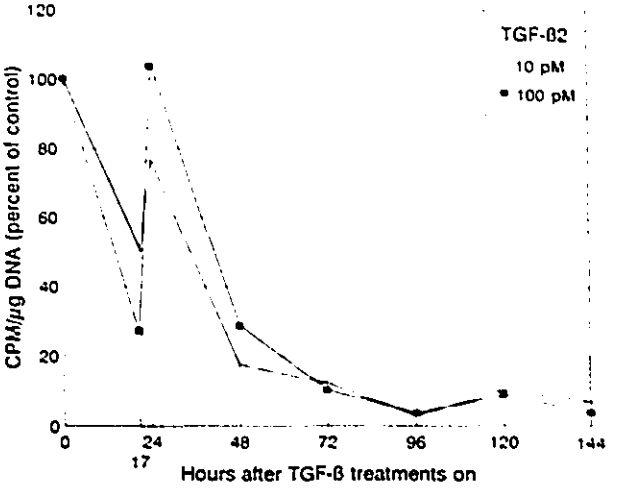


Fig. 8. Culture of MAC-T cells in TGF-β2 containing medium with 1% FBS for 144 h. Samples were taken every 24 h with an additional sample time at 17 h (first cell cycle). CPM/µg DNA for all samples except 24 h (see text) are significantly less ($P < .05$) than control (no TGF-β2 with serum).

and III TGF-β receptors in these cell types, and have shown that preincubation of epithelial cells with TGF-β isoforms results in a downregulation of TGF-β receptors, with TGF-β2 being more effective than TGF-β1.

Few potent growth inhibitors of mammary cells have been identified, in sharp contrast to a number of mammary cell mitogens: GH, IGF-I, IGF-II, EGF, TGF-α, aFGF, bFGF, estrogens, progesterone, and insulin (Akers, 1990) that have been documented. Control of mitogenesis in the mammary gland is particularly important since excessive inhibition would limit mammary development, while absence of mammogenic regulators could result in uncontrolled growth and tumor formation. The data from this study and Daniel and Robinson (1992), demonstrated that TGF-β1 and TGF-β2 inhibit growth of mammary epithelial cells and fibroblasts. With regard to mammary epithelial cell growth, MAC-T cells exhibited an ED_{50} at very low concentrations (4 pM), similar to that of CCl-64 Mv 1 Lu (mink lung epithelial cells), which are used as a TGF-β bioassay system because of their exquisite sensitivity (Ikeda et al., 1987). Moreover, inhibition was not a result of cytotoxicity as concentrations 1,000-fold higher (4 nM) than the ED_{50} (4 pM) did not cause cell death and since the inhibition was reversible following TGF-β removal.

TGF-β growth inhibition properties may be influenced by serum. For example, serum contains α_2 macroglobulin which inhibits the binding of TGF-β to its receptor (Danielpour and Sporn, 1990; Philip et al., 1994). However, when MAC-T or MF-2 cells were cultured in serum-free, BSA-free media and stimulated with IGF-I, growth inhibition by TGF-β2 was similar to that in serum-containing media (compare Figs. 1 and 2).

It was not surprising that MAC-T cells were growth inhibited by TGF-β, since epithelial cells are generally growth inhibited by TGF-β although the extreme sensitivity of MAC-T cells to TGF-β was unexpected. On the other hand, stromal cells are often unresponsive or stimulated by TGF-β in vitro (Roberts and Sporn, 1990) and in vivo (Daniel et al., 1989). We have demonstrated that mammary fibroblasts are growth inhibited by TGF-β, although they are considerably less sensitive to TGF-β than epithelial cells.

In the present study, using an affinity labelling technique, we have identified three high affinity binding components on MAC-T and MF-2 cells. While these binding complexes have the same apparent Mr and binding pattern as type I, II, and III TGF-β receptors, they display isoform specificities that are distinct from those normally observed. In general, type I and II receptors bind TGF-β1 with a much higher affinity than TGF-β2 and the type III receptors bind TGF-β1 and TGF-β2 with similar affinities (Chiefetz et al., 1990; Roberts and Sporn, 1992). In contrast, the type I and type II receptors detected on MAC-T and MF-2 cells have equal or slightly higher affinity for TGF-β2 than for TGF-β1 when labelled with ^{125}I-TGF-β1 or ^{125}I-TGF-β2; the type III receptors on these cell types displayed approximately a 5-fold higher affinity for TGF-β2 than TGF-β1 when labelled with ^{125}I-TGF-β1 or ^{125}I-TGF-β2.

Cell surface TGF-β binding components, with the same Mr as type I and II receptors, which display high affinity for TGF-β2 have previously been reported (Cheifetz et al., 1990). In addition, there are several reports describing the presence of subsets of the type III receptors which display a greater affinity for TGF-

β2 than for TGF-β1 (Segarini et al., 1987; Mitchell and O'Connor-McCourt, 1991; Mitchell et al., 1992). These differences in the affinity labelled binding characteristics in different cell types can be due to post-translational modifications or due to interaction with other proteins.

The isoform specific binding of TGF-β to receptors on MAC-T and MF-2 cells may reflect differential roles of TGF-β1 and -β2 on these cells. Although both isoforms were equipotent in the growth inhibitory response, TGF-β2 was more potent than TGFβ1 in the downregulation of receptors on these cells. A recent study by Robinson and colleagues (1991) has demonstrated that the patterns of gene expression and the distribution of TGF-β isoforms in the mouse mammary gland vary with the physiological state of the animal. For example, TGF-β2 was expressed only during pregnancy and TGF-β1 and -β3 were expressed in both non-pregnant and pregnant states, while no TGF-β isoform expression was detected during lactation. However, there is no information concerning TGF-β receptor changes in the mammary with various reproductive states. Whether the expression of TGF-β receptors parallels the expression of ligands observed during various physiological states remains to be determined.

Since ligand-dependent receptor-downregulation is often associated with attenuated cellular response (Norris, 1985), we measured thymidine incorporation/μg DNA daily for 144 h in MAC-T cells cultured in FBS (1%) with 0, 10, or 100 pM TGF-β2. TGF-β2 caused at least 50% inhibition across all times, in fact growth continued to decline until 72 h, after which ^{3}H-TdR/μg DNA stabilized at only 5% the level of cells not treated with TGF-β. Since cells were synchronized (to maximize early differences in ^{3}H-TdR) and because the MAC-T cell cycle is approximately 17 h (Huynh et al., 1991) little difference in ^{3}H-TdR/μg DNA was evident when comparing TGF-β treated or untreated cells at 24 h. ^{3}H-TdR in MAC-T cells in 1% FBS is maximal at approximately 17 h; however, by 24 h most serum-supplemented cells have progressed through the S phase into G_2 (Lu and Means, 1993), while serum-starved and growth inhibited cells have been arrested in G_0/G_1. Thus, ^{3}H-TdR (occurring during the S phase) is minimal at this time. By 48 h, however, the cells have escaped synchronization, since after the third progression (51 h) the cell cycle is close to random (Freshney, 1994). Thus, all time points except 24 h (Fig. 8) yield valid comparisons as a high percentage of cells are potentially in S-phase.

To permit long term culture of epithelium and fibroblasts, we have immortalized several lines with the SV-40 Large-T-antigen (LT). Non-transfected mammary epithelial cells typically survive only 10–25 passages, whereas MAC-T cells (with the LT) have maintained growth and morphological properties after 350 passages (Huynh et al., 1991). Although oncogene "immortalization" may alter many cellular functions, MAC-T cells retain many properties of in vivo mammary epithelial cells, such as the presence of tight junctions, gap junctions, as well as the ability to differentiate (synthesis of caseins, lactose, and lipids) in response to prolactin and appropriate extracellular matrices (Huynh et al., 1991; MacDonald et al., 1994). Likewise, LT transfected mammary fibroblasts are growth arrested when cultured in the absence of appropriate hormone/growth factor stimulation. However, several studies using a variety of cell types have shown that transfection with LT results in a loss of growth inhibition to TGF-β (Hosobuchi and Stampfer, 1989; Laiho et al., 1990; Pietenpol et al., 1990b; Missero et al., 1991). TGF-β induced inhibition of DNA synthesis has been shown to involve retention of the retinoblastoma gene product, pRB, in an underphosphorylated state which arrests cells in middle or late G1 phase of the cell cycle (Laiho et al., 1990). The LT binds hypophosphorylated pRB, thereby releasing cells from normal growth arrest (Weinberg, 1991). An elegant series of experiments have demonstrated TGF-β may function through suppression of c-myc gene expression in some cell types (Pietenpol et al., 1990a). In these cells, a TGF-β control element in the promoter of c-myc appears to require hypophosphorylated pRB for TGF-β mediated suppression of c-myc and subsequent suppression of growth (Pietenpol et al., 1991; Moses, 1992). Thus, high levels of LT could tie up much hypophosphorylated pRB and induce resistance to TGF-β growth inhibition.

In the present study, we have shown that incorporation of the LT transgene did not negate TGF-β inhibitory properties in either parenchymal or stromal cells of the bovine mammary gland. First, the MAC-T cell line was growth inhibited by extremely low concentrations of TGF-β (ED_{50} = 4 pM). Secondly, there were no differences in growth inhibition (whether measured by DNA or thymidine assays) between normal and LT transfected fibroblasts. Moreover, one line, MF-T3, that developed a transformed phenotype subsequent to LT transfection remained growth inhibited by the same concentrations of TGF-β that effectively inhibit normal (non-transfected) fibroblasts (Table 1). Our results are consistent with others, who have shown that transformation by oncogenes such as HPV E7 or LT which bind pRB was not able to induce TGF-β resistance (Valverius et al., 1989; Braun et al., 1990; Woodworth et al., 1990). Furthermore, it has been reported that TGF-β induced fibroblast growth inhibition may function independently of c-myc expression (Moses, 1992) and the mammary gland in general appears to be resistant to transformation induced by LT (Choi et al., 1987, 1988; Wolff et al., 1992).

In summary, our studies have shown the cell specific regulation of bovine mammary cell growth by TGF-β1 and TGF-β2 and have analyzed TGF-β receptors on two cell types of the bovine mammary gland. Other researchers have documented the synthesis and secretion of TGF-β isoforms in lactating and non-lactating tissue (Cox and Burke, 1991; Maier et al., 1991; Tokuyama and Tokuyama, 1993). Thus, the presence of TGF-β synthesis and secretion by bovine mammary cells, together with our results on the characterization of TGF-β receptors and on the potent inhibitory effects of TGF-β on bovine mammary cells, provide a basis for further studies to define the role of TGF-β in bovine mammary development, involution, and lactation.

ACKNOWLEDGMENTS

The authors thank Elaine de Heuvel and Cathy Teng for excellent technical assistance and extend our gratitude to Lisa Gloutney for preparing this manuscript

for print. TGF-β1 (used for radiolabelling) was a gift from Bristol-Meyer Squibb.

LITERATURE CITED

Akers, R.M. (1990) Lactation physiology: A ruminant animal perspective. Protoplasma, *159*:96–111.

Braun, L., Durst, M., Mikumo, R., and Gruppuso, P. (1990) Differential response of nontumorigenic and tumorigenic human papillomavirus type 16-positive epithelial cells to transforming growth factor β1. Cancer Res., *50*:7324–7332.

Cheifetz, S., Hernandez, H., Laiho, M., ten Dijke, P., Iwata, K., and Massague, J. (1990) Distinct transforming growth factor-β receptor subsets as determinants of cellular responsiveness to three TGF-β isoforms. J. Biol. Chem., *265*:20533–20538.

Choi, Y., Henrard, D., Lee, and Ross, S.R. (1987) The mouse mammary tumor virus long terminal repeat directs expression in epithelial and lymphoid cells of different tissues in transgenic mice. J. Virol., *61*:3013–3019.

Choi, Y., Lee, I., and Ross, S.R. (1988) Requirement for the simian virus 40 small tumor antigen in tumorigenesis in transgenic mice. Mol. Cell. Biol., *8*:3382–3390.

Cox, D.A., and Burk, R.R. (1991) Isolation and characterization of milk growth factor, a transforming-growth-factor-β2-related polypeptide, from bovine milk. Eur. J. Biochem., *197*:353–358.

Daniel, C.W., and Robinson, S.D. (1992) Regulation of mammary growth and function by TGF-β. Mol. Reprod. Dev., *32*:145–151.

Daniel, C.W., Silberstein, G.B., Van Horn, K., Strickland, P., and Robinson, S. (1989) TGF-β1-induced inhibition of mouse mammary ductal growth: Development specificity and characterization. Dev. Biol., *135*:20–30.

Danielpour, D., and Sporn, M.B. (1990) Differential inhibition of transforming growth factor β1 and β2 by α2 macroglobulin. J. Biol. Chem., *265*:6973–6977.

Franzen, P., ten Dijke, P., Ichijo, H., Yamashita, H., Schulz, P., Heldin, C.H., and Miyazono, K. (1993) Cloning of TGF-β type I receptor that forms a heteromeric complex with the type II TGF-β receptor. Cell, *75*:681–692.

Freshney, R.I. (1994) Specialized techniques. In: Culture of Animal Cells: A Manual of Basic Technique. R.I. Freshney, ed. Wiley-Liss Inc., New York, Vol. 3, pp. 369–404.

Hosobuchi, M., and Stampfer, M.R. (1989) Effects of transforming growth factor-β on growth of human mammary epithelial cells in culture. In Vitro Cell. Dev. Biol., *25*:705–713.

Huynh, H.T., Robitaille, G., and Turner, J.D. (1991) Establishment of bovine mammary epithelial cells (MAC-T): An in vitro model for bovine lactation. Exp. Cell Res., *197*:191–199.

Ikeda, T., Lioubin, M.N., and Marquardt, H. (1987) Human transforming growth factor type β2: Production by a prostatic adenocarcinoma cell line, purification, and initial characterization. Biochemistry, *26*:2406–2410.

Jhappan, C., Geiser, A.G., Kordon, E.C., Bagheri, D., Henninghausen, L., Roberts, A.B., Smith, G.H., and Merlino, G. (1993) Targeting expression of a transforming growth factor β1 transgene to the pregnant mammary gland inhibits alveolar development and lactation. EMBO J., *12*:1835–1845.

Jin, Y., Cox, D.A., Knecht, R., Raschdorf, F., and Cerletti, N. (1991) Separation, purification, and sequence identification of TGF-β1 and TGF-β2 from bovine milk. J. Prot. Chem., *10*:565–575.

Joyce, M.E., Roberts, A.B., Sporn, M.B., and Bolander, M.E. (1990) Transforming growth factor-β and the initiation of chondrogenesis and osteogenesis in rat femur. J. Cell Biol., *110*:2195–2207.

Labarca, C., and Paigen, K. (1980) A simple, rapid, and sensitive DNA assay procedure. Anal. Biochem., *102*:344–352.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assemblage of the head of bacteriophage T4. Nature, *227*:680–685.

Laiho, M., DeCaprio, J.A., Ludlow, J.W., Livingston, D.M., and Massague, J. (1990) Growth inhibition by TGF-β linked to suppression of retinoblastoma protein phosphorylation. Cell, *62*:175–185.

Lin, H.Y., Wang, Z.F., Ng-Eaton, E., Wainberg, R.A., and Lodish, H.F. (1992) Expression cloning of the TGF-β type II receptor, a functional transmembrane serine/threonine kinase. Cell, *68*:775–785.

Lopez-Casillas, F., Cheifetz, S., Doody, J., Andres, J., Lane, W.S., and Massague, J. (1991) Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-β receptor system. Cell, *67*:785–795.

Lu, K.P., and Means, A.R. (1993) Regulation of the cell cycle by calcium and calmodulin. Endocr. Rev., *14*:40–58.

MacDonald, E.A., Xia, L., Monardes, H., and Turner, J.D. (1994) Neutrophil function in vitro: Diapedesis and phagocytosis. J. Dairy Sci., *77*:628–638.

Maier, R., Schmid, P., Cox, D., Bilbe, G., and McMaster, G.K. (1991) Localization of transforming growth factor-β1, -β2 and -β3 gene expression in bovine mammary gland. Mol. Cell. Endocrinol., *82*:191–198.

Massague, J. (1990) The transforming growth factor-β family. Ann. Rev. Cell Biol., *6*:597–641.

McKiernan, S.H., and Bavister, B.D. (1992) Different lots of bovine serum albumin inhibit or stimulate in vitro development of hamster embryos. In Vitro, *28A*:154–156.

Missero, C., Filvaroff, E., and Paolo Dotto, G. (1991) Induction of transforming growth factor β1 resistance by the E1A oncogene requires binding to a specific set of cellular proteins. Biochemistry, *88*:3489–3493.

Mitchell, E.J., and O'Connor-McCourt, M.D. (1991) A transforming growth factor-β (TGF-β) receptor from human placenta exhibits a higher affinity for TGF-β2 than for TGF-β1. Biochemistry, *30*:4350–4356.

Mitchell, E.J., Fitz-Gibbon, L., and O'Connor-McCourt, M.D. (1992) Subtypes of betaglycan and of type I and type II transforming growth factor-β (TGF-β) receptors with different affinities for TGF-β1 and TGF-β2 are exhibited by human placental trophoblast cells. J. Cell. Physiol., *150*:334–343.

Moses, H.L. (1992) TGF-β regulation of epithelial cell proliferation. Mol. Reprod. Dev., *32*:179–184.

Norris, D.O. (1985) An introduction to vertebrate endocrinology. In: Vertebrate Endocrinology. D.O. Norris, ed. Lea & Febiger, Philadelphia, pp. 1–25.

Otsuka, H., Dubbs, D.R., and Kit, S. (1979) Variant lines of mouse kidney cells transformed by an SV40-TSA mutant with growth properties of wild-type transformed-cells at non-permissive temperature. J. Gen. Virol., *42*:373–386.

Pelton, R.W., Dickinson, M.E., Moses, H.L., and Hogan, B.L.M. (1990) In situ hybridization analysis of TGF-β3 RNA expression during mouse development: Comparative studies with TGF-beta-1 and beta-2. Development, *110*:609–620.

Philip, A., and O'Connor-McCourt, M.D. (1991) Interaction of transforming growth factor-β1 with α_2-macroglobulin. J. Biol. Chem., *266*:22290–22296.

Philip, A., Bostedt, L., Stigbrand, T., and O'Connor-McCourt, M. (1994) Binding of transforming growth factor β (TGF-β) to pregnancy zone protein (PZP): Comparison to the TGF-β–α 2-macroglobulin interaction. Eur. J. Biochem., *221*:687–693.

Pietenpol, J.A., Stein, R.W., Moran, E., Yaciuk, P., Schlegel, R., Lyons, R.M., Pettelkow, M.R., Munger, K., Howley, P.M., and Moses, H.L. (1990a) TGF-β1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. Cell, *61*:777–785.

Pietenpol, J.A., Holt, J.T., Stein, R.W., and Moses, H.L. (1990b) Transforming growth factor-β1 suppression of c-myc gene transcription: Role in inhibition of keratinocyte proliferation. Proc. Natl. Acad. Sci. U.S.A., *87*:3758–3762.

Pietenpol, J.A., Munger, K., Howley, P.M., Stein, R.W., and Moses, H.L. (1991) Factor-binding element in the human c-myc promoter involved in transcriptional regulation by transforming growth factor β1 and by the retinoblastoma gene product. Proc. Natl. Acad. Sci. U.S.A., *88*:10227–10231.

Roberts, A.B., and Sporn, M.B. (1990) The transforming growth factor-betas. In: Peptide Growth Factors and Their Receptors. M.B. Sporn and A.B. Roberts, eds. Springer-Verlag, Heidelberg, Germany, pp. 421–472.

Roberts, A.B., and Sporn, M.B. (1992) Differential expression of the TGF-β isoforms in embryogenesis suggests specific roles in developing and adult tissues. Mol. Reprod. Dev., *32*:91–98.

Robinson, S.D., Silberstein, G.B., Roberts, A.B., Flanders, K.C., and Daniel, C.W. (1991) Regulated expression and growth inhibitory effects of transforming growth factor-β isoforms in mouse mammary gland development. Development, *113*:867–878.

Robinson, S.D., Roberts, A.B., and Daniel, C.W. (1993) TGF-β suppresses casein synthesis in mouse mammary explants and may play a role in controlling milk levels during pregnancy. J. Cell Biol., *120*:245–251.

SAS Institute Inc. (1988) SAS/STAT User's Guide. Release 6.03. Cary, NC.

Segarini, P.R., Roberts, A.B., Rosen, D.M., and Seyedin, S.M. (1987) Membrane binding characteristics of two forms of transforming growth factor-β. J. Biol. Chem., *262*:14655–14662.

Silberstein, G.B., and Daniel, C.W. (1987) Reversible inhibition of mammary gland growth by transforming growth factor-β. Science, *237*:291–293.

Southern, P.J., and Berg, P. (1982) Transformation of mammalian

cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Gen., *1*:327–341.
Tokuyama, Y., and Tokuyama, H. (1993) Purification and identification of TGF-β2-related growth factor from bovine colostrum. J. Dairy Res., *60*:99–109.
Valverius, E.M., Walker-Jones, D., Bates, S.E., Stampfer, M.R., Clark, R., McCormick, F., Dickson, R.B., and Lippman, M.E. (1989) Production of and responsiveness to transforming growth factor-β in normal and oncogene-transformed human mammary epithelial cells. Cancer Res., *49*:6269–6274.
Wakefield, L.M., Smith, D.M., Masui, T., Harris, C.C., and Sporn, M.B. (1987) Distribution and modulation of the cellular receptor for transforming growth factor-beta. J. Cell Biol., *105*:965–975.
Wang, Z.F., Lin, H.Y., Ng-Eaton, E., Downward, J., Lodish, H.F., and Weinberg, R.A. (1991) Expression cloning and characterization of the TGF-β type III receptor. Cell, *67*:797–805.
Weinberg, R.A. (1991) Tumor suppressor genes. Science, *254*:1138–1146.
Wolff, J., Wong, C., Cheng, H., Poyet, P., Butel, J.S., and Rosen, J.M. (1992) Differential effects of the simian virus 40 early genes on mammary epithelial cell growth, morphology, and gene expression. Exp. Cell Res., *202*:67–76.
Woodward, T.L., Akers, R.M., and Turner, J.D. (1994) Lack of mitogenic response to EGF, pituitary and ovarian hormones in bovine mammary epithelial cells. Endocr. J., *2*:529–535.
Woodworth, C.D., Notario, V., and DiPaolo, J.P. (1990) Transforming growth factor beta 1 and 2 transcriptionally regulate human papillomavirus (HPV) type 16 early gene expression in HPV-immortalized human genital epithelial cells. J. Virol., *64*:4767–4775.
Zar, J.H. (1984) Multiple comparisons. In: Biostatistical Analysis. J.H. Zar, ed. Prentice Hall Inc. Englewood Cliffs, NJ, pp. 194–195.
Zhao, X., McBride, B.W., Politis, I., Huynh, H.T., Akers, R.M., Burton, J.H., and Turner, J.D. (1992) Receptor binding and growth-promoting activity of insulin-like growth factor-1 in a bovine mammary epithelial cell line (MAC-T3). J. Endocr., *134*:307–312.

SUMMARY 3

This chapter analyzed the effects of TGF-β on bovine mammary cell proliferation, defined the presence of cellular receptors and examined their regulation. Mammary epithelial and fibroblast lines from chapter 1 were used. Cellular proliferation was stimulated by FBS, insulin or IGF-I as described in chapter 2. This report is the first to describe potent growth inhibitory effects to TGF-β in the bovine mammary gland. Mammary epithelial cells were growth arrested by TGF-β at very low concentrations, in fact the concentrations were among the lowest ever reported for any cell type in any species. TGF-β growth inhibitory effects were reversible. Additionally, this study determined that fibroblasts were growth inhibited by TGF-β, though not growth arrested.

The pathway involved in TGF-β growth inhibition is currently under investigation, however, in several cell types, it appears to involve the cell cycle regulators, pRB or p53. The SV-40 Large-T-antigen, previously used to immortalize the MAC-T bovine mammary epithelial cell line, has been shown to bind both pRB and p53 and may interfere with their regulation of cell cycle progression. In this study we showed that TGF-β action in bovine mammary cells is not inhibited by transfection with the SV-40 Large-T-antigen. Furthermore, even when fibroblasts were transformed following Large-T-antigen transfection, they did not lose responsiveness to TGF-β.

With regard to TGF-β receptors, we found that fibroblasts and epithelial cells contained type I, II and III receptors and unexpectedly TGF-β2 had an equal or greater affinity for these receptors than TGF-β1. Additionally, these receptors were downregulated by TGF-β1 or TGF-β2. However, downregulation of receptors did not affect cellular inhibition induced by TGF-β. Therefore, this chapter was the first study to identify TGF-β as a potent reversible growth inhibitor of bovine mammary epithelial cells and fibroblasts.

CHAPTER 4

Inhibiton of cellular proliferation and modulation of insulin-like growth factor binding proteins by retinoids in a bovine mammary cell line

T.L. Woodward, H.T. Huynh, J.D. Turner, and X. Zhao.

Accepted for publication in The Journal of Cellular Physiology (1996)

ABSTRACT

Retinoids are potent inhibitors of growth and tumor progression in many mammary carcinoma cell lines, though regulation of growth in non-tumorigenic mammary epithelial cells by retinoids is less clear. Here, we have characterized the inhibition of MAC-T (a non-transformed bovine mammary epithelial cell line) cellular proliferation by retinoids and their role in regulating insulin-like growth factor binding proteins (IGFBPs). Retinoic acid (RA) (100 nM) was a potent inhibitor of MAC-T cell proliferation. Retinol was 10-100 times less effective. Neither retinoid could completely arrest growth at non-cytotoxic concentrations. Retinoic acid inhibited cellular proliferation by 1 h ($P<.05$), but inhibition was 5-fold greater by 24 h ($P<.01$). This second stage of growth inhibition (after 12 h) was dependent upon protein synthesis. However, RA induced inhibition of cellular proliferation did not persist, with thymidine incorporation increasing toward control levels by 4 d in culture. Retinoic acid was less effective in inhibiting thymidine incorporation when cells were stimulated with insulin, des(1-3) IGF-I or Long(R^3) IGF-I when compared to cells stimulated with native IGF-I or serum. Inhibition of proliferation by RA was associated with increased levels of IGFBP-2 in conditioned media and in plasma membrane preparations. Treatment with insulin or des(1-3) IGF-I resulted in the appearance of IGFBP-3 in conditioned media and on the cell surface. However, RA significantly reduced IGFBP-3 levels in conditioned media, and eliminated IGFBP-3 associated with the plasma membrane. Thus, RA is a potent but transient inhibitor of bovine mammary epithelial cell proliferation and this growth

inhibition is correlated with increased IGFBP-2 accumulation and inhibition of IGF-I stimulated IGFBP-3 protein secretion.

INTRODUCTION

Retinoids inhibit the proliferation of many mammary carcinoma cell lines (Fontana et al., 1991; Adamo et al., 1992; Decensi et al., 1993; Halter et al., 1993; Shemer et al., 1993). However, our understanding of retinoid action in normal mammary epithelium is limited. Retinol is transported in the circulation by retinol binding proteins (Creek et al., 1993) and intracellularly retinol and retinoic acid (RA) are bound to a distinct set of cellular retinol binding proteins (CRBPs) and cellular retinoic acid binding proteins (CRABPs) (Wolf and Phil, 1991; Napoli, 1993; Giguere, 1994). Although retinoids have been reported to alter cellular events independent of receptor binding (Samokyuszyan et al., 1984; Pitts et al., 1986; Moore et al., 1992), retinoid acid receptors have been identified and shown to regulate transcription of genes containing retinoic acid response elements (RARE) (Chambon et al., 1991; Richard and Zingg, 1991; Marshall et al., 1994; Penricks et al., 1994). Furthermore, even though RA has been recognized as a growth inhibitor for decades, only more recent studies have probed into the mechanism of RA action and have demonstrated the involvement of RA in transcriptional regulation of several genes which directly or indirectly result in growth inhibition, tumor suppression and modulation of differentiation and cell-to-cell communication (De Luca, 1991; Giguere, 1994; Hossain and Bertram, 1994; Man, 1994; Ruberte, 1994).

Insulin-like growth factor binding proteins (IGFBPs) are one family of proteins that are regulated by RA and at least one member (IGFBP-6) contains a RARE within its promoter (Zhu et al., 1993). Six IGFBPs have been cloned and sequenced and nearly all of IGF-I transported extracellularly is bound to one of these high affinity binding proteins (Jones and Clemmons, 1995). Also, a new IGFBP, mac25, has been described and likely contains a RARE in its promoter (Swisshelm et al., 1995). Previously, we have shown IGF-I (including des(1-3) IGF-I and Long(R^3) IGF-I) to be among the most potent stimulators of bovine mammary epithelial cell growth (Woodward et al., 1994). In fact, IGF-I was a considerably stronger mitogen in MAC-T cells or primary bovine mammary epithelial cells than estrogen, progesterone, growth hormone, prolactin, insulin, epidermal growth factor, TGF-α, TGF-β or hydrocortisone (Woodward et al., 1994, 1995). IGFBPs, on the other hand, have been shown to potentiate or attenuate the effects of IGF-I (for review, Clemmons et al., 1995). IGFBPs can augment or inhibit IGF-I mediated cellular proliferation depending upon 1) the type of IGFBP, 2) the cell type, and 3) the cell environment, i.e. extracellular matrix, serum, hormones, etc. Furthermore, IGFBP-3 has recently been shown to be a ligand for a cell surface receptor in a breast cancer cell line. Upon binding to this receptor IGFBP-3 inhibits cellular proliferation, independent of IGF-I (Oh et al., 1992; 1993 a,b).

In several breast cancer cell lines, RA blocks IGF-I stimulation of cellular proliferation. In MCF-7 cells this inhibition of proliferation has been correlated with increased levels of IGFBP-4 and the appearance of IGFBP-3 in conditioned media (Fontana et al., 1991;

Adamo et al., 1992; Sheikh et al., 1992). Interestingly, growth inhibition and IGFBP modulation by RA appear to preferentially affect estrogen receptor positive breast carcinoma lines (Fontana et al., 1991; Sheikh et al., 1993a). These cell lines already have altered steady-state IGFBP expression when compared to estrogen receptor negative lines (Clemmons et al., 1990; Sheikh et al., 1993a); further supporting the interaction of the steroid hormone receptor superfamily and IGFBPs in mammary epithelium.

In the present study we examined the proliferation of a non-transformed bovine mammary epithelial cell line (MAC-T) to retinol and RA in media without serum and without serum albumin, and in IGF-I spiked or serum-containing media. We have also described the distinct alteration in IGFBP profiles in conditioned media and plasma membrane preparations by RA with or without insulin and IGF-I. Our last objective of this study was to analyze these data and more clearly define the mechanism(s) of RA induced growth inhibition and contrast this with other known growth inhibitors in mammary epithelium.

MATERIALS AND METHODS

Materials and cell culture

All enzymes, hormones, media, serum and culture reagents were obtained from GIBCO BRL (Burlington, Ont.), Becton Dickinson (St. Laurent, Que) or Sigma Chemical Co. (St. Louis, MO), unless otherwise noted. Recombinant human IGF-I, des(1-3) IGF-I and

Long(R^3) IGF-I were purchased from GroPep Pty. Ltd. (Adelaide, Australia). Retinol and all-trans retinoic acid (RA) were purchased from Fluka (Ronkonkoma, NY). Retinol and RA were solubilized in 100% ethanol at 37 C under nitrogen in the dark and always used within 24 h. Controls always contained ethanol at concentrations equal to those with retinoids. Final ethanol concentrations never exceeded 0.03%, unless otherwise noted.

The MAC-T cell line is an established bovine mammary epithelial cell line, clonal in origin and immortalized by low level constitutive expression of the SV-40 large-T-antigen (Huynh et al., 1991). Additionally, MAC-T cells are non-transformed as they show contact inhibition, are non-tumorigenic in nude mice, do not form foci in soft agar or on plastic, are responsive to FBS and exogenous growth factors, growth arrested by TGF-β1 or TGF-β2 and secrete α- and β-caseins (Huynh et al., 1991; Woodward et al., 1994, 1995). MAC-T cells were maintained for routine culture in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS in a humidified, 37 C, 5% CO_2 incubator as described previously (Woodward et al., 1994).

Quantification of cell proliferation

Cellular proliferation was measured by assaying both total DNA and incorporation of methyl-^{3}H-thymidine (^{3}H-TdR) (ICN, Irvin, CA; SA = 64 Ci/mmol) as previously described (Woodward et al., 1994). All cellular proliferation experiments had a minimum of 9 replicates per treatment with all experiments being repeated at least twice.

Positive (with FBS or in the absence of an inhibitor) and negative growth controls (serum-free or TGF-β1 supplemented) were used in all experiments to assure validity of results. To stimulate cell proliferation, FBS (1%), IGF-I (30 ng/ml), des(1-3) IGF-I (30 ng/ml), Long(R^3) IGF-I (30 ng/ml) or insulin (3 μg/ml) were used. Briefly, for ^{3}H-TdR, 1 X 10^4 MAC-T cells/cm^2 were plated, attached overnight, and serum-starved either 48 h and treatments applied, or treatments applied in FBS directly after plating. Thymidine incorporation was determined as described previously (Woodward et al., 1994). For DNA assays, the treatment protocol was identical to ^{3}H-TdR assay except for a 72 h treatment period, after which cells were lysed, harvested in sample buffer containing 1 μg/ml Hoescht 33258 dye and the absorbance measured fluorometrically as described previously (Woodward et al., 1993). For long term (7 d) thymidine experiments, cells were plated at a density of 1 X 10^3 cells/cm^2. Cells were refed with fresh media and treatments every 24 h during all experiments.

Cytotoxicity assay

Cytotoxicity of retinoids was measured by culturing MAC-T cells in retinol or RA for 72 h. Media and treatments were changed daily. Subsequently, total cell number and live cell number, measured by trypan blue exclusion, were quantitated by hemocytometer (9 replicates/treatment). Final ethanol concentrations in the cytotoxicity assay never exceeded 0.3%.

Plasma membrane preparations

MAC-T cells were cultured as described above for conditioned media sample collection.

Enriched plasma membrane was prepared, using a modified protocol described by Pollak and coworkers (1990), by rinsing MAC-T cells in cold PBS 2X, lysing cells in hypotonic buffer (5 ml/plate: 1 mM EDTA, 1 mM sodium bicarbonate, inhibitors as described in Western ligand blotting section, except NaF was omitted), and homogenized on ice for 10 s (Polytron PT100, 1/2 speed). Homogenate was first centrifuged at 1000 X g for 10 min to remove nuclei and supernatant was transferred to new tubes and centrifuged again at 15,000 X g for 5 min to remove lysosomal contamination (Darnell et al., 1990). The supernatant was then centrifuged at 50,000 X g for 40 min to pellet the membrane proteins. The membrane pellet was resuspended in sample buffer with inhibitors. Samples were then adjusted to equal protein concentrations by BCA assay (Chromatographic, Brocksville, Ont.).

Western ligand blotting

MAC-T cells (1.8 X 10^4 cells/cm^2) were plated in DMEM containing 10% FBS overnight. Prior to treatment, cells were maintained in serum-free DMEM for 48 h. Cells were cultured in indicated treatments for 48 h and conditioned media was harvested for Western ligand blotting analysis. In some samples, protease inhibitors (see below) were immediately added upon media collection. Conditioned media were then centrifuged at 2000 X g at 4 C for 5 min to remove cells. Conditioned medium samples were either frozen (-80 C) or analyzed immediately. Conditioned media was either ran unconcentrated or concentrated 5-10 fold on SDS-PAGE gels as indicated. Media were concentrated 5-10 fold using 10,000 molecular weight cut off low protein binding

cellulose filters (Millipore, Mississauga, Ontario, Canada). Samples were adjusted by protein content following concentration. Conditioned medium samples were separated using SDS-PAGE, 12% acrylamide, according to Laemmli (1970) under non-reducing conditions. Samples were prepared in the presence of the following inhibitors: phenylmethylsulfonyl fluoride (2 mM), leupeptin (3 μM), aprotinin (16 μM), EDTA (2 mM), NaF (50 mM) and $NaVO_4$ (500 μM). Samples were electrotransferred onto nitrocellulose filters and probed with ^{125}I-IGF-I (S.A.= 2000 Ci/mmol) (Amersham, Oakville, Ont.), as previously described by Hossenlopp (1986). Filters were exposed to Kodak X-Omat film with Cronex lighting-plus intensifying screens (DuPont) at -80 C. Filters were also exposed overnight to 2 d in a PhosphorImager cassette and subsequently scanned for band intensity in a PhosphorImager (Molecular Dynamics, San Fransico, CA). Some autoradiographs were recorded by videocamera and the image was digitized on a PowerMac 7100AV. Band intensity on the digitized image was quantified using NIH-Image version 1.57 (1995) software.

Statistical analysis

The general linear models procedure (PROC GLM) of SAS (1988) was used to analyze dependent variables. Data from proliferation assays were analyzed using Dunnett's test (Zar, 1984), when comparing treatments to control. Comparisons between treatments utilized non-orthogonal contrasts to obtain F values. These critical F values were then replaced by the Bonfernoni F test (Zar, 1984).

RESULTS

Growth response of MAC-T cells to retinoids

Retinoic acid inhibited cellular proliferation at 100 nM in both DNA and ^{3}H-TdR assays (Figures 1a and 2b). In contrast, retinol inhibited ^{3}H-TdR in MAC-T cells only at a high concentration (10 μM) (Figure 1b). Similarly, using total DNA as an indice of cellular proliferation, retinol inhibited growth at 1 and 10 μM (Figure 2d). Neither retinoid was able to completely arrest growth without significant cytotoxic effects. Based on a trypan-blue exclusion assay, RA and retinol were 100% cytotoxic after 72 h culture at 100 μM and 1 mM, respectively. Interestingly, both retinoids were able to modestly increase total DNA ($P<=.05$) of MAC-T cells at 100 nM, when cultured in serum-free, non supplemented DMEM for 72 h (Figures 2a and 2c). Retinoic acid increased ^{3}H-TdR in serum-free MAC-T cells, also, though results were not significant ($P>.10$) (data not shown).

Time course of retinoic acid induced growth inhibition

In the presence of 1% FBS, RA significantly inhibited ^{3}H-TdR into MAC-T cells, and this inhibition was observed as early as 1 h after RA addition ($P<.05$) (Figure 3a). Inhibition was persistant between 2 and 12 h, then further decreased to levels of less than 50% of control cells and this growth inhibition was significantly greater than that observed by 12 h (12 h vs 24 h, $P<.01$) (Figure 3a). In the presence of 10 μM cycloheximide, RA inhibited MAC-T cellular proliferation only by 20% or less ($P<.05$)

and growth inhibition was not different across times (Figure 3b). Thus, it appears that RA inhibits growth in a phasic manner, with the first phase occurring rapidly (in less than 1 h) and the second and much more potent inhibition not occurring until after 12 h and requiring protein synthesis. Furthermore, RA maintained its inhibition of MAC-T cellular proliferation up to 3 d after treatments began (Figure 3c). Proliferation, however, was less inhibited on d 4 and 5 and was not different from control by d 6. Interestingly, on d 7 RA treated cells incorporated more ^{3}H-thymidine than control cells ($P<.05$) (Figure 3c). Cells were not growth inhibited in control (1% FBS-no RA) treatments by topo-inhibition, as ^{3}H-TdR counts were much higher than previous days (CPM: d3=29,300, d6=54,673, d7=76,270). Also, a previous experiment with MAC-T cells under identical conditions has shown that TGF-β2 could maintain complete growth inhibition of ^{3}H-TdR, while control cells incorporated thymidine at rates 20 fold higher for at least 6 days, thus demonstrating growth was not impaired in control cells (Woodward et al., 1995).

Mitogen dependent retinoic acid growth inhibition

Retinoic acid (1 μM) inhibited ^{3}H-TdR of MAC-T cells by approximately 50% (Figure 1b, 3a and 4) when cells were cultured in 1% FBS. However, cells cultured in IGF-I were growth inhibited (^{3}H-TdR) 64% by RA (Figure 4). Whereas, when stimulated by insulin, des(1-3) IGF-I or long(R^3) IGF-I, cells were only growth inhibited by RA 20%, 27% and 15%, respectively (Figure 4). Interestingly, the growth inhibition caused by RA in the presence of mitogens with no or very low affinity for IGFBPs (insulin, des(1-

3) IGF-I or Long(R^3) IGF-I) was similar to growth inhibition by RA in cycloheximide treated MAC-T cells.

IGFBPs in retinoic acid treated MAC-T cells

The major IGFBP secreted into media by MAC-T cells when cultured in serum-free, hormone/growth factor-free conditions is a single strong band at 30 kDa (Figure 5a). We have previously demonstrated this band to be IGFBP-2 (Romagnolo et al., 1994). When MAC-T cells were cultured in serum-free DMEM, all other IGFBP bands are at or below the level of detection in unconcentrated conditioned media. Again in serum-free DMEM, RA caused a dose dependent accumulation of IGFBP-2 in the media and maximal levels were observed at a concentration of 1 μM and at a higher concentration (10 μM) IGFBP-2 accumulation was reduced (Figure 5a). The optimal time of IGFBP-2 accumulation in the conditioned media of MAC-T cells was between 36 and 72 h for all treatments (Figures 5b and 5c). Thus subsequent experiments used RA at concentrations of 1 μM or less and cells were cultured for 48 h to optimize IGFBP accumulation and minimize degradation of binding proteins that is inherent with extended culture periods.

IGFBP-2 was stimulated 2-3 fold by insulin or des(1-3) IGF-I, 5 fold by RA and 7-8 fold by RA + insulin or RA + des(1-3) IGF-I (Figure 6 a,b). Insulin or des(1-3) IGF-I treatment of serum-free cultured MAC-T cells resulted in the appearance of a 40-45 kDa doublet, previously identified as IGFBP-3 (Romagnolo et al., 1994). Unexpectedly, RA was a potent inhibitor of insulin or des(1-3) IGF-I induced IGFBP-3 accumulation into

conditioned media (Figure 6a,c and 7). In fact, RA reduced IGFBP-3 in conditioned media of MAC-T cells stimulated by insulin or des(1-3) IGF-I by 80%. Interestingly, RA alone induced a faint band at 40-45 kDa when cultured in serum-free media (Figure 6 a,c and 7).

When samples were concentrated 5-10 fold by passage through a 10,000 kDa cut-off low protein binding filter, visualization of IGFBP-3 was substantially enhanced. Retinoic acid (1 μM) could completely block IGFBP-3 induced by a low concentration of insulin (300 ng/ml). Attenuation of IGFBP-3 induced by a high insulin concentration (3 μg/ml) by RA was also observed (Figure 7). Additionally, a faint 24 kDa IGFBP was also observed in concentrated samples (Figure 7). This IGFBP is probably IGFBP-4. It is not likely a breakdown product of higher molecular weight IGFBPs, because no differences were seen between samples prepared in the presence of proteolytic and phosphatase inhibitors when compared to samples without inhibitors (data not shown). The autoradiograph in figure 7 was prepared from samples containing inhibitors.

Crude plasma membrane preparations contained an IGFBP profile similar to conditioned media (Figure 8). IGFBP-2 was increased by insulin, des(1-3) IGF-I or RA with a strong synergistic action when RA was combined with insulin or des(1-3) IGF-I. One unique feature of the plasma membrane preparations was that IGFBP-3 induced by insulin (3 μg/ml) or des(1-3) IGF-I (30 ng/ml) was completely lost when treated with RA (1 μM). Also, there was no detectable IGFBP-3 in RA treated cells. The plasma membrane

preparations also revealed a more distinct doublet for IGFBP-3 when treated with insulin or des(1-3) IGF-I than conditioned media, though this may represent differences in total IGFBP-3 protein loaded, when compared to conditioned media preparations.

DISCUSSION

In this investigation we have demonstrated that the retinoids, RA and retinol, inhibit cellular proliferation of MAC-T cells, a non-transformed bovine mammary epithelial cell line. Similarly, others have shown that RA inhibits proliferation of breast cancer cell lines (Lacroix and Lippman, 1980; Fontana et al., 1991; Sheikh et al., 1993). More recently, RA has been shown to inhibit proliferation of normal rat mammary epithelium when these non-clonal primary epithelial cells were cultured in basement membrane extracted from the Englebreth-Holm-Swarm sarcoma (Lee et al., 1995). The present study, however, is the first to demonstrate the potent growth inhibitory properties of RA in a non-transformed clonal mammary epithelial line devoid of stromal components, exogenous extracellular matrices and in the absence of serum and serum albumin. Furthermore, we have examined IGFBP secretion and cell surface associated IGFBP in MAC-T cells treated with RA, since RA induced growth inhibition was markedly reduced when MAC-T cells were cultured in IGF-I analogues that show reduced affinity for IGFBPs, suggesting that RA inhibits growth through a pathway involving IGFBPs. Specifically, we have shown RA to increase IGFBP-2 5-fold and eliminate IGF-I induced

IGFBP-3 in conditioned media and on plasma membranes of MAC-T cells. These results are in contrast to results reported in breast cancer cells, as Chen and colleagues (1994) and Martin and coworkers (1995) reported that RA enhanced IGFBP-3 secretion into the media in the MCF-7 mammary tumor cell line.

Retinoid induced growth inhibition

Inhibition of mammary tumor growth by RA is not novel, however, as RA has proven effective in retarding the incidence, appearance and growth of many mammary tumors in both in vitro and in vivo studies spanning at least 20 years (Moon et al., 1976; Lotan and Nicolson, 1977; Lotan, 1979; Lacroix and Lippman, 1980; Fraker et al., 1984; Marth et al., 1984; Fontana et al., 1990). It is of great interest to determine if and how normal mammary epithelium may also be affected by preventive or therapeutic retinoid treatment, though only a few studies have examined RA effects on non-transformed mammary epithelial cells. A manuscript by Lotan (1979) originally found no significant change in growth of a nonmalignant myoepithelial line and minimal inhibition (13%) in the normal breast epithelial line, HBL-100 following 7 d of culture in RA containing media (1 μM). However, the HBL-100 cell line has been reported to lack prolactin and estrogen receptors and does not secrete casein (Laherty et al., 1990), whereas the MAC-T cell line is more representative of functional mammary epithelium since it has been shown to morphologically and functionally (synthesize and secrete α- and β-caseins in response to prolactin) differentiate in culture.

Sixteen years after Lotan's study (1979), Lee and colleagues (1995), have shown non-clonal primary mammary epithelial cells from the rat are growth inhibited following culture in RA containing media for 5 d - 25 d. Our results confirm data by Lee and coworkers (1995), in that we also found RA to be a potent inhibitor of mammary epithelial cell proliferation. In addition to examining the mechanism of RA induced growth inhibition (see RA modulation of IGFBPs, below), the present study, for the first time, analyzed how RA affects cellular proliferation after short exposure times (1 h to 24 h) as well as in long term culture (1d to 7 d). Short term growth analyses are particularly relevant in light of findings by Moore and coworkers (1992) that retinoids can substantially alter Golgi apparatus morphology and enhance Golgi apparatus function in normal mammary epithelial cells after only 5 min retinoid exposure.

Using both ^{3}H-TdR and total DNA as measures of cell proliferation, we have determined MAC-T cells are growth inhibited by RA in as little as 1 h and maximally growth inhibited after 3 d in culture. This is in contrast to the study by Lee and coworkers (1995), which determined maximal inhibition of growth by RA in rat mammary epithelium occurred following 21 d of culture. In contrast, when MAC-T cells were cultured in RA for periods exceeding 5 d, cellular inhibition by RA was lost. Possible explantions for descrepancies between these results include: 1) Substantial variation of RA effects in breast tumor lines has been attributed to status of estrogen receptors and estrogen responsiveness (Fontana et al., 1991; Sheikh et al., 1993a) and whether these cells possess cellular retinoic acid binding proteins (Marth et al., 1984; Ueda et al., 1985), 2) MAC-T cells were growth stimulated by insulin, IGF-I or 1% FBS (serum

albumin was never added) and cultured in the absence of exogenous matricies, whereas a combination of fatty-acid free BSA, EGF, hydrocortisone, prolactin, insulin, transferrin, ascorbic acid and progesterone were used in growth media to culture these rat mammary cells grown inside EHS matrix in Lee's study. This is especially relevant since FBS and serum albumin have been reported to bind retinoic acid (Smith et al., 1973; Lacroix and Lippman, 1980) and alter its action. Moreover, we have previously demonstrated that several BSA preparations including fatty-acid free BSA significantly alter mammary epithelial cell proliferation (Woodward et al., 1994).

In the absence of protein synthesis, however, MAC-T cells are only minimally (11%) growth inhibited after 24 h culture in RA (1 μM). Moreover, when MAC-T cells are treated with RA (1 μM) for 1-4 h growth is inhibited 15-18%. Interestingly, HBL-100 cells are similarly growth inhibited by RA (1 μM). Since protein synthesis is required for RA to maximally inhibit growth in MAC-T cells, it is likely that RA inhibits growth by at least 2 mechanisms. The first of which occurs within 1 h of treatment and is protein synthesis independent. The second requires protein synthesis and adequate time, 24 h, (likely for accumulation of sufficient quantity of protein) to exhibit maximal growth inhibition.

RA modulation of IGFBPs and mechanisms of RA inhibition

Several studies have demonstrated that breast cancer cells inhibited by RA show marked increases in IGFBP-3 accumulation (Adamo et al., 1992; Sheikh et al., 1993b; Martin

et al., 1995). The appearance of IGFBP-3 occurs in conditioned media or plasma membrane preparations of MAC-T cells when treated with the mitogens, insulin or IGF-I. In fact, IGFBP-3 quantities in conditioned media and on cell membranes are similar to IGFBP-2 quantities (the major IGFBP secreted by MAC-T cells) following insulin/IGF-I treatment. However, RA causes a substantial decrease in insulin/IGF-I induced IGFBP-3 accumulation in conditioned media, and elimination of IGFBP-3 bound to plasma membrane preparations. Chen and colleagues (1994) demonstrated that IGFBP-3 enhanced DNA synthesis in MCF-7 cells. Interestingly, these authors have also reported that MCF-7 cells are growth inhibited by RA and show substantial increased IGFBP-3 secretion by RA. This study, which used MCF-7 cells that were transfected with an IGFBP-3 expression vector, found that increased IGFBP-3 secretion by these cells resulted in 1) enhanced IGF-I binding to the cells, 2) increased DNA synthesis in the transfected cells by IGF-I when compared to their non-transfected counterparts and 3) a decrease in IGF-I desensitization of its own receptor. Thus, since IGFBP-3 has been reported to enhance IGF-I mitigated DNA synthesis in MCF-7 cells, it would appear that increased IGFBP-3 secretion by these cells in response to RA would not be a mechanism by which RA induces growth inhibition, but possibly a compensatory mechanism to overcome RA mediated growth inhibition (Adamo et al., 1992).

In contrast, several lines of evidence indicate that changes in both the type and concentration of IGFBPs following RA treatment may regulate MAC-T cell growth. Substantial growth inhibition in MAC-T cells occurs only after 17-24 h, the time when

IGFBPs are first detectable in conditioned media. Additionally, in the absence of protein synthesis, MAC-T cells are only slightly growth inhibited by RA. Interestingly, IGFBP accumulation in conditioned media in response to RA is not persistent over time and new IGFBP accumulation is substantially lower following 108 h of culture than 72 h of culture. Similarly, inhibition of proliferation is diminished following 4 d of RA treatment. Moreover, we have previously shown IGFBP-3 to potentiate the growth stimulatory effects of IGF-I in a MAC-T cell line transfected with an IGF-I expression vector (Romagnolo et al., 1994). Thus, RA induced growth inhibition is correlated with RA induced IGFBP-3 downregulation.

In addition to RA causing a decrease in IGFBP-3 in conditioned media, RA prevents IGFBP-3 binding to cell membranes in MAC-T cells. IGFBP-3, by binding to the plasma membrane, may help localize IGF-I near its receptor and subsequently potentiate IGF-I action. Increased cell surface binding of IGFBP-3 has been correlated with a decrease in affinity for IGF-I and an increase in IGF-I mediated cellular proliferation (Conover, 1992; Jones and Clemmons, 1995). In fibroblasts, as much as 97% of cell surface IGF-I is bound to plasma membrane associated IGFBP-3 (Clemmons et al., 1987). Our data has shown substantial IGFBP-3 bound to the cell surface following treatment with mitogens, insulin or IGF-I, and hence during periods of maximal mitogenesis. These data refute the hypothesis that increases in IGFBP-3 in conditioned media upon IGF-I/insulin treatment are a result of release of cell surface associated IGFBP-3, because little to no cell surface IGFBP-3 exists until after insulin/IGF-I

treatment. Likewise, it appears RA does not promote cell surface binding of IGFBP-3 at the expense of IGFBP-3 in conditioned media, since IGFBP-3 is completely lacking in plasma membrane preparations from RA treated cells. Thus, shifts in IGFBP from conditioned media to cell membranes (or cell membranes to conditioned media) in response to RA treatment as suggested by Adamo and colleagues (1992) and demonstrated by Shemer et al., (1993) are not apparent in MAC-T cells.

The role of IGFBP-2 in modulating IGF-I mediated cell proliferation is, perhaps, more complicated since it is upregulated by insulin/IGF-I and strongly upregulated by RA, while RA + insulin or IGF-I results in an additive IGFBP-2 upregulation. Purified IGFBP-2 has been shown to inhibit cell proliferation in several in vitro studies (Jones and Clemmons, 1995). Morevover, Clemmons and coworkers (1990) have demonstrated that rapidly proliferating ER-negative breast carcinoma cell lines secrete primarily IGFBP-3, whereas ER-positive lines, which grow slower, secrete primarily IGFBP-2. Although exogenous IGF-I is a modest stimulus for IGFBP-2 secretion in MAC-T cells, we have previously demonstrated that in MDIGF-I MAC-T cells (MAC-T cells transfected with an inducible IGF-I expression vector) there is shift from IGFBP-2 expression to IGFBP-3 expression upon IGF-I induced expression (Romagnolo et al., 1994). Furthermore, this shift in expression from IGFBP-2 to IGFBP-3 is correlated with a large increase in DNA synthesis. Thus, it is plausible that IGFBP-2 is only present in sufficient quantities to interfere with MAC-T cell proliferation when stimulated by RA (5-6-fold) or RA + IGF-I/insulin (7-8-fold). Also, since conditioned media from MAC-T cells treated with RA

contains approximately 25-fold more IGFBP-2 than all other IGFBPs, IGFBP-2 may preferentially sequester IGF-I at the expense of other IGFBPs (that may potentiate IGF-Is' actions), regardless of binding affinities of IGF-I to the various IGFBPs. Furthermore, when MAC-T cells are stimulated with IGF analogues that do not bind to IGFBPs, RA can only inhibit growth 15-27% compared with 64% growth inhibition if native IGF-I is used to stimulate growth. This data in light of the lack of IGFBP-3 and all other IGFBPs following RA treatment makes IGFBP-2 a likely candidate for RA induced growth inhibition.

Retinoic acid targeting of the IGF-I pathway is particularly pertinent since, it is well known that IGF-I is a potent mitogen in breast tumor cell lines as well as normal murine, human and bovine mammary epithelial lines (Yee et al., 1991; Zhao et al., 1992; Hadsell et al., 1994) and its actions can be acutely regulated by IGFBPs that are also secreted by these cells (Fielder et al., 1992; Sheikh et al., 1993b; Chen et al., 1994; Romagnolo et al., 1994;). This is especially relevant in ruminant mammary epithelium, which unlike its murine and human counterparts shows little to no response to EGF (Woodward et al., 1994; Moorby et al., 1995). In fact, MAC-T cells are very sensitive to IGF-I (Zhao et al., 1992; Woodward et al., 1994), but show minimal or no growth stimulation to ovarian steroids, growth hormone, TGF-α or EGF (Woodward et al., 1994). Therefore, alteration of IGF-I induced cellular proliferation is a likely target for growth inhibitors.

Interestingly, we have previously demonstrated the potent mitostatic effects of TGF-β on

MAC-T cells (Woodward et al., 1995) and several laboratories have shown retinoic acid to work through a TGF-β pathway, by increasing TGF-β proteins or TGF-β receptors (Falk et al., 1991; Kim et al., 1992; Poli et al., 1992, Roberts and Sporn, 1992). In fact, Lee et al, (1995) who have recently demonstrated RA inhibits proliferation of non-clonal rat mammary epithelium postulated RA may function by inducing TGF-β protein. However, several important differences exist between RA and TGF-β induced growth inhibition in the MAC-T cell line. First, TGF-β can cause growth arrest ($\geq$95% growth inhibition) of MAC-T cells, compared with only a maximum of 50% growth inhibition by RA. Secondly, TGF-β maintains growth inhibition for at least 6 d following treatment addition (Woodward et al., 1995), compared to reduced growth inhibition on d 4 and no inhibition by d 6 for RA. Also, TGF-β is not cytotoxic at concentrations 100-fold higher than concentrations needed for maximal growth inhibition (Woodward et al., 1995). On the other hand, maximal growth inhibition of RA occurs at cytotoxic levels. Finally, and perhaps the most dramatic difference between TGF-β and RA induced growth inhibition of MAC-T cells is TGF-β has no demonstratable effect on IGFBP profiles in the presence or absence of insulin/IGF-I. Others have suggested the modulation of IGFBPs in response to RA is a compensation mechanism for RA induced growth inhibition (Adamo et al., 1992; Sheikh et al., 1993b). However, since TGF-β1 (a more potent growth inhibitor than RA) has no effect on IGFBP-2 or IGFBP-3, the alterations in IGFBP expression are not a result of growth inhibition in itself.

Figure 9 is a model of RA action in MAC-T cells. a) represents MAC-T cells cultured

in IGF-I containing media and b) IGF-I +RA containing media. The model demonstrates that upon RA treatment of MAC-T cells occurs an increase in IGFBP-2 and a decrease (elimination) in IGFBP-3 from both media and the cell surface. We propose that these binding proteins alter IGF-I availability to the receptor or alter ligand-receptor mediated cellular proliferation following treatment with RA.

These studies provide compelling evidence that retinoids and especially RA are potent, but transient, inhibitors of mammary epithelial cell proliferation. Additionally, data herewithin supports the hypothesis that RA induced growth inhibition occurs, in part, as a result of an increase in IGFBP-2 in conditioned media and plasma membrane preparations and may also involve a decrease or elimination of IGFBP-3 from the conditioned media and plasma membrane preparations, respectively. Future studies detailing how RA effects: 1) IGF receptors, 2) IGFBP synthesis and secretory pathways and 3) alterations in RARs or CRABPs in short and long term culture in normal mammary epithelium are necessary to fully understand how retinoids may alter function of normal breast tissue when used in breast cancer trials.

ACKNOWLEDGEMENTS

The authors would like to thank Elaine deHeuvel for excellent technical assistance, Joseé Plamondon for assistance with PhosphorImagery and Dr. Robin Beech for assistance in densitometry. This work was supported by the National Sciences and Engineering Research Council of Canada (NSERC; OGP0155423 to X.Z. and OPG036727 to J.D.T.).

LITERATURE CITED

Adamo, M.L., Z. Shao, F. Lanau, J.C. Chen, D.R. Clemmons, C.T. Roberts, D. LeRoith, and J.A. Fontana. 1992. Insulin-like growth factor-I (IGF-I) and retinoic acid modulation of IGF-binding proteins (IGFBPs): IGFBP-2,-3,-4 gene expression and protein secretion in a breast cancer cell line. Endocrinology, 131:1858-1866.

Chambon, P., A. Zelent, M. Petkovich, C. Mendelsohn, P. Leroy, A. Krust, P. Kastner, and N. Brand. 1991. The family of retinoic acid nuclear receptors. In: Retinoids 10 Years On. Saurat, J.H., ed., Karger, Basel, pp. 10-27.

Chen, J., Z. Shao, M.S. Sheikh, A. Hussain, D. LeRoith, C.T. Roberts, and J.A. Fontana. 1994. Insulin-like growth factor-binding protein enhancement of insulin-like growth factor-I (IGF-I) mediated DNA synthesis and IGF-I binding in a human breast carcinoma cell line. J. Cell. Phys., 158:69-78.

Clemmons, D.R., V.K.M. Han, R.G. Elgin, and A.J. D'Ercole. 1987. Alterations in the synthesis of a fibroblast surface associated 35 K protein modulates binding of somatomedin-C/insulin-like growth factor I. Mol. Endocr., 1:339-347.

Clemmons, D.R., C. Camacho-Hubner, E. Coronado, and C.K. Osborne. 1990. Insulin-like growth factor binding protein secretion by breast carcinoma cell lines: correlation with estrogen receptor status. Endocrinology, 127:2679-2686.

Conover, C.A. 1992. Potentiation of insulin-like growth factor (IGF) action by IGF-binding protein-3: studies of underlying mechanism. Endocrinology, 130:3191-3199.

Creek, K.E., P. St. Hilaire, and J.R. Hodam. 1993. A comparison of the uptake, metabolism and biological effects of retinol delivered to human keratinocytes either free or bound to serum retinol-binding protein. J. Nutr. 123:356-361.

Darnell, J., H. Lodish, and D. Baltimore. 1990. The study of cell organization and subcellular structure. In: Molecular Cell Biology. J. Darnell, H. Lodish and D. Baltimore, eds. Scientific American Book, Inc., NY, pp. 132-133.

Decensi, A., F. Formelli, R. Torrisi, and A. Costa. 1993. Breast cancer chemoprevention: studies with 4-HPR alone and in combination with tamoxifen using circulating growth factors as potential surrogate endpoints. J. Biol. Chem. 17G :226-233.

DeLuca, L.M. 1991. Retinoids and their receptors in differentiation, embryogenesis and neoplasia. FASEB J., 5:2924-2933.

Falk, L.A., F. DeBenedelli, N. Luhrey, M.C. Birchenall-Roberts, L.W. Ellingsworth, C.R. Fallynek, and F.W. Russelli. 1991. Induction of transforming growth factor-β1 (TGF-β1), receptor expression and TGF-β1 protein production in retinoic acid-treated HL-60 cells: possible TGF-β1-mediated autocrine inhibition. Blood, 77:1248-1255.

Fielder, P.L., G. Thordarson, A. English, R.G. Rosenfeld, and F. Talamantes. 1992. Expression of a lactogen-dependent insulin-like growth factor-binding protein in cultured mouse mammary epithelial cells. Endocrinology, 131:261-267.

Fontana, J.A., D. Miranda, and A.B. Mezu. 1990. Retinoic acid inhibition of human breast carcinoma proliferation is accompanied by inhibition of the synthesis of a M_r 39,000 protein. Cancer Res., 50:1977-1982.

Fontana, J.A., A. Burrows-Mezu, D.R. Clemmons, and D. LeRoith. 1991. Retinoid modulation of insulin-like growth factor-binding proteins and inhibition of breast carcinoma proliferation. Endocrinology, 128:1115-1122.

Fraker, L.D., S.A. Halter, and J.T. Forbes. 1984. Growth inhibition by retinol of a human breast carcinoma cell line in vitro and in athymic mice. Cancer Res., 44:5757-5763.

Giguere, V. 1994. Retinoic acid receptors and cellular retinoid binding proteins: complex interplay in retinoid signalling. Endocr. Rev., 15:61-79.

Hadsell D.L., C.A. Gibson, and C.R. Baumrucker. 1994. Insulin-like growth factor (IGF) binding to a murine mammary epithelial cell line. J. Cell. Phys., 161:435-440.

Halter, S.A., A.R. Winnier, and C.L. Arteaga. 1993. Pretreatment with vitamin A inhibits transforming growth factor-α stimulation of human mammary carcinoma cells. J. Cell. Physiol., 156:80-87.

Hossain, M.Z., and J.S. Bertram. 1994. Retinoids suppress proliferation, induce cell spreading, and up-regulate connexin43 expression only in postconfluent 10T1/2 cells: implications for the role of gap junctional communication. Cell Growth and Differ., 5:1253-1261.

Hossenlopp, P., D. Seurin, B. Segovia-Guinson, S. Hardouin, and M. Binaux. 1986. Analysis of serum insulin-like growth factor binding proteins using Western blotting: use of the method for titration of the binding proteins and competitive binding studies. Anal. Bioch., 154:138-143.

Huynh, H.T., G. Robitaille, and J.D. Turner. 1991. Establishment of bovine mammary epithelial cells (MAC-T): An in vitro model for bovine lactation. Exp. Cell Res., 197:191-1991.

Jones, J.I., and D.R. Clemmons. 1995. Insulin-like growth factors and their binding proteins: biological actions. Endocr. Rev., 16:3-34.

Kim, H.J., N.J. Bugdan, L.J. D'Augustine, L.I. Guld, and G.F. Bryce. 1992. Effect of topical retinoic acids on the level of collagen mRNA during the repair of UVB-induced dermal damage in the hairless mouse and the possible role of TGF-β as a mediator. J. Inves. Dermat., 98:359-363.

Lacroix, A., and M.E. Lippman. 1980. Binding of retinoids to human breast cancer cell lines and their effects on cell growth. J. Clin. Invest., 65:586-591.

Laemelli, U.K. 1970. Cleavage of structural proteins during the assemblage of the head of bacteriophage T4. Nature, 227:680-685.

Laherty, R.F., W.X. Balcavage, C. Goff, T. Alvager, S. Ghosh, R.W. Geib, M. King, and D. Personett. 1990. HBL-100 cells do not secrete casein and lack prolactin and estradiol receptors. In Vitro Cell. & Devel. Biol., 26:933-935.

Lee, P.P., M.T. Lee, K.M. Darcy, K. Shudo, and M.M. Ip. 1995. Modulation of normal mammary epithelial cell proliferation, morphogenesis, and functional differentiation by retinoids: a comparison of the retinobenzoic acid derative RE80 with retinoid acid. Endocrinology, 136:1707-1717.

Lotan, R., and G.L. Nicolson. 1977. Inhibitory effects of retinoic acid or retinyl acetate on the growth of untransformed, transformed, and tumor cells in vitro. J. Natl. Cancer Inst., 59:1717-1722.

Lotan, R. 1979. Different susceptibilities of human melanoma and breast carcinoma cell lines to retinoic acid-induced growth inhibition. Cancer Res., 39:1014-1019.

Man, T. 1994. Solid tumors chemoprevention with retinoids. Leukemia, 8:1785-1790.

Marshall, H., M. Studer, H. Popperl, S. Aparicio, A. Kuroiwa, S. Brenner, and R. Krumlauf. 1994. A conserved retinoic acid response element required for early expression of the homeobox gene Hoxb-1. Nature, 370:567-571.

Marth, C., I. Mayer, and G. Daxenbichler. 1984. Effect of retinoic acid and 4-hydroxytamoxifen on human breast cancer cell lines. Bioch. Pharm., 33:2217-2221.

Martin, J.L., J.A. Coverlay, S.T. Pallison, and R.C. Baxler. 1995. Insulin-like growth factor-binding protein-3 production by MCF-7 breast cancer cells: stimulation by retinoic acid and cyclic adenosine monophosphate and differential effects of estradiol. Endocr., 136:1219-1226.

Moon, R.C., C.J. Grubbs, and M.B. Sporn. 1976. Inhibition of 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis by retinyl acetate. Cancer Res., 36:2626-2630.

Moorby, C.D., J.A. Taylor, and I.A. Forsyth. 1995. Transforming growth factor-alpha: receptor binding and action on DNA synthesis in the sheep mammary gland. J. Endocr., 144:165-171.

Moore, D.M., H. Spring, M. Trendelenburg, M. Montag, B.A. Mollenhauer, H.H. Mollenhauer, and D.J. Moore. 1992. Retinol stimulates golgi apparatus activity in cultured bovine mammary gland epithelial cells. J. Nutr., 122:1248-1253.

Napoli, J.L. 1993. Biosynthesis and metabolism of retinoic acid: roles of CRBP and CBP in retinoic acid: roles of CRBP and CBP in retinoic acid homeostasis. J. Nutr., 123:362-366.

Oh, Y., H.L. Muller, H. Pham, G. Lamson, and R.G. Rosenfeld. 1992. Non-receptor mediated, post-transcriptional regulation of insulin-like growth factor binding protein (IGFBP)-3 in Hs578T human breast cancer cells. Endocrinology, 131:3123-3125.

Oh, Y., H.L. Muller, G. Lamson, and R.G. Rosenfeld. 1993a. Insulin-like growth factor (IGF)-independent action of IGF-binding protein-3 in Hs578T human breast cancer cells. J. Biol. Chem., 268:14964-14971.

Oh, Y., H.L. Muller, H. Pham, and R.G. Rosenfeld. 1993b. Demonstration of receptors for insulin-like growth factor binding protein-3 on Hs578T human breast cancer cells. J. Biol. Chem., 268:26045-26048.

Oldham, E.R., R.J. Eberhart, and L.D. Muller. 1991. Effects of supplemental vitamin A or β-carotene during the dry period and early lactation on udder health. J. Dairy Sci., 74:3775-3781.

Pemricks, S.M., D.A. Lucas, and J.F. Grippo. 1994. The retinoid receptors. Leukemia, 8:1797-1806.

Pitts, J.D., A.E. Hamilton, E. Kam, R.R. Burk, and J.P. Murphy. 1986. Retinoic acid inhibits junctional communication between animal cells. Carcinogenesis, 7:1003-1010.

Poli G., A.L. Kinter, J.S. Justement, P. Bressler, J.H. Kehrl, and A.S. Fauci. 1992. Retinoic acid mimics transforming growth factor beta in the regulation of human immunodeficiency virus expression in monocytic cells. Proc. Natl. Acad. Sci., 89:2689-2693.

Pollak, M.N., C. Polychronakos, and M. Richard. 1990. Insulin-like growth factor I: a potent mitogen for human osteogenic sarcoma. J. Natl. Cancer Inst., 82:301-305.

Roberts, A.B., and M.B. Sporn. 1992. Mechanistic interrelationships between two superfamilies: the steroid/retinoid receptors and transforming growth facotr-β. Cancer Surv., 14:205-220.

Romagnolo, D., R.M. Akers, J.C. Byatt, E.A. Wong, and J.D. Turner 1994. IGF-I-Induced IGFBP-3 potentiates the mitogenic actions of IGF-I in mammary epithelial MD-IGF-I cells. Molec. and Cell. Endocr., 102:131-139.

Ruberte, E. 1994. Nuclear retinoic acid receptors and regulation of gene expression. Arch. of Toxic., 16:105-111.

Samokyszyn, V.M., B.F. Sloane, K.V. Honn, and L.J. Marnett. 1984. Cooxidation of 13-cis-retinoic acid by prostaglandin H synthase. Bioch. and Bioph. Res. Comm., 124:430-436.

Sankaran, L., and Y.J. Topper. 1982. Effect of vitamin A deprivation on maintenance of rat mammary tissue and the potential of the epithelium for hormone-dependent milk protein synthesis. Endocrinology, 111:1061-1067.

SAS Institute Inc. 1988. SAS/STAT user's guide. Rel 6.03 Cary, NC.

Sheikh, M.S., Z. Shao, J. Chen, D.R. Clemmons, C.T. Roberts, D. LeRoith, and J.A. Fontana. 1992. Insulin-like growth factor binding protein-5 gene expression is differentially regulated at a post-transcriptional level in retinoic acid-sensitive and resistant MCF-7 human breast carcinoma cells. Bioch. and Bioph. Res. Comm., 188:1122-1130.

Sheikh, M.S., Z. Shao, A. Hussain, J. Chen, C.T. Roberts, D. LeRoith, and J.A. Fontana. 1993a. Retinoic acid and estrogen modulation of insulin-like growth factor binding protein-4 gene expression and the estrogen receptor status of human breast carcinoma cells. Bioch. and Bioph. Res. Comm., 193:1232-1238.

Sheikh, M.S., Z. Shao, A. Hussain, D.R. Clemmons, J. Chen, C.T. Roberts, D. LeRoith, and J.A. Fontana. 1993b. Regulation of insulin-like growth factor binding protein-1, 2, 3, 4, 5, and 6: synthesis, secretion, and gene expression in estrogen receptor-negative human breast carcinoma cells. J. Cell. Phys., 155:556-567.

Shemer, J., A. Yaron, H. Werner, Z.M. Shao, M.S. Sheikh, J.A. Fontana, D. LeRoith, and C.T. Roberts. 1993. Regulation of insulin-like growth factor (IGF) binding protein-5 in the T47D human breast carcinoma line by IGF-I and retinoic acid. J. Clinic. Endocr. & Metab., 77:1246-1250.

Smith, J.E., P.O. Milch, Y. Muto, and D.S. Goodman. 1973. The plasma transport and metabolism of retinoic acid in the rat. Biochem. J., 132:821-827.

Swisshelm, K., K. Ryan, K. Tsuchiya, R. Sager. 1995. Enhanced expression of an insulin growth factor-like binding protein (mac25) in senescent human mammary epithelial cells and induced expression with retinoic acid. Proc. Natl. Acad. Sci., 92:4472-4476.

Ueda, H., M. Ono, Y. Hagino, and M. Kuwano. 1985. Isolation of retincic acid-resistant clones from human breast cancer cell line MCF-7 with altered activity of cellular retinoic acid-binding protein. Cancer Res., 45:3332-3338.

Wolf, G., and D. Phil. 1991. The intracellular vitamin A-binding proteins: an overview of their functions. Nutr. Rev., 49:1-12.

Woodward, T.L., W.E. Beal, and R.M. Akers. 1993. Cell interactions in initiation of mammary epithelial proliferation by oestradiol and progesterone in prepubertal heifers. J. Endocr., 136:149-157.

Woodward, T.L., R.M. Akers, and J.D. Turner. 1994. Lack of mitogenic response to EGF, pituitary and ovarian hormones in bovine mammary epithelial cells. Endocrine, 2:529-535.

Woodward, T.L., N. Dumont, M. O'Connor-McCourt, J.D. Turner, and A. Philip. 1995. Characterization of transforming growth factor-β (TGF-β) growth regulatory effects and receptors on bovine mammary cells. J Cell. Phys., 165:339-348.

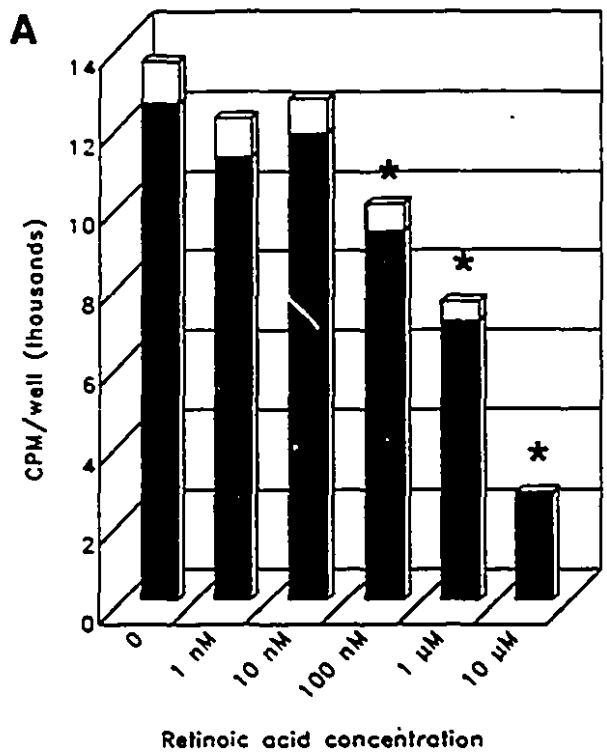
Yee, D., N. Rosen, R.E. Favoni, and K.J. Cullen. 1991. The insulin-like growth factors, their receptors, and their binding proteins in human breast cancer. Cancer Trtmt. and Res., 53:93-106.

Zar, J.H. 1984. Multiple comparisons. In: Biostatistical Analysis. J.H. Zar, ed. Prentice Hall Inc. Englewood Cliffs, NJ, pp. 194-195.

Zhao, X., B.W. McBride, I. Politis, H.T. Huynh, R.M. Akers, J.H. Burton, and J.D. Turner. 1992. Receptor binding and growth-promoting activity of insulin-like growth factor-I in a bovine mammary epithelial cell line (MAC-T3). J. Endocr., 134:307-312.

Zhu, X., N. Ling, and S. Shimasaki. 1993. Structural characterization of the rat insulin-like growth factor binding protein-6 gene. Bioch & Bioph. Res. Comm., 191:1237-1243.

Figure 1: Inhibition of MAC-T cell proliferation by retinoic acid (a) and retinol (b) as measured by incorporation of ^{3}H-thymidine into MAC-T cells following 17 h culture in retinoids (1 nM to 10 μM). All treatments included 1% FBS. Data are expressed as CPM/well $\pm$ SEM. Asterisks indicate significant difference ($P<.05$) from control (no retinoids).


A
CPM/well (thousands)
0
2
4
6
8
10
12
14
*
*
*
0
1 nM
10 nM
100 nM
1 μM
10 μM
Retinoic acid concentration

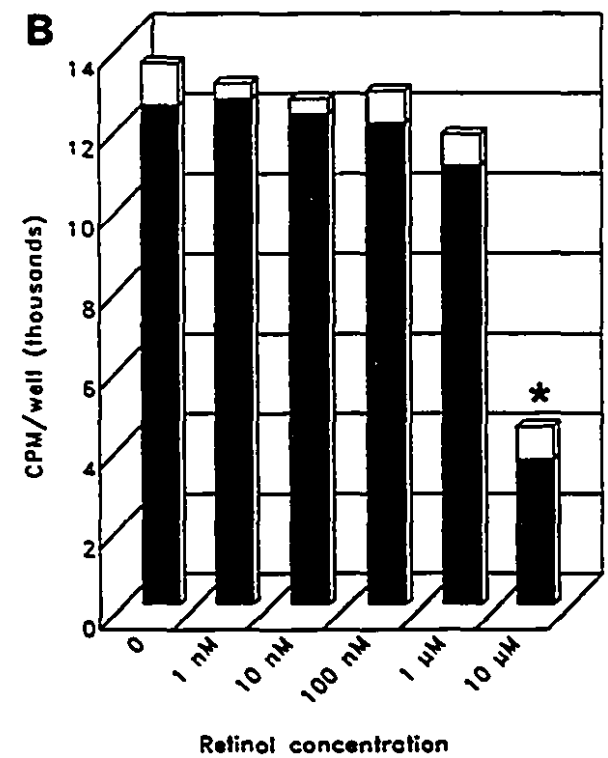
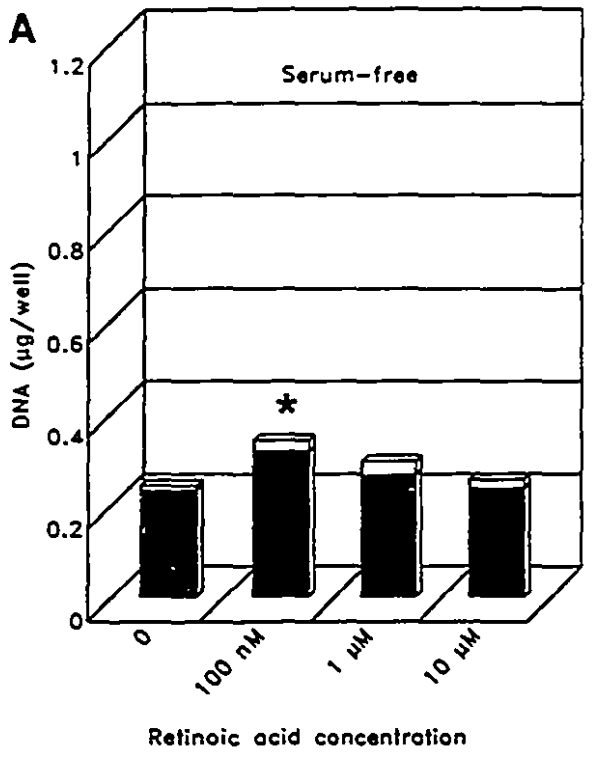
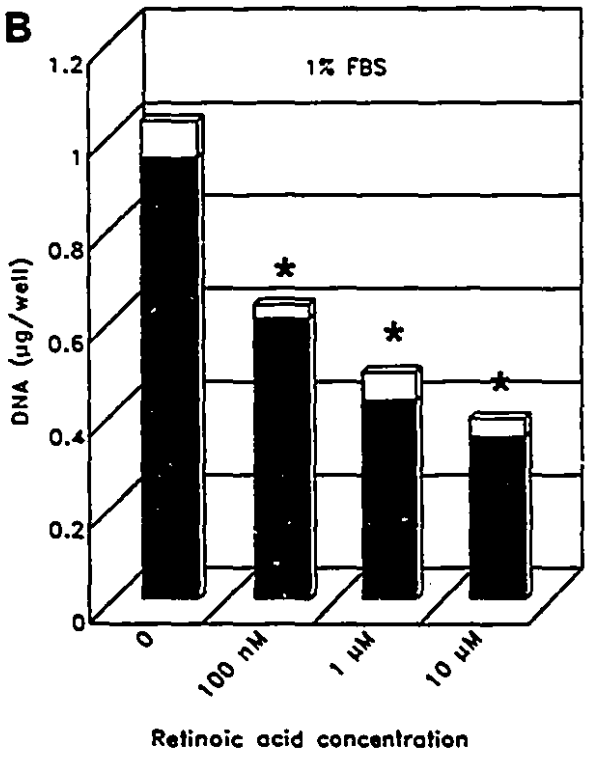
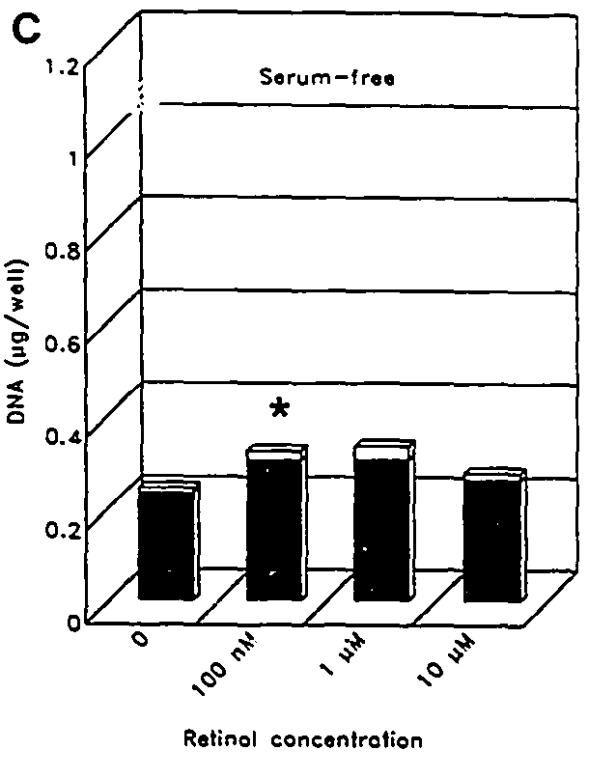

B
CPM/well (thousands)
0
2
4
6
8
10
12
14
*
0
1 nM
10 nM
100 nM
1 μM
10 μM
Retinol concentration

Figure 2 Effect of retinoic acid (a,b) and retinol (c,d) on cell growth (as measured by total DNA) following 72 h culture in no serum (a,c) or 1% FBS (b,d). Both retinoids inhibited growth in the presence of FBS (b,d) but slightly increased growth in serum-starved MAC-T cells (a,c). Data are expressed as μg DNA/well $\pm$ SEM. Asterisks indicate significant difference ($P < .05$) from control (no retinoids).


A
Serum-free
DNA (µg/well)
1.2
1
0.8
0.6
0.4
0.2
0
*
0
100 nM
1 µM
10 µM
Retinoic acid concentration


B
1% FBS
DNA (µg/well)
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
0
100 nM
1 µM
10 µM
Retinoic acid concentration


C
Serum-free
DNA (µg/well)
1.2
1
0.8
0.6
0.4
0.2
0
*
0
100 nM
1 µM
10 µM
Retinol concentration

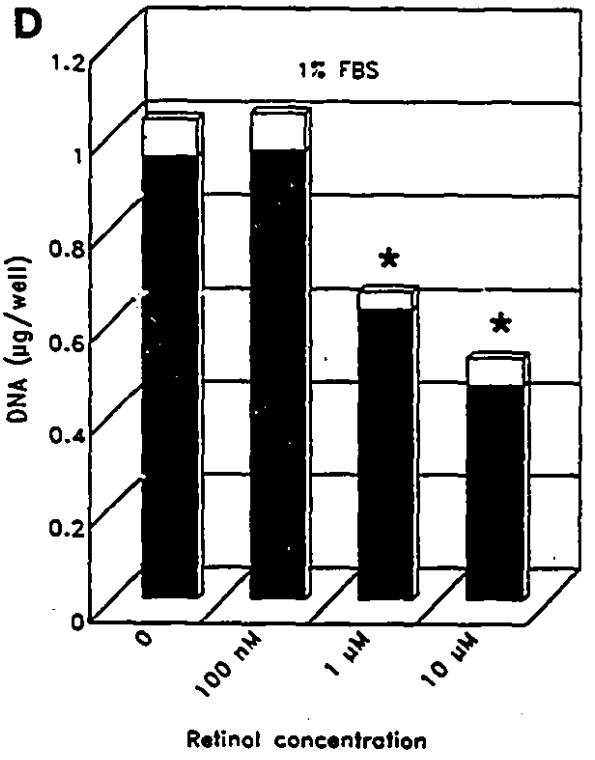
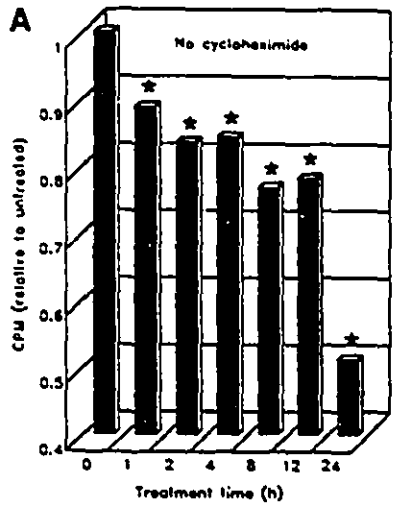
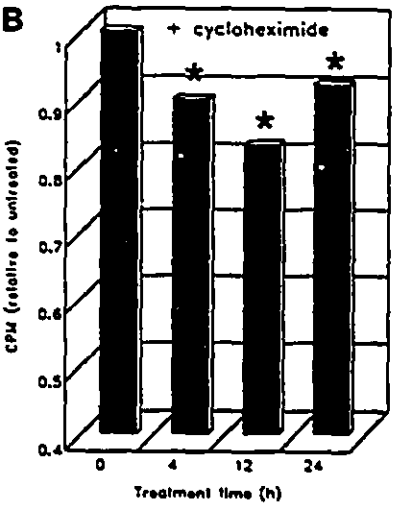

D
1% FBS
DNA (µg/well)
1.2
1
0.8
0.6
0.4
0.2
0
*
*
0
100 nM
1 µM
10 µM
Retinol concentration

Figure 3 Time course of effect of 1 μM retinoic acid on incorporation of ^{3}H-thymidine into DNA of MAC-T cells in the presence (a,c) or absence (b) of protein synthesis. (a) 0 to 24 h; (b) 0 to 24 h with 10 μM cycloheximide or (c) 0-7 d culture. Cells for short term (a,b) culture were plated at 1 X 10^4 cells/cm^2 and at 1 X 10^3 cells/cm^2 for long term culture (c). Data are expressed as CPM relative to untreated (i.e. adjusted so cells not treated with retinoic acid (untreated) = 1.0). In figures a and b all points except 0 h are significantly different than control (no retinoids) ($P<.05$). In figure c, asterisks indicate significant difference ($P<.05$) from control (no retinoids).


A
No cycloheximide
CPM (relative to untreated)
1
0.9
0.8
0.7
0.6
0.5
0.4
0 1 2 4 8 12 24
Treatment time (h)


B
+ cycloheximide
CPM (relative to untreated)
1
0.9
0.8
0.7
0.6
0.5
0.4
0 4 12 24
Treatment time (h)

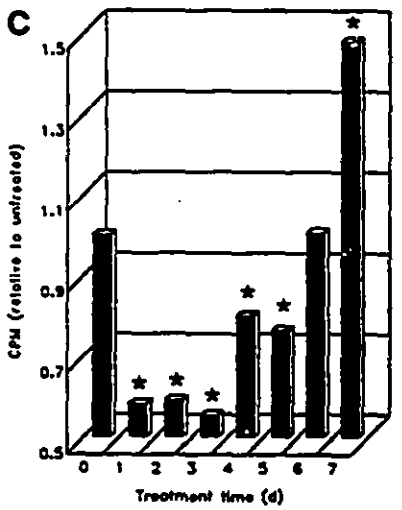

C
CPM (relative to untreated)
1.5
1.3
1.1
0.9
0.7
0.5
0 1 2 3 4 5 6 7
Treatment time (d)

Figure 4 Inhibition of MAC-T cell proliferation by retinoic acid (1 μM) as measured by incorporation of ^{3}H-thymidine into MAC-T cells growth stimulated by 1% FBS, bovine insulin (3 μM), IGF-I (30 ng/ml), 30 ng/ml des(1-3) IGF-I (30 ng/ml) or Long(R^3) IGF-I (30 ng/ml). Retinoic acid is least effective at inhibiting proliferation of mitogens that show a reduced affinity for IGFBPs (insulin, des(1-3) IGF-I and Long(R^3) IGF-I). Data are expressed as CPM/well. Percent differences between RA treated and control are presented in parentheses. Solid bars = control, Open bars = retinoic acid treated.

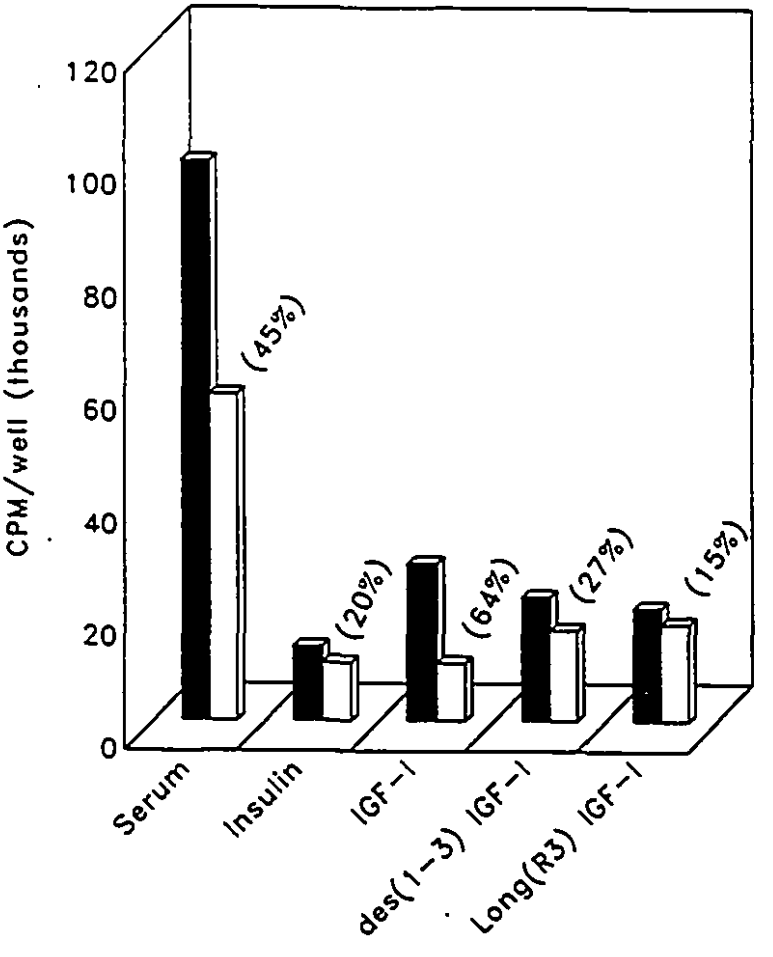
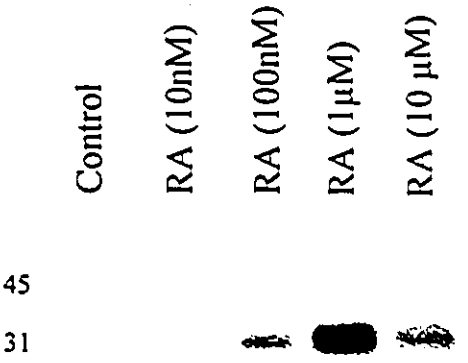
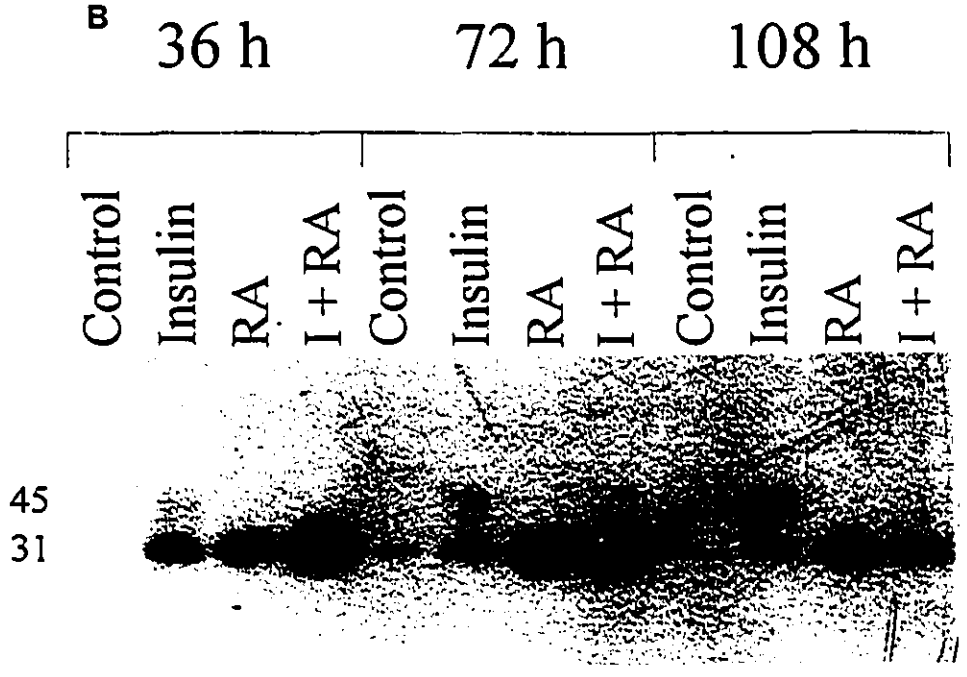

CPM/well (thousands)
120
100
80
60
40
20
0
(45%)
(20%)
(64%)
(27%)
(15%)
Serum
Insulin
IGF-I
des(1-3) IGF-I
Long(R3) IGF-I

Figure 5 Western ligand blots probed with ^{125}I-IGF-I for IGFBPs secreted into conditioned media of MAC-T cells a) following 48 h treatment with increasing concentrations of retinoic acid (0 to 10 μM), or (b) time course of maximal IGFBP secreted into conditioned media of MAC-T cells after treatment with retinoic acid (1 μM), insulin (3 μg/ml) or retinoic acid + insulin for 36 h, 72 h and 108 h; (c) densitometric reading of Western blot in (b).

A


Control
RA (10nM)
RA (100nM)
RA (1μM)
RA (10 μM)
45
31

B


36 h
72 h
108 h
Control
Insulin
RA
I + RA
Control
Insulin
RA
I + RA
Control
Insulin
RA
I + RA
45
31

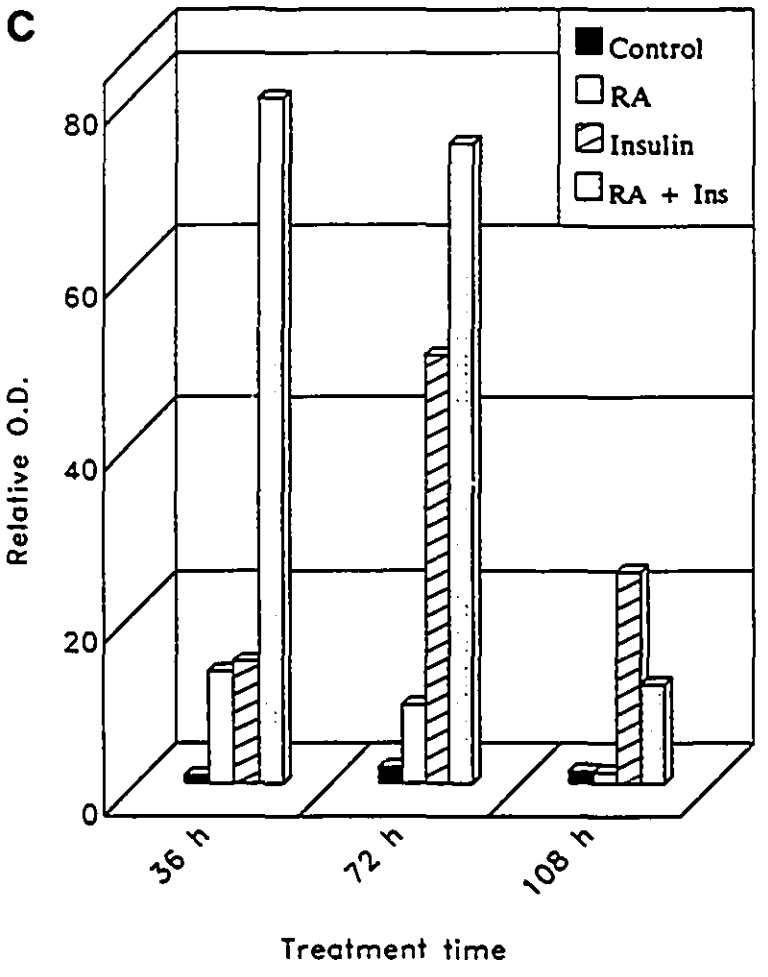
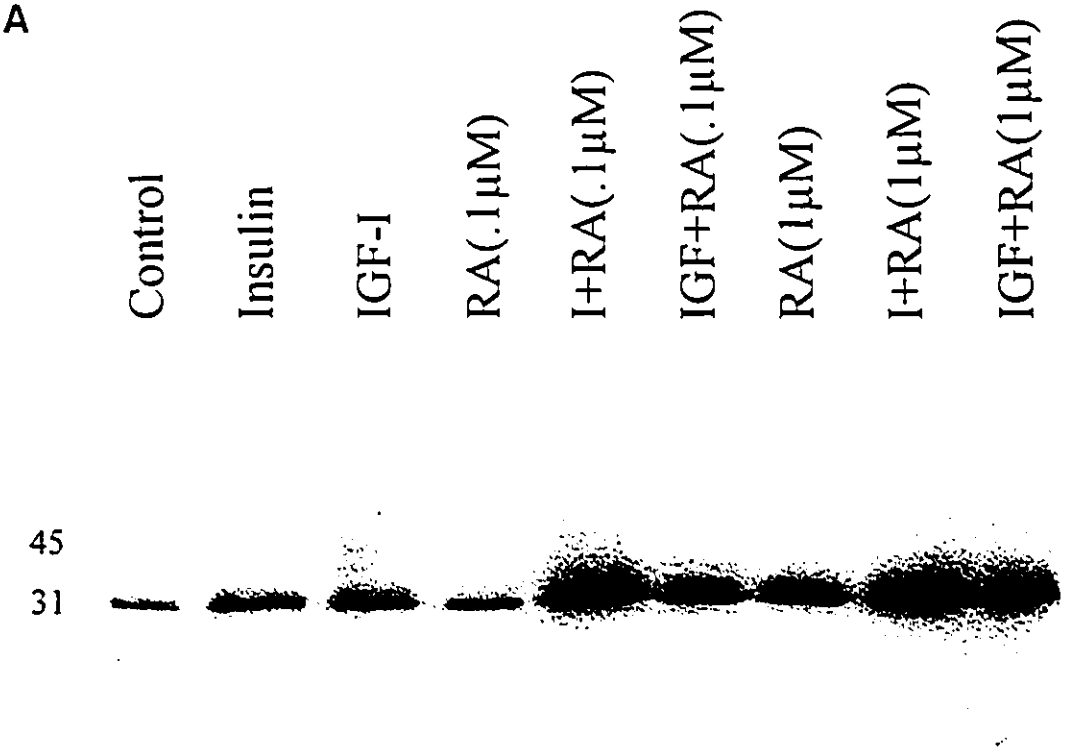
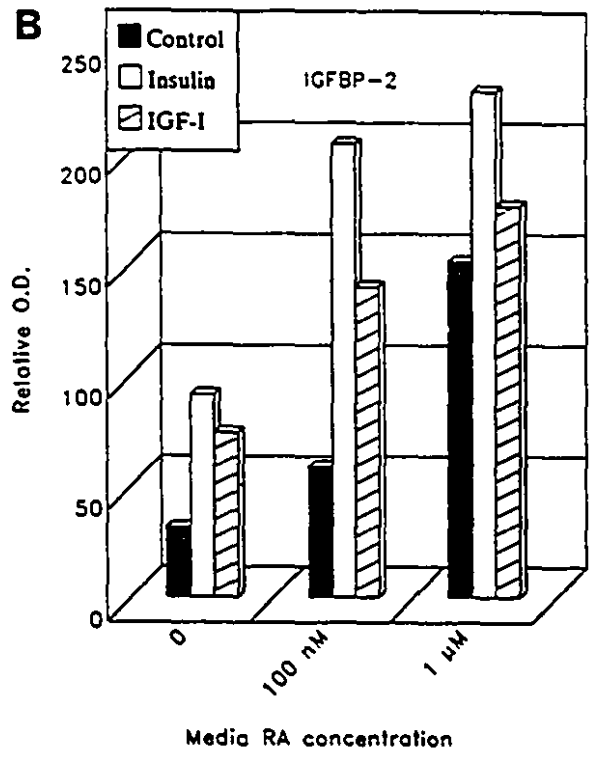

C
Control
RA
Insulin
RA + Ins
80
60
40
20
0
Relative O.D.
36 h
72 h
108 h
Treatment time

Figure 6 (a) Western ligand blot of unconcentrated conditioned media from MAC-T cells for IGFBPs in response to insulin (3 μg/ml), des(1-3) IGF-I (30 ng/ml) or RA (.1 or 1 μM); (b) densitometric analysis of lower molecular weight band (IGFBP-2) and (c) upper molecular weight bands (IGFBP-3).

A


Control
Insulin
IGF-I
RA(.1μM)
I+RA(.1μM)
IGF+RA(.1μM)
RA(1μM)
I+RA(1μM)
IGF+RA(1μM)
45
31


B
Control
Insulin
IGF-I
IGFBP-2
250
200
150
100
50
0
Relative O.D.
0
100 nM
1 μM
Media RA concentration

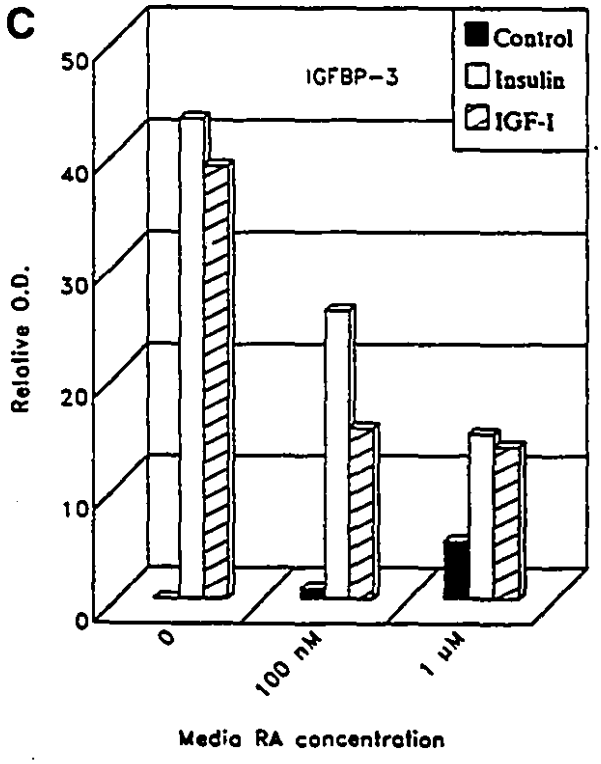

C
Control
Insulin
IGF-I
IGFBP-3
50
40
30
20
10
0
Relative O.D.
0
100 nM
1 μM
Media RA concentration

Figure 7 Western ligand blot of concentrated media from MAC-T cells for IGFBPs following treatment with retinoic acid, insulin and des(1-3) IGF-I as indicated. 5-10 fold concentrated samples were used. Protease inhibitors (as described in Materials and Methods) were added upon harvesting media.

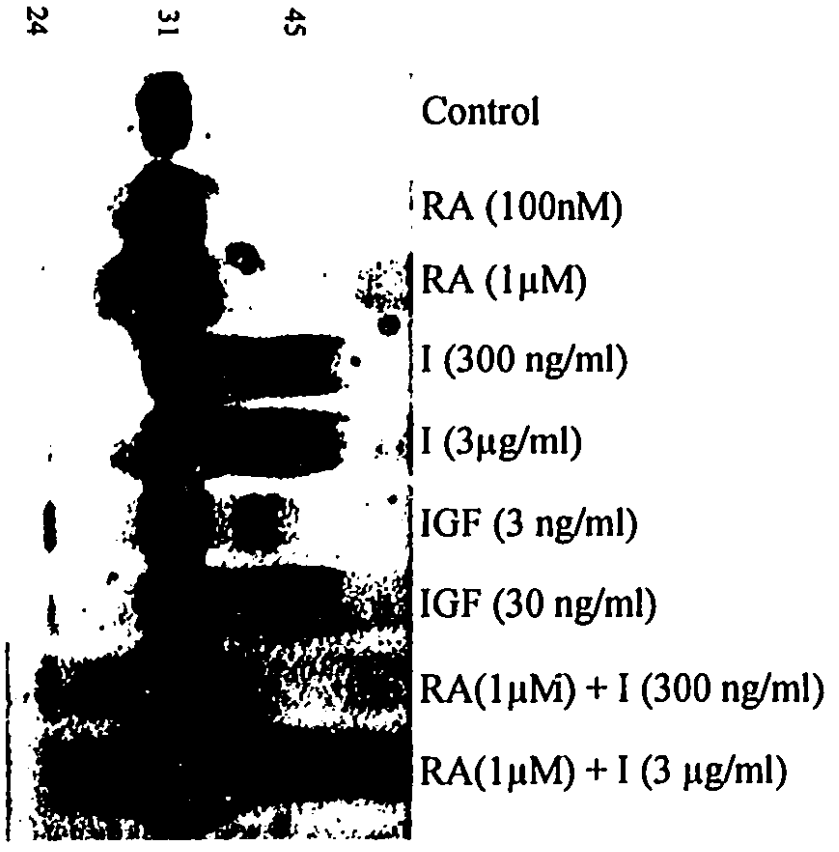
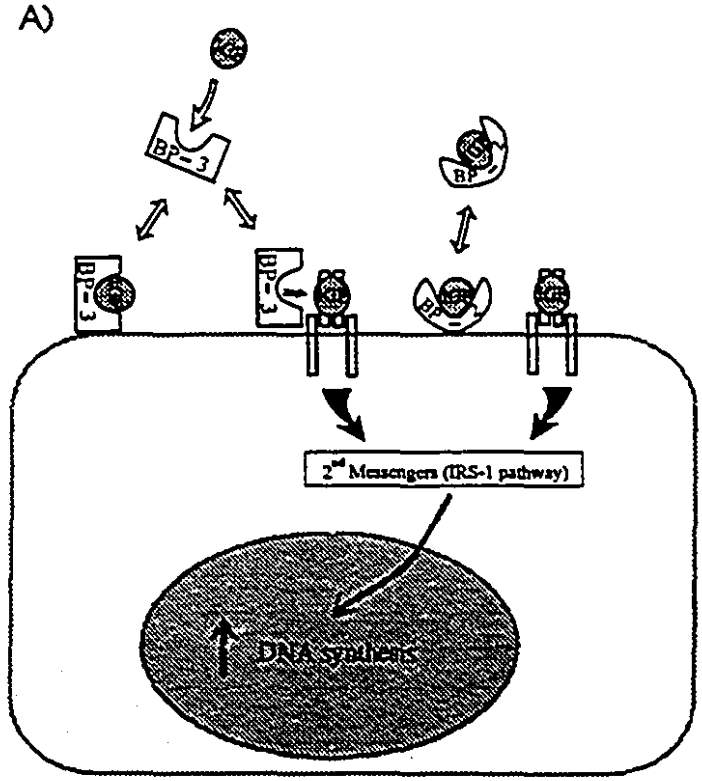

24
31
45
Control
RA (100nM)
RA (1μM)
I (300 ng/ml)
I (3μg/ml)
IGF (3 ng/ml)
IGF (30 ng/ml)
RA(1μM) + I (300 ng/ml)
RA(1μM) + I (3 μg/ml)

Figure 8 Western ligand blot of plasma membrane preparations from MAC-T cells for IGFBPs. Note: the conditioned media used for the Western ligand blot in figure 7 is from the cells used to obtain plasma membrane preparations in this Western blot. Cells were cultured in retinoic acid (1 μM), insulin (3 μg/ml) or des(1-3) IGF-I (30 ng/ml).

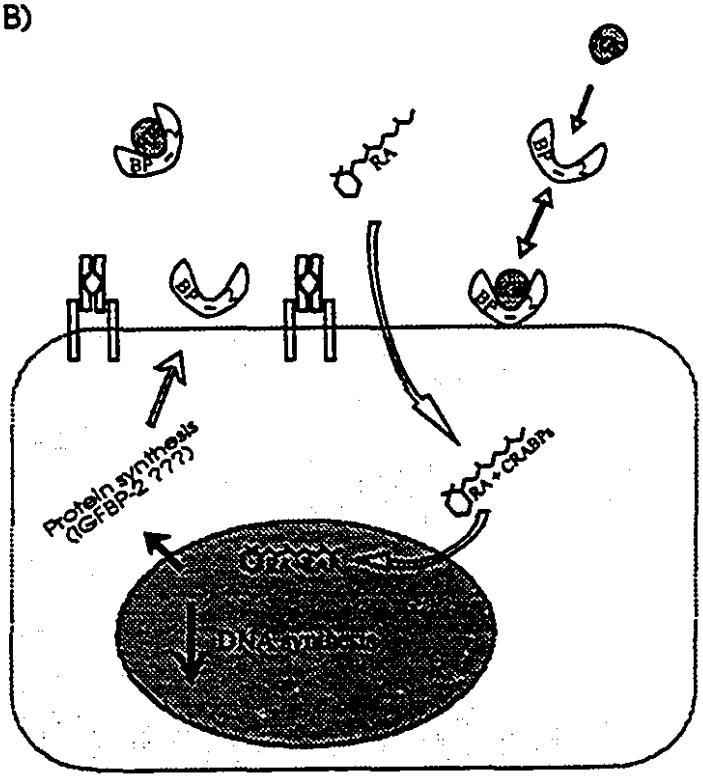
Control
RA
Ins
RA + Ins
IGF-I
RA + IGF-I
45
31

Figure 9 A model of RA effects in MAC-T cells. MAC-T cells are growth stimulated by IGF-I (a) and this stimulation of growth is concurrent with IGFBP-3 binding to the cell surface and in the conditioned media. However, when RA is added (b) DNA synthesis is inhibited (following protein synthesis) and this inhibition is correlated with elimination of cell surface associated IGFBP-3 and conditioned media IGFBP-3 and increased IGFBP-2 on the plasma membrane and in conditioned media.

A)


BP-3
BP-3
BP-3
BP-3
2nd Messengers (IRS-1 pathway)
DNA synthesis

B)


BP-3
BP-3
BP-3
BP-3
RA
Protein synthesis (IGFBP-2 ???)
RA + CRABP
DNA synthesis

SUMMARY 4

Chapter 4 examined the effects of the retinoids, retinol and retinoic acid (RA), on mammary epithelial cell proliferation. Additionally, this study examines the mechanism of retinoid mediated inhibition of mammary cell proliferation. This was the first report to identify retinoic acid as a potent inhibitor of proliferation in a non-transformed mammary epithelial cell line in any species.

Retinoic acid growth inhibition of mammary epithelial cell proliferation was substantially reduced when cells were growth stimulated by IGF-I analogues (Chapter 2) that have a low affinity for insulin-like growth factor binding protein (IGFBP). Also, RA was not able to substantially inhibit cellular proliferation in the absence of protein synthesis. This led us to examine the role of RA in regulating IGFBP secretion and cell membrane binding. This study, for the first time in non-transformed cells, found that IGFBPs appear to be responsible for much of retinoic acid's growth inhibitory properties. Retinoic acid stimulated IGFBP-2 and inhibited IGF-I induced IGFBP-3. IGFBP-3 has previously been shown to potentiate the effect of IGF-I on proliferation of MAC-T cells, whereas IGFBP-2 is generally thought to sequester IGF-I, preventing receptor binding.

In addition to examining retinoic acid's mechanism of action, we compared growth inhibition by RA to TGF-β (described in chapter 3). We found that unlike several

reports, TGF-β and retinoic acid inhibit mammary epithelial cell proliferation by independent pathways. This study therefore, identified a second potent growth regulator of bovine mammary epithelial cell proliferation that functions independently of TGF-β.

CHAPTER 5

Regulation of Gap Junctional Communication and Connexin43 Protein Expression in Bovine Mammary Fibroblast and Epithelial Cells

ABSTRACT

Recent studies have demonstrated acute modulation of gap junction protein expression in the mammary gland between nonpregnant, pregnant, and lactating mice. The expression of connexin43 (Cx43) was examined in primary, clonal and immortalized bovine mammary fibroblasts and epithelial cells by Western blot and immunocytochemical analyses. Gap junctional intercellular communication (GJIC) was accessed following microinjection of Lucifer Yellow dye. All epithelial and fibroblast cells expressed Cx43. All cells had abundant punctate cell surface labelling of Cx43, though fibroblasts and SV-40 Large-T-antigen immortalized epithelial cells (MAC-T) had increased cytoplasmic perinuclear Cx43 labelling. Fibroblasts, but not epithelium, expressed a transformed phenotype after stable transfection with the SV-40 Large-T-antigen. Transformed fibroblasts expressed little Cx43, lacked cell surface labelling for Cx43 and lacked gap junctional communication. Retinoic acid (RA), TGF-β1 and cAMP each inhibited cellular proliferation of MAC-T cells and all increased total Cx43 expression, especially phosphorylated Cx43 species. Concentrations of growth inhibitors that induced maximal Cx43 expression did not always cause maximal growth inhibition. Unlike RA or TGF-β, cAMP significantly modulated the intracellular distribution of Cx43, resulting in less perinuclear Cx43 and increased cell surface Cx43. None of the growth inhibitors examined were able to increase GJIC in the well coupled MAC-T cells, even when the cells were serum-starved. These data suggest that bovine mammary parenchymal and stromal cells have abundant regulatable gap junctions and immortalized cells may be appropriate models of the bovine mammary gland when a normal phenotype is retained.

INTRODUCTION

Gap junctions are channels that allow for the direct passage of small molecules (Ca^{2+}, amino acids, second messengers) from one cell to another. A gap junction channel is composed of a hemichannel, connexon, from one cell that pairs with a hemichannel from an adjacent cell. Each connexon is formed by six polypeptides, the connexins, arranged in a hexamer around the central pore (Beyer et al., 1993). The connexins are a family of at least 12 proteins, which are differentially expressed in nearly all cell types (Casico et al., 1995; Laird and Saez, in press). Many cellular functions have been ascribed to gap junction channels. Researchers have demonstrated the importance of gap junctional intercellular communication (GJIC) in normal development, with disruption of GJIC at early stages in embryo development causing specific developmental defects and death (Warner et al., 1984; Reaume et al., 1995). Gap junctions also allow for the passage of ions in electrically excitable tissues such as nervous tissue, smooth muscle and cardiac muscle (Beyer, 1993). Additionally, GJIC is believed to play an important role in regulating cellular proliferation and differentiation in the embryo and adult by transmitting secondary messengers, metabolites and ions from one cell to another (Beyer, 1993; Wolburg and Rohlmann, 1995). Consequently, abnormalities in GJIC or loss of GJIC have been demonstrated in cancer and disease states (Holder et al, 1993).

Recently, Monaghan and colleagues (1994) have demonstrated marked increases in connexin26 expression in the mouse mammary gland during progression from virgin to pregnancy to lactation. Furthermore, these workers found that abruptly following

lactation, during involution, connexin26 expression is greatly diminished. Pozzi and coworkers (1995) have confirmed these results, also in the mouse, finding highest expression of both Cx32 and Cx26 during lactation, however these researchers could not detect either of these connexins in human mammary tissue by immunocytochemistry. Both studies suggest that connexin expression may be critical to normal mammogenesis, lactogenesis and involution. However in these reports and others, considerable differences exist concerning which connexins are synthesized or expressed in murine and human mammary cells (Lee et al., 1992; Wilgenbus et al., 1992; Tomasetto et al., 1993; Monaghan et al., 1994; Pozzi et al., 1995). Thus far, only rodents have been examined for alterations in connexin expression and GJIC in nonpregnant, pregnant, lactating and involuting mammary glands.

In addition to modulation of gap junctional communication during mammary gland maturation and differentiation, many independent studies have demonstrated that gap junctions regulate cellular proliferation, while loss of gap junctional communication causes cellular dysfunction and may lead to tumorigenesis (Lowenstein, 1967; Eghbali et al., 1991; Zhu et al., 1992; Ruch, 1994; Mesnil et al., 1995). Several key observations have provided compelling evidence that gap junctions are involved in control of cellular proliferation: 1) Tumor promoters, such as TPA have been shown to block gap junctional communication (Oh et al., 1991). Additionally, many tumor cell lines, such as the rat C6 glioma cells, human SKHep 1 hepatoma cells and human mammary 21MT-2 cells, lack gap junctional communication, (Eghbali et al., 1991; Naus et al., 1991; Lee et al., 1992) 2) Transfection of communication-deficient tumor cell lines with

connexin26, connexin32 or connexin43 results in both a decreased rate of cellular proliferation and a reversal of the neoplastic phenotype as shown by in vivo an in vitro studies (Eghbali et al., 1991; Zhu et al., 1992; Ruch, 1994; Mesnil et al., 1995), 3) Subtractive hybridization for mRNAs expressed in normal breast epithelial cells, but not in breast tumor cells led to the identification of connexin26 as a class II tumor suppressor (Lee et al., 1992). However, results are still not conclusive as independent laboratories studying GJIC in the breast have demonstrated 1) GJIC is not eliminated in all breast tumor cells (Enomoto et al., 1992; Laird et al., 1995), 2) GJIC is only downregulated in tumor cells when the cells are highly metastatic (Nicolson et al., 1988), and GJIC may actually be enhanced in early passage carcinomas (Eldridge et al., 1989).

In the present work, we first describe the expression of connexin43 in several well characterized clonal lines of bovine mammary epithelial cells and fibroblasts (Huynh et al., 1991; Woodward et al., 1994; Woodward et al., 1995) by Western blot analysis and immunocytochemistry. Alterations in Cx43 protein quantity and maturation and GJIC in simian virus-40 Large-T-antigen (SV-40 LT) transfected epithelial and fibroblast cells are contrasted with their non-transfected counterparts. Finally, increased Cx43 expression, Cx43 immunolocalization and GJIC are accessed following treatment with the previously identified inhibitors of bovine mammary epithelial cell proliferation: 1) retinoic acid, 2) TGF-β_1, and 3) dibutyrl cAMP.

MATERIALS AND METHODS

Materials, cells, and culture conditions

All hormones, enzymes, media, serum and culture reagents were obtained from GIBCO BRL (Burlington, Ont.), Becton Dickinson (St. Laurent, Que.) or Sigma Chemical Co. (St. Louis, MO), unless otherwise noted. All-trans retinoic acid (RA) was purchased from FLUKA (Ronkonkoma, NY). RA was solubilized in 100% ethanol at 37° C under nitrogen in the dark and always used within 24 h. Controls always contained equal concentrations of ethanol. TGF-β_1 was a gift from Bristol-Meyer Squibb. All glass and plasticware were silanized (Sigmacote; Sigma) prior to TGF-β exposure. TGF-β was diluted in a 5 mM HCl/15μM BSA solution as previously described (Woodward et al., 1995).

A bovine mammary epithelial cell line (MAC-T) obtained from a mid-lactational bovine animal and immortalized by stable transfection with the SV-40 Large-T-antigen (LT) (Huynh et al., 1991) was used. Two primary mammary cell populations, one fibroblast and one epithelial, isolated from pregnant bovine non-lactating animals were previously described (Woodward et al., 1994). Additionally several epithelial cell lines (MEB-E) and fibroblasts (MFB-3, MFB-T3) were isolated from mammary glands of non-pregnant or pregnant bovine animals (MFB-3hUnf, MFB-Pr) (Woodward et al., 1995). MFB-T3 cells also stably express the LT as previously described; however, unlike MAC-T cells, MFB-T3 cells display a transformed phenotype (Woodward et al., 1995). All cells except primary cell populations were clonal. Cell type was confirmed by immunocytochemical

analysis for vimentin (Boehringer-Mannheim, Indianapolis, IN), cytokeratin (pan-specific, Boehringer-Mannheim), smooth muscle actin (Enzo Diagnostics, Farmingdale, NY), and fibronectin (Sigma Chem. Co.) (Woodward et al., 1994; 1995). All cells were maintained for routine culture in 10% FBS supplemented DMEM in a humidified 37° C, 5% CO_2 incubator.

Western blotting

Cells were cultured in 100 mm tissue culture dishes, scraped with a rubber policeman in the presence of protease inhibitors (see below) and pelleted at 500 X g for 5 min, trypsin was deactivated with serum, repelleted and pellet rinsed with cold PBS. An aliquot of resuspended cells was removed for protein assay (BCA assay, Pierce, Rockford, IL) and hemocytometric counting. Samples were adjusted to equal cell number by hemocytometric analysis or to equal protein by BCA assay as previously described (Sorensen and Brodbeck, 1986). Cells were recentrifuged and pellet was resuspended at 1 X 10^7 cells/ml in 1 ml sample buffer, 2 mM PMSF, 3 μM leupeptin, 16 μM aprotinin, 2 mM EDTA, 50 mM sodium floride, 500 μM sodium orthovanadate, 100 μl β-mercaptoethanol, 360 μl H_2O and 500 μl 2X loading buffer (Woodward et al., 1995), on ice and sample sonicated 10 s. Proteins were electrophoretically separated as per Laemmli (1970) using a 10% polyacrylamide gel with an acrylamide:bis acrylamide ratio of 30:0.4. Gels in Figures 1 and 4 were ran for 2-2.5 hours at 28 mA, while the gel in Figure 6 was ran at 24 mA for 3 h to enhance separation of Cx43 species. Samples were electrotransferred onto nitrocellulose filters and probed with CT-360 rabbit polyclonal site directed antibody for the carboxyl terminal of Cx43, as we have previously reported

(Laird et al., 1995). Filters were exposed, overnight to 2 d, in a PhosphorImager cassette and subsequently scanned for band intensity in a PhosphorImager (Molecular Dynamics, San Fransico, CA). All results obtained from Western blotting were repeated with similar results.

Immunocytochemistry

Fixed cells were labelled for Cx43, vimentin, smooth muscle actin, or cytokeratin as previously described (Laird et al., 1995). Briefly, cells grown on glass coverslips were fixed in 100% ethanol, blocked of nonspecific binding sites with 10% horse sera or 2% bovine serum albumin, rinsed in PBS and immunolabelled. Cells were labelled with 1-5 μg anti-Cx43 antibody or 1:100 dilutions of cytoskeletal antibodies for 1 h. Cells were rinsed 6X for 30 min in PBS and incubated 1 h in secondary antibody, goat anti-mouse or donkey anti-rabbit rhodamine and FITC conjugated antibodies. Coverslips were rinsed, mounted and analyzed on a Zeiss LSM 410 inverted confocal microscope as described previously (Laird et al., 1995). All immunocytochemistry experiments were repeated at least twice.

Cell communication assay

When determining gap junctional communication in all cell types, cells were cultured on glass coverslips in 1% FBS prior to microinjection procedure. However, when testing growth inhibitors, cells were cultured in serum-free media or 1% FBS supplemented DMEM for 24 h prior to and during growth inhibitor addition. Individual cells were pressure microinjected with 5% aqueous Lucifer yellow CH (Sigma Chemical Co.).

Microinjected dye was allowed to spread for 10 min before cells were fixed in 3.7% formaldehyde in PBS. Cells were subsequently viewed on a Zeiss axiophot fluorescent microscope or a Zeiss LSM 410 confocal microscope. At least 12 microinjected cells were analyzed for all treatments.

RESULTS

Connexin expression and GJIC in non-transformed bovine mammary cells

Bovine mammary epithelial cells, whether primary non-clonal, clonal nontransfected (MEB-E), or clonal SV-40 Large-T-antigen immortalized (MAC-T), all expressed Cx43 protein as measured by Western blot analysis (Figure 1). No major differences in Cx43 abundance or phosphorylation status were observed between fibroblasts (lanes A,B) and epithelial cells (lanes C,D,E). There were differences in Cx43 protein quantities between different fibroblast cell lines, notably primary cells (lane A) had less total Cx43 than MFB-3 (lane B). Variations in Cx43 species (phosphorylated versus unphosphorylated) or quantity were minimal between primary (lane C), clonal (lane D) and immortalized epithelial cells (lane E). Moreover, quantities of Cx43 in fibroblasts or epithelial cells, as measured by PhosphorImager analysis of Western blots were similar whether cell lysates were normalized to cell number or to total protein (data not shown). Cx32 and Cx26 were not identified in any bovine mammary epithelial or fibroblasts cell lysates (M.A. Sia, D.W. Laird, and T.L. Woodward, unpublished observations).

Immunofluorescent labelling of MEB-E cells for Cx43, revealed high levels of connexin43 at the cell surface (Figure 2 A). In MAC-T cells connexin43 labelling was also evident at the cell surface (Figure 2 B). In contrast to the MEB-E non transfected epithelial cells, MAC-T cells had considerably higher perinuclear pools of Cx43. Data presented by Sia and colleagues (1995) using colocalization and protein traffik blocking drugs, indicate a small Cx43 pool exists within the medial or trans Golgi apparatus of the MAC-T cells, though most of the perinuclear Cx43 pool has been transported past the trans Golgi apparatus. When clonal non-transfected MFB-C fibroblasts were immunolabelled for Cx43, punctate staining were observed between opposing cells. (Figure 2 C).

Gap junctional communication was measured by transfer of microinjected Lucifer Yellow dye (LY). In MEB-E epithelial cells (Figure 3 A), MAC-T epithelial cells (Figure 3 B) or MFB-C fibroblast cells (Figure 3 C) microinjected LY dye always spread to at least third order cells.

Connexin expression and GJIC in transformed bovine mammary fibroblasts

Mammary fibroblasts, MFB-TC, exhibited a transformed phenotype following stable transfection with a SV-40 Large-T-antigen expressing vector (Woodward et al., 1995). Western blotting and PhosphorImager analysis revealed a striking downregulation of total Cx43 protein (Figure 4 A, B). Moreover, phosphorylated species of Cx43 in transformed fibroblasts (lane C) were downregulated more than unphosphorylated species, when compared to either non-transfected clonal MFB-C fibroblasts (lane A) or

primary fibroblasts (lane B), Figure 4 A, B.

Immunolocalization of Cx43 in transformed fibroblasts (MFB-TC) revealed no Cx43 at location of cell-cell contact. Instead punctate Cx43 was restricted to perinuclear location (Fig 5 A, arrows). Additionally, dye transfer in MFB-TC was substantially reduced when compared to non-transfected MFB-C cells (Figure 5 B, C). Lucifer Yellow never transferred to more than one cell (i.e. not all first order cells, only to one cell in contact with the injected cell) and most often did not leave the microinjected cell.

Regulation of connexin expression and GJIC by growth inhibitors

Previously, our laboratory and others have demonstrated TGF-β_1, retinoic acid, and cyclic AMP inhibits cellular proliferation of MAC-T cells (Woodward et al., 1995, Woodward et al., In Press; Hund and Sheffield, 1991, respectively). Dose response curves revealed maximal growth inhibition by RA and TGF-β occurred at 1 μM and 40 pM, respectively. When MAC-T cells were treated with TGF-β_1, total Cx43 protein was increased 2-2.6 fold as measured by PhosphorImager analysis of Western blots (Table 1 A). Similarly, retinoic acid (100 nM) enhanced faster migrating Cx43 species 1.9 fold and phosphorylated Cx43 species 2.7 fold (Figure 6 A, B). Higher concentrations of retinoic acid (10 μM to 100 μM, where RA begins to cause cytotoxicity) inhibited phosphorylated Cx43 species by up to 70% versus untreated cells. Interestingly, RA was not cytotoxic at 1 μM, though Cx43 expression was reduced when compared to 100 nM RA.

Despite a preferential increase in phosphorylated Cx43 species, TGF-β (100 pM) and RA (100 nM) did not appear to alter cellular distribution of Cx43 as observed by immunocytochemical analysis (data not shown). Moreover, TGF-β (100 pM) or RA (100 nM) did not enhance microinjected LY spread (Figure 7 A, B, D). However, treatment with RA at 10 μM, which is beginning to exert cytotoxic effects but is not lethal, limited LY dye spread to first order cells or less (Figure 7 E) and resulted in the disappearance of intercellular punctate Cx43 labelling (Figure 8 A, B). In 10 μM RA treated MAC-T cells, Cx43 immunostaining was dispersed throughout the cytoplasm (Figure 8 B). Concomitant with the loss of GJIC by cytotoxic concentrations (lanes E, F) of RA was a decrease in phosphorylated species of Cx43 (46 kD and 44 kD), and a modest increase in Cx43 at 42 and 43 kD (Figure 6).

Similar to TGF-β and RA, dibutyrl cyclic AMP (dbcAMP) increased total Cx43 by approximately 2-3 fold (Table 1 B). Slower migrating Cx43 species were increased by dbcAMP 2.8 fold (1 mM), while faster migrating forms only increased 1.6 fold. Unlike RA or TGF-β, dbcAMP caused a dramatic redistribution of Cx43 (Figure 9). Prior to dbcAMP treatment, MAC-T cells had a large pool of perinuclear Cx43. Following dbcAMP (200 μM / 12 h) treatment, this intracellular pool is dramatically reduced. Moreover, a consistent increase in cell surface (intercellular) punctate Cx43 was present following dbcAMP treatment (Figure 9). Functional coupling, as measured by spread of LY was not significantly altered, however (Figure 7 C).

DISCUSSION

Loss of connexin expression and gap junctional communication has been associated with breast tumorigenesis by in vitro and in vivo studies (Nicolson et al., 1988; Lee et al., 1992; Wilgenbus et al., 1992; Tomasetto et al., 1993). Recent murine studies have demonstrated a developmentally specific regulation of connexin expression in the mammary gland; with peak connexin expression during lactation and minimal connexin expression in virgin mammary glands or during involution (Monaghan et al., 1994; Pozzi et al., 1995). Interestingly, gap junction expression, regulation and function have not been examined in the bovine mammary gland, where normal mammary development and function have been fervently studied for most of this century. Using a series of bovine mammary epithelial and fibroblast cell lines in this study, we establish the expression of Cx43 in all these cells, and characterize their distinct cell specific immunolocalization. Additionally, our results indicate that expression of the SV-40 Large-T-antigen and subsequent cellular 'immortalization' does not, in itself, limit connexin gene expression or gap junctional communication. However, Large-T-antigen transfected cells that display a transformed phenotype do have reduced connexin43 protein, altered Cx43 immunolocalization and decreased gap junctional communication. Finally, the present study examines the correlation between growth inhibition (by retinoic acid, TGF-β and cAMP) and gap junction upregulation in bovine mammary epithelial cells.

Studies concerning gap junction proteins and their regulation in the ruminant mammary

gland are lacking. In recent years, however, normal mouse, rat and human mammary cells have been examined for connexin expression and gap junctional communication (Wilenbus et al., 1992; Monaghan et al., 1994; Pozzi et al., 1995). In these species, some discrepancies still exist between which connexins are expressed. Wilenbus and colleagues (1992) reported that normal breast epithelium and normal breast connective tissue only show immunoreactivity for Cx43, not Cx26 or Cx32. In mouse mammary gland tissue, Monaghan and coworkers (1994) found no immunoreactivity for Cx43 or Cx32 in any stage of mammogenesis or lactogenesis. However, these researchers did find immunoreactivity to Cx26 in mammary tissue during pregnancy and lactation. Another laboratory, recently reported the presence of Cx26 and Cx32 in mouse and rat mammary gland, but only during lactation (Pozzi et al., 1995). Additionally, Cx43 was present in all stages of rodent mammary examined and in the human breast (nonpregnant), though immunolocalization revealed junctions between myoepithelial cells and not secretory epithelial cells (Pozzi et al., 1995).

Herein, we have identified by Western blot and immunocytochemical analyses the presence of Cx43: 1) in low passage primary epithelial cells and 2) fibroblasts, 3) in clonal epithelial and 4) fibroblast cell lines, in 5) SV-40 Large-T-antigen transfected epithelial cells and in 6) SV-40 transfected and phenotypically transformed fibroblast cells from the bovine mammary gland. Interestingly, little difference in Cx43 total protein was observed between primary cells and non transformed cell lines, indicating that Cx43 expression is not a result of adaptation to culture.

Mammary epithelial cells (MAC-T) and MFB-TC fibroblasts were transfected with the SV-40 Large-T-antigen to allow for extended passage in culture (immortalization) without senescence or retarded proliferation (Huynh et al., 1991; Woodward et al., 1995). The mechanism by which SV-40 Large-T-antigen confers 'immortality' can also alter growth and differentiation responsiveness as well as induce a transformed phenotype (Weinberg, 1991; Moses, 1992; Weinberg, 1995). MAC-T cells, however, do not express a transformed phenotype, are growth responsive and growth inhibited and retain normal epithelial properties (Huynh et al., 1991; MacDonald et al., 1994; Woodward et al., 1994; Woodward et al., 1995). Similarly, MAC-T cells expressed Cx43 at levels comparative to primary epithelial cells or clonal non transfected epithelial cells (MEB-E). Immunolocalization of Cx43 did, however, reveal increased perinuclear pools of Cx43 when compared to nontransfected counterparts. Cx43 is still abundant at the cell surface in MAC-T cells, and gap junctional communication is at least equal to GJIC in non-transfected epithelial cells.

Much data has suggested that tumorigenesis in the breast and other tissues may inhibit normal gap junctional communication (Nicolson et al., 1988; Holder et al., 1993; Neveu et al., 1994; Wolburg and Rohlmann, 1995). In 1989, Eldridge and colleagues reported that immortalization, but not transformation, of human mammary epithelial cells by benzo(a)pyrene induced decreased intercellular communication. Additionally, these researchers found that tumorigenic mammary epithelial cells communicated to a greater extent than normal or immortalized cells and hypothesized that 'immortalization' of tumor cells may be responsible for the reduced gap junctional communication often found

in tumor cells. Loss of GJIC in keratinocytes 'immortalized' by infection (not transfection) with the oncogenic simian-virus 40 was also reported by Steinberg and Defendi (1981). Additionally, Rosen and colleagues (1988) reported that SV-40 infection and transformation decreased GJIC in human fibroblasts. We have previously reported that identical transfections with SV-40 Large-T-antigen often led to cellular transformation in bovine mammary fibroblasts, whereas no epithelial cells expressed a transformed phenotype (Woodward et al., 1995). The nontransformed MAC-T cell line were well coupled by gap junctions, despite the expression of the Large-T-antigen. However, bovine mammary fibroblasts transfected with the Large-T-antigen and expressing a transformed phenotype did show decreased connexin expression and loss of GJIC (Huynh et al., 1991; Woodward et al., 1995). Thus, our results are contradictory to those of Eldridge and coworkers (1989), since we found immortalization in itself does not inhibit connexin expression or GJIC, but induction of a tumorigenic phenotype did inhibit connexin expression and arrest GJIC.

Loss or diminished gap junctional communication in tumor cells may correspond to loss of control of cellular proliferation in the tumor state. Several reports have indicated that gap junctional communication may play an important role in regulating cellular proliferation (Zhu et al., 1992; Hossain and Bertram, 1994; Mesnil et al., 1995). Many studies have now demonstrated inhibition of cellular proliferation in tumor cells transfected with gap junctional proteins (Eghbali et al., 1991; Mehta, 1991; Rose et al., 1993; Bond et al., 1994; Lin et al., 1995; Mesnil et al., 1995). Gap junctional communication and/or connexin expression has been shown to be inhibited by tumor-

promoting agents that increase cell proliferation (Krutovskikh et al., 1995) and enhanced by agents that inhibit cellular proliferation (Chiba et al., 1994; Hossain and Bertram, 1994; Ren et al., 1994). Researchers have also found similar correlations between growth inhibition and gap junctional communication in non-transformed cells (Chiba et al., 1994; Ren et al., 1994; Zhang and Thorgeirsson, 1994).

These studies specifically address three agents that inhibit cellular proliferation and analyze how they affect gap junctions and their regulation. Previously, we and others have shown retinoic acid, cAMP and TGF-β1 inhibit cellular proliferation of mammary cells (Hund and Sheffield, 1991; Woodward et al., 1995; In press). Other laboratories have demonstrated that retinoic acid enhances connexin expression or GJIC (Chiba et al., 1994; Hossain and Bertram, 1994; Bex et al., 1995). cAMP has also been shown to enhance GJIC (Mehta et al., 1992). Results concerning TGF-β effects on GJIC are less definitive, with studies demonstrating inhibition of GJIC (Albright et al., 1991; Chandross et al., 1995) or stimulation of GJIC (Albright et al., 1991; Chiba et al., 1994). However, TGF-β often exhibits opposing effects in dissimilar cells, generally stimulating mesenchymal cell proliferation and inhibiting parenchymal cell proliferation (Roberts and Sporn, 1990). We have demonstrated that all three growth inhibitors examined: cAMP, retinoic acid, and TGF-β enhance total Cx43 protein expression in the non-transformed MAC-T cell line. Though, only cAMP was able to substantially alter immunolocalization of Cx43, causing increased punctate cell surface Cx43 labelling and decreased cytoplasmic and perinuclear Cx43.

Despite elevated connexin43 expression following treatment with RA, cAMP or TGF-β, none of these growth inhibitors significantly enhanced transfer of microinjected Lucifer Yellow. Recent evidence from our laboratory indicates that induction of quiescence by serum starvation did not inhibit gap junctional communication in mammary cells, despite substantial connexin downregulation (Sia et al., 1995). Therefore, we propose that upregulation (or downregulation) of total connexin expression, increased maturation of connexin and even increased cell membrane expression of gap junction proteins do not necessarily alter GJIC in well coupled cells.

When growth inhibition affected cellular cytotoxicity, connexin expression, phosphorylation and gap junctional communication were all inhibited. We have previously reported that retinoic acid begins to cause cellular cytotoxicity at 10 μM in MAC-T cells, though significant cell death does not occur until 100 μM (Woodward et al., In Press). At concentrations of 10 or 100 μM, RA decreased connexin43 expression, disproportionately decreased phosphorylated Cx43 species, eliminated cell surface Cx43 and arrested dye transfer in MAC-T cells. Although many studies have shown toxins inhibit gap junctional communication at lethal doses, others have shown, as reported here, sub-lethal concentrations of cytotoxic agents to inhibit GJIC (Tateno et al., 1993; Hu and Cotgreave, 1995). In fact, Cx43 expression was maximal in MAC-T cells following RA treatment at 100 nM, while Cx43 quantities dropped at the non-cytotoxic concentration of 1 μM. TGF-β was not cytotoxic at concentrations 100-fold higher than those that arrest MAC-T cell growth (40 pM) (Woodward et al., 1995). Cx43 expression increased as TGF-β1 concentrations were increased up until 10 pM TGF-β1,

with no significant change occurring at higher TGF-β concentrations. Therefore, TGF-β upregulation of Cx43 nearly paralleled TGF-β inhibition of growth.

Since allometric mammary gland growth and development occur during puberty, pregnancy and lactation (Sheffield, 1988), gap junctional communication and its regulation may be particularly relevant in the adult mammary gland, as others have demonstrated the necessity of GJIC in normal embryonic development (Warner et al., 1984; McLachin and Kidder, 1986; Reaume et al., 1995). We have provided the first evidence in bovine mammary epithelial and fibroblast cells of functional gap junction proteins (Cx43), that can be acutely regulated by growth inhibitors. However, this regulation by growth inhibitors does not always significantly effect function, i.e., GJIC. We have also determined that immortalization of cells does not inhibit GJIC, whereas oncogenic transformation may strongly inhibit connexin expression, maturation, and function.

ACKNOWLEDGEMENTS:

The authors would like to thank Bristol-Meyer Squibb for TGF-β_1, Josee Plamondon for assistance in PhosphorImager use and Michael Sia for his help in several assays. This work was supported by a Quebec Merit Fellowship to T.L.W. and a Medical Research Council Grant to D.W.L. (Grant MT-12241).

LITERATURE CITED

Albright, C.D., P.M. Grimley, R.T. Jones, J.A. Fontana, K.D. Keenan, and J.H. Resau. 1991. Cell-to-cell communication: a differential response to transforming growth factor-β in normal and transformed (BEAS-2B) human bronchial epithelial cells. Carcinogenesis, 12:1993-1999.

Bex, V., T. Mercier, C. Chaumontet, I. Gaillardsanchez, B. Flechon, F. Mazet, O. Traub, and P. Martel. 1995. Retinoic acid enhances connexin43 expression at the post-transcriptional level in rat liver epithelial cells. Cell Bioch. Funct., 13:69-77.

Beyer, E.C. 1993. Gap Junctions. Int. Rev. Cytol., 137C:1-37.

Bond, S.L., J.F. Bechberger, N.K. Khoo, and C.C. Naus. 1994. Transfection of C6 glioma cells with connexin32: the effects of expression of a nonendogenous gap junction protein. Cell Growth Diff., 5:179-186.

Casico, M., N.M. Kumar, R. Safarik, and N.B. Gilula. 1995. Physical characterization of gap junction membrane connexons (hemi-channels) isolated from rat liver. J. Cell Biol., 270:1863-18648.

Chandrass, K.J., M. Chanson, D.C. Spray, and J.A. Kessler. 1995. Transforming growth factor-beta-1 and forskolin modulate gap junctional communication and cellular phenotype of cultured Schwann cells. J. Neurosci., 15:262-273.

Charles, A.C., C.C.G. Naus, D. Zhu, G.M. Kidder, E.R. Dirksen, and M.J. Sanderson. 1992. Intercellular calcium signalling via gap junctions in glioma cells. J. Cell Biol., 118:195-201.

Chiba, H., N. Sawada, M. Oyamada, T. Kojima, K. Iba, S. Ishii, and M. Mori. 1994. Hormonal regulation of connexin43 expression and gap junctional communication in human osteoblastic cells. Cell Struct. Funct., 19:173-177.

Eghbali, B., J.A. Kessler, L.M. Reid, C. Roy, and D.C. Spray. 1991. Involvement of gap junctions in tumorigenesis: transfection of tumor cells with connexin32 cDNA retards growth in vivo. Proc. Natl. Acad. Sci. 88:10701-10705.

Eldridge, S.R., T.W. Martens, C.A. Sattler, and M.N. Gould. 1989. Association of decreased intercellular communication with the immortal but not the tumorigenic phenotype in human mammary epithelial cells. Cancer Res., 49:4326-4331.

Enomoto, K., K. Furuya, S. Yamagishi, and T. Maeno. 1992. Mechanically induced electrical and intracellular calcium responses in normal and cancerous mammary cells. Cell Calcium, 13:501-511.

Holder, J.W., E. Elmore, and J.C. Barrett. 1993. Gap junction function and cancer. Cancer Res., 53:3475-3485.

Hossain, M.Z. and J.S. Bertram. 1994. Retinoids suppress proliferation, induce cell spreading, and up-regulate connexin43 expression only in postconfluent 10T1/2 cells: implications for the role of gap junctional communication. Cell Growth Diff., 5:1253-1261.

Hu, J., and I.A. Cotgreave. 1995. Glutathione depletion potentiates 12-O-tetradecanoyl phorbol-13-acetate (TPA)-induced inhibition of gap junctional intercellular communication in WB-F344 rat liver epithelial cells: relationship to intracellular oxidative stress. Chemico-Biol. Interactives, 95:291-307.

Hund, B., and L.G. Sheffield. 1991. Cyclic AMP mediated growth response of mammary epithelial cells (MAC-Ts) J. Dairy Sci., 74:143. Abstract.

Huynh, H.T., G. Robitaille, and J.D. Turner. 1991. Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. Exp. Cell Res.,197:191-199.

Krutovskikh, V.A., M. Mesnil, G. Mazzoleni, and H. Yamasaki. 1995. Inhibition of rat liver gap junction intercellular communication by tumor-promoting agents in vivo: association with aberrant localization of connexin proteins. Lab. Invest., 72:571-577.

Laird, D.W., and J.C. Saez. 1995. Posttranscriptional events in the expression of gap junctions. In: Advances in Molecular Cell Biology, Vol.18: In Press.

Laird, D.W., M. Castillo, and L. Kasprak. 1995. Gap junction turnover, intracellular trafficking and phosphorylation of connexin43 in brefeldin A-treated rat mammary tumor cells. J. Cell Biol., In Press.

Lamelli, U.K. 1970. Cleavage of the structural proteins during the assemblage of the head of bacteriophage T4. Nature, 227:680-685.

Lee, S.W., C. Tomasetto, D. Paul, K. Keyomarsi, and R. Sager. 1992. Transcriptional downregulation of gap-junction proteins blocks junctional communication in human mammary tumor cell lines. J. Cell Biol., 118:1213-1221.

Lin, Z., Z. Zhang, Y. Han, C.C. Naus, K.R. Yu, and H. Holtzer. 1995. Functional expression of gap junction gene Cx43 and the myogenic differentiation of rhabdomyosarcoma cells. Sci. In China. 38B:305-312.

Loewenstein, W.R. 1967. Intercellular communication and tissue growth. J. Cell Biol., 33:225-234.

Mehta, P.P., A. Hotz-Wagenblatt, B. Rose, D. Shalloway, and W.R. Loewenstein. 1991. Incorporation of the gene for a cell-cell channel protein into transformed cells leads to normalization of growth. J. Memb. Biol., 124:207-225.

Mehta, P.P., M. Yamamoto, and B. Rose. 1991. Transcription of the gene for the gap junction protein connexin43 and expression of functional cell-to-cell channels are regulated by cAMP. Mol. Biol. Cell, 3:839-850.

Mesnil, M., V. Krutovskikh, C. Piccoli, C. Elfgang, O. Traub, K. Willecke, and H. Yamasaki. 1995. Negative growth control of HeLa cells by connexin genes: connexin species specificity. Cancer Res., 55:629-639.

Monaghan, P., N. Perusinghe, G. Carlile, and W.H. Evans. 1994. Rapid modulation of gap junction expression in mouse mammary gland during pregnancy, lactation, and involution. J. Histoch. Cytoch., 42:931-938.

Moses, H.L. 1992. TGF-β regulation of epithelial cell proliferation. Mol. Reprod. Dev., 32:179-184.

Naus, C.C.G., J.F. Bechberger, S.Caveney, and J.X. Wilson. 1991. Expression of gap junction genes in astrocytes and C6 glioma cells. Neurosc. Lett., 126:33-36.

Naus, C.C.G., S. Hearn, D. Zhu, B.J. Nicoloson, and R.R. Shivers. 1993. Ultrastructural analysis of gap junctions in C6 glioma cells transfected with connexin43 cDNA. Exp. Cell Res., 206:72-84.

Neveu, M., J.R. Hully, K.L. Babcock, E.L. Hertzberg, B.J. Nicholson, D.L. Paul, and H.C. Pitot. 1994. Multiple mechanisms are responsible for altered expression of gap junction genes during oncogenesis in rat liver. J. Cell Sci., 107:83-95.

Nicolson, G.L., K.M. Dulski, and J.E. Trosko. 1988. Loss of intercellular junctional communication correlates with metastatic potential in mammary adenocarcinoma cells. Proc. Natl. Acad. Sci., 85:473-476.

Oh, S.Y., C.G. Grupen, and W.C. Murray. 1991. Phorbol ester induces phosphorylation and downregulation of connexin43 in WB cells. Bioch. Biophy. Acta 1094:243-245.

Pozzi, A., B.Risek, D.T. Kiang, N.B. Gilula, N.H. Kumar. 1995. Analysis of multiple gap junction gene products in the rodent and human mammary gland. Exp. Cell Res., 220:212-219.

Reaume, A.G., P.A. de Sousa, S. Kulkarni, B.L. Langille, D. Zhu, T.C. Davies, S.C. Juneja, G.M. Kidder, J. Rossant. 1995. Cardiac malformation in neonatal mice lacking connexin43. Science, 267:1831-1834.

Ren, P., A.W. Feijter, D.L. Paul, and R.J. Ruch. 1994. Enhancement of liver cell gap junction protein expression by glucocorticoids. Carcinogenesis. 15:1807-1813.

Rose, B., P.P. Mehta, and W.R. Loewenstein. 1993. Gap-junction protein gene suppresses tumorigenicity. Carcinogenesis, 14:1073-1075.

Rosen, A., P.A. van der Merwe, and J.S. Davidson. 1988. Effects of SV40 transformation on intercellular gap junctional communication in human fibroblasts. Cancer Res., 48:3485-3489.

Roberts, A.B., and M.B. Sporn. 1990. The transforming growth factor-betas. In: Peptide Growth Factors and Their Receptors. M.B. Sporn and A.B. Roberts, eds. Springer-Verlag, Heidelberg, Germany, pp.421-472.

Ruch, R.J. 1994. The role of gap junctional intercellular communication in neoplasia. Annals Clin. Lab. Sci., 24:216-231.

Sheffield, L.G. 1988. Organization and growth of mammary epithelia in the mammary gland fat pad. J. Dairy Sci., 71:2855-2874.

Sorensen, K., and U. Brodbeck. 1986. A sensitive protein assay method using microtiter plates. Experientia, 42:161-162.

Stauffer, K.A. 1995. The gap junction proteins β_1-connexin (connexin-32) and β_2-connexin (connexin-26) can form heteromeric hemichannels. J. Biol. Chem., 270:6768-6772.

Tateno, C., S. Ito, M. Tanaku, and A. Yoshitake. 1993. Effects of pyrethroid insecticides on gap junctional intercellular communications in Balb/c3T3 cells by dye-transfer assay. Cell Biol. Toxic., 9:215-221.

Tomasetto, C., M.J. Neveu, J. Daley, P.K. Horan, and R. Sager. 1993. Specificity of gap junction communication among human mammary cells and connexin transfectants in culture. J. Cell Biol., 122:157-167.

Traub O., J. Look, R. Dermietzel, F. Brummer, D. Hulser, K. Willecke. 1989. Comparative characterization of the 21-kD and 26-kD gap junction proteins in murine liver and cultured hepatocytes. J. Cell Biol., 108:1039-1051.

Warner, A.E., S.C. Guthrie, and N.B. Gilula. 1984. Antibodies to gap-junctional protein selectively disrupt junctional communication in the early amphibian embryo. Nature, 311:127-131.

Weinberg, R.A. 1991. Tumor suppressor genes. Science, 254:1138-1146.

Weinberg, R.A. 1995. The retinoblastoma protein and cell cycle control. Cell, 81:323-330.

Wilgenbus, K.K., C.J. Kirkpatrick, R. Knuechel, K. Willecke, and O. Traub. 1992. Expression of Cx26, Cx32 and Cx43 gap junctional proteins in normal and neoplastic human tissues. Int. J. Cancer, 51:552-529.

Wolburg, H., and A. Rohlmann. 1995. Structure-function relationships in gap junctions. Int. Rev. Cyt., 157:315-373.

Woodward, T.L., R.M. Akers, and J.D. Turner. 1994. Lack of mitogenic response to EGF, pituitary and ovarian hormones in bovine mammary epithelial cells. Endocr. J., 2:529-535.

Woodward, T.L., N. DuMont, M. O'Connor-McCourt, J.D. Turner, and A. Philip. 1995. Characterization of transforming growth factor-β growth regulatory effects and receptors on bovine mammary cells. J. Cell. Phys., 165:339-348.

Woodward, T.L., J.D. Turner, H.T. Huynh, and X. Zhao. 1996. Inhibition of cellular proliferation and modulation of insulin-like growth factor binding proteins by retinoids in a bovine mammary epithelial cell line. J. Cell. Phys., In Press.

Zhang, M., and S.S. Thorgeirsson. 1994. Modulation of connexins during differentiation of oval cells into hepatocytes. Exp. Cell Res., 213:37-42.

Zhu, D., G.M. Kidder, S. Caveney, and C.C. Naus. 1992. Growth retardation in glioma cells cocultured with cells overexpressing gap junction protein. Proc. Natl. Acad. Sci., 89:10218-10221.

Figure 1: Western blot analysis of Cx43 in bovine mammary cells: (A) primary fibroblasts, (B) clonal fibroblasts (MFB-C), (C) primary epithelium, (D) clonal epithelium (MEB-E) and (E) clonal SV-40 Large-T-antigen immortalized epithelial cells (MAC-T). Note the presence of Cx43 species at 42, 44 and 46 kD in all cell lysates. Lysates were adjusted to cell number.

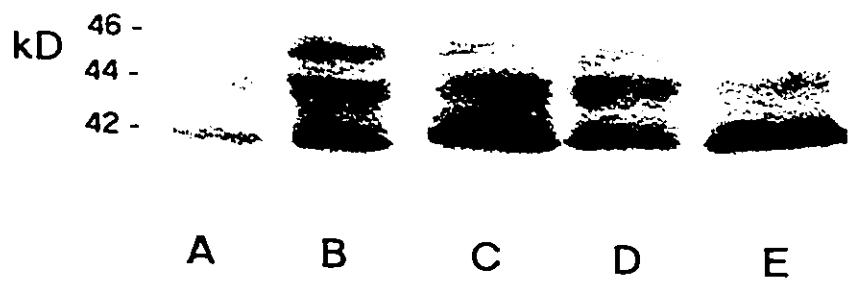

kD
46 -
44 -
42 -
A
B
C
D
E

Figure 2: Immunolocalization of Cx43 in bovine mammary cells: (A) clonal non-transfected MEB-E epithelial cells, (B) clonal immortalized MAC-T epithelial cells, (C) clonal non-transfected MFB-C fibroblasts cells. Note intercellular punctate labelling in all cell types, and increased perinuclear vesicular labelling in MAC-T cells. Bars = 10 μm.

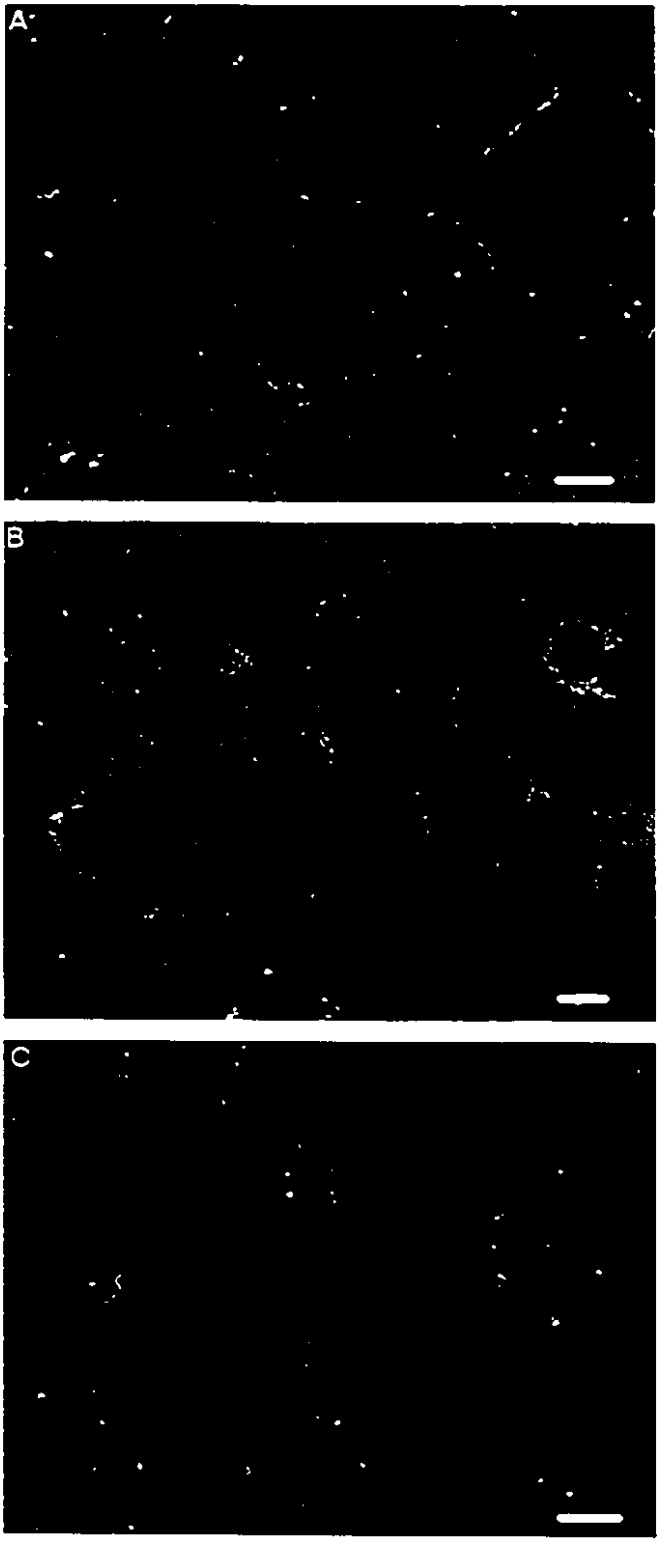
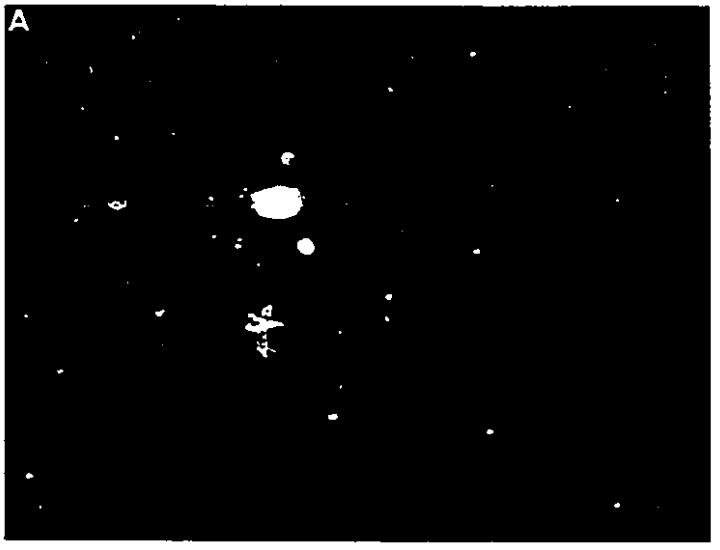
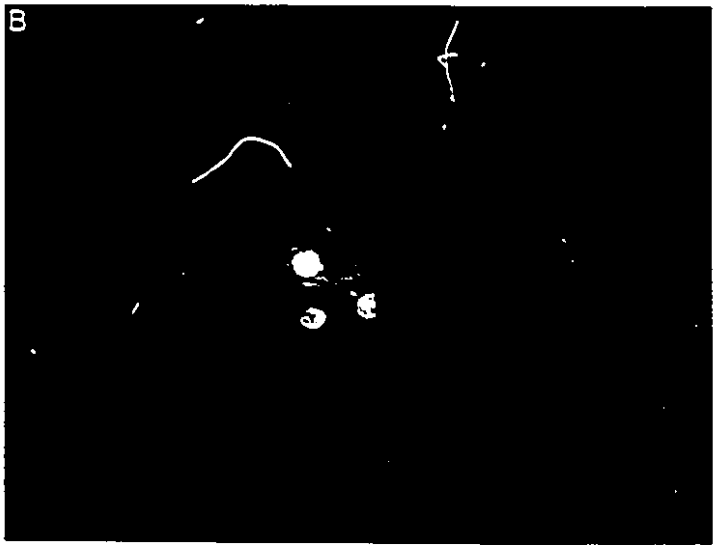

A
B
C

Figure 3: Dye coupling in (A) MEB-E epithelial cells, (B) MAC-T epithelial cells and (C) MFB-C fibroblast cells. Cells were microinjected with Lucifer Yellow and dye was allowed to spread for 10 min before fixation. Note that dye spread to at least 3rd order cells in all cell types.


A


B

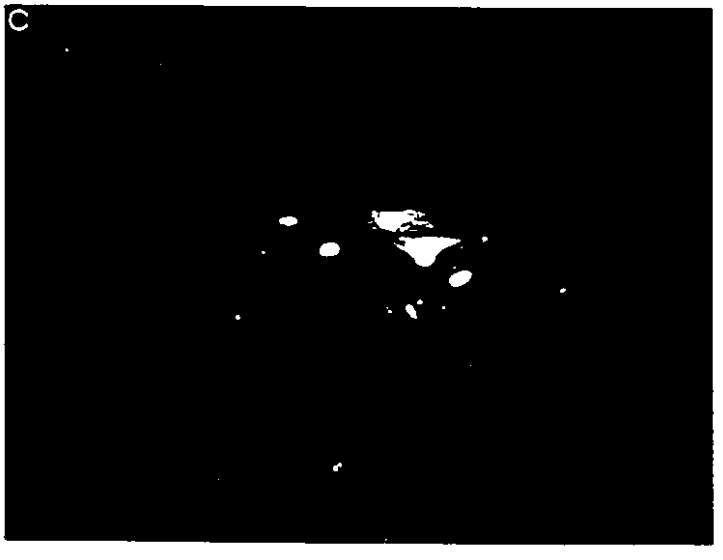

C

Figure 4: Western blot (Fig. A) and PhosphorImager (Fig. B) analyses of total Cx43 in bovine mammary fibroblasts. Primary fibroblast populations (lane B) are compared to clonal non-transfected MFB-C fibroblasts (lane A) and SV-40 Large-T-antigen transfected MFB-TC fibroblasts (lane C) displaying a transformed phenotype. Note the large reduction in the phosphorylated Cx43 species and the downregulation of total Cx43 in transformed fibroblasts. Lysates were adjusted to cell number.

A

B

44-46 kD	4.0	3.2	0.4
42-43 kD	4.3	3.7	1.4
Lane	A	B	C

Figure 5: Immunolocalization of Cx43 (A) and dye transfer (B) in transformed bovine mammary MFB-TC fibroblasts. Note the lack of Cx43 immunostaining at locations of cell-cell apposition and the appearance of punctate intracellular staining in MFB-TC cells (A, arrows). Lucifer Yellow failed to transfer from the microinjected cell (asterisk) to any neighboring cells (B) as observed by phase contrast microscopy (C). Bars = 10 μm.


A


B


C

Figure 6: Western blot (Fig. A) and PhosphorImager (Fig. B) analyses of total Cx43 in bovine mammary MAC-T epithelial cells treated with all-trans retinoic acid (RA). Note an increase in all species of Cx43 up to 100 nM RA (lane C). However, concentration above 100 nM RA result in a decrease in phosphorylated species.

A

B

44-46 kD	1.0	2.0	2.7	1.7	0.5	0.3
42-43 kD	1.0	1.8	1.9	1.3	1.4	1.6
Lane	A	B	C	D	E	F

Figure 7: Dye transfer in MAC-T cells cultured in 1% FBS, untreated (A), or treated with 100 pM TGF-β_1 (B), 200 μM dibutyrl cAMP (C), 100 nM all trans RA (D) or 10 μM RA (E). Cells were microinjected with Lucifer Yellow and dye was allowed to spread for 10 min before fixation. Note there is little difference in dye transfer except when cytotoxic concentrations of RA (E) were used, where dye transfer was completely eliminated. Cells were imaged under identical conditions.

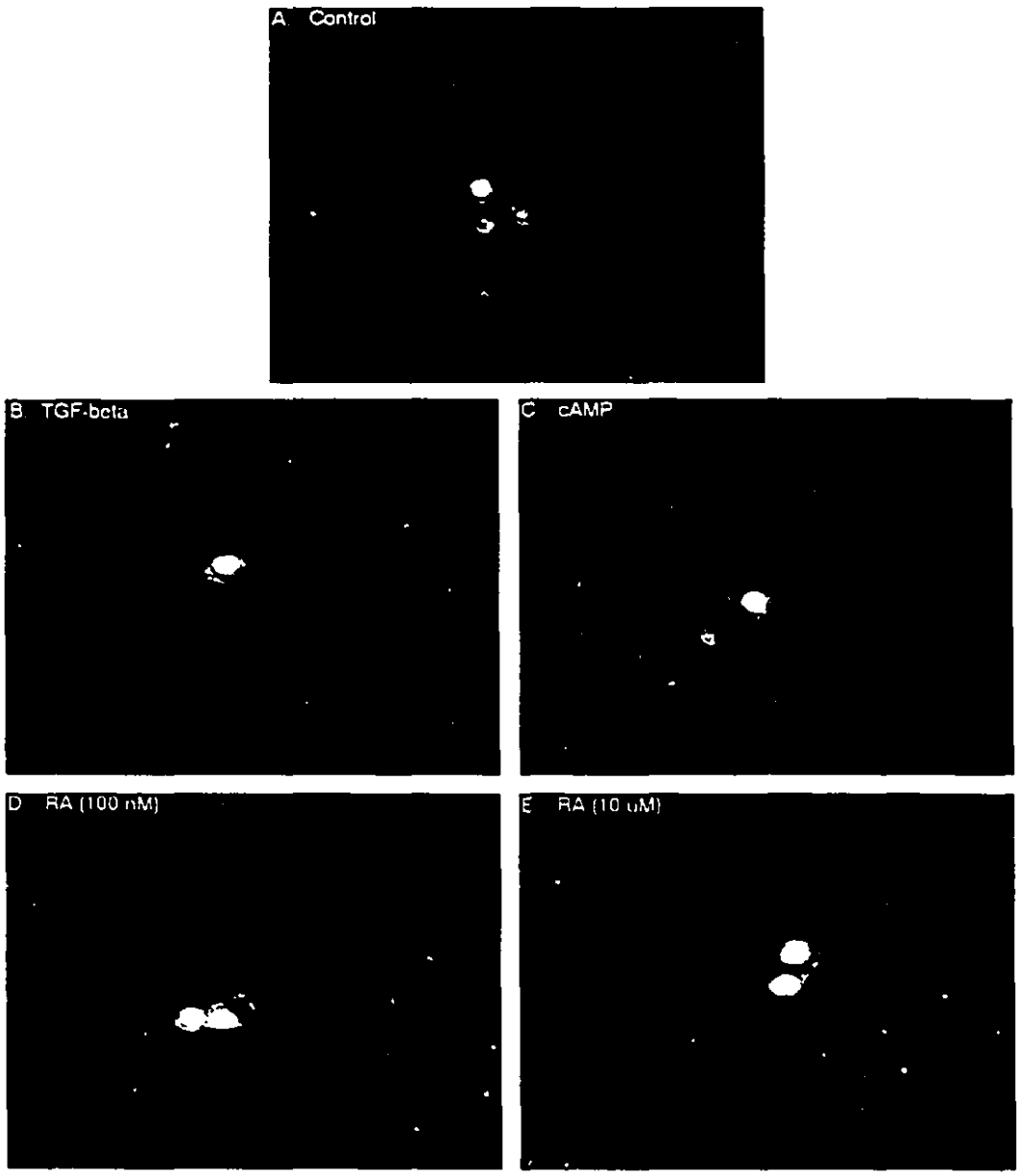
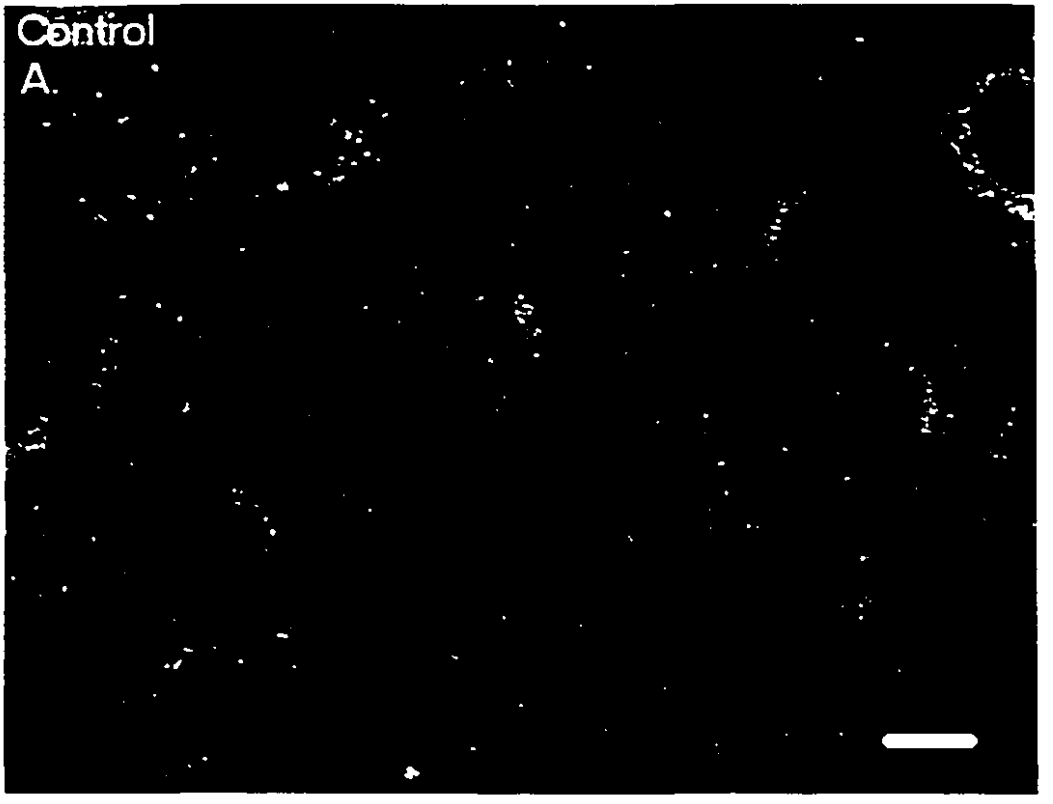

A Control
B TGF-beta
C cAMP
D RA (100 nM)
E RA (10 uM)

Figure 8: Immunolocalization of Cx43 in untreated (A) and all trans RA (10 μM) treated (B) MAC-T cells. Note intercellular punctate labelling in untreated MAC-T cells (A) is completely eliminated in 10 μM RA treated cells (B). Also, there is diffuse intracellular labelling throughout the cytoplasm of RA treated cells. Cells were imaged under identical conditions. Bars = 10 μm.


Control
A.

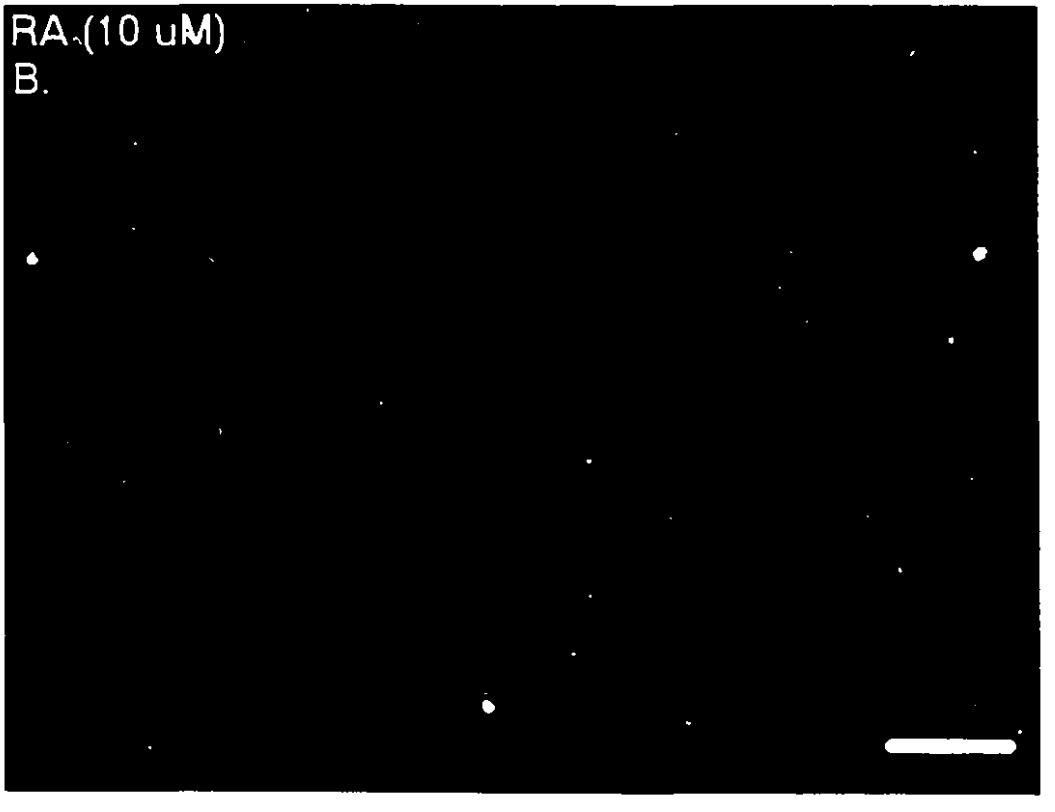
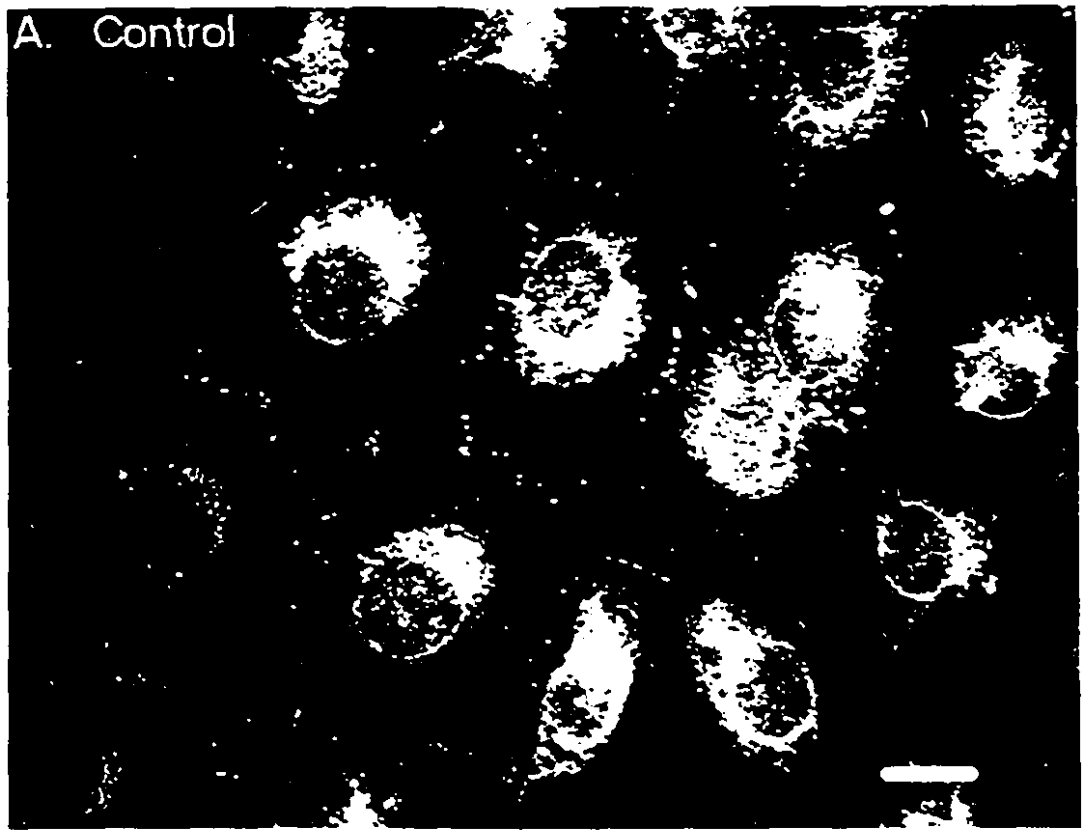

RA (10 uM)
B.

Figure 9: Immunolocalization of Cx43 in untreated (A) and dibutyrl cAMP (B) treated MAC-T cells. Note perinuclear Cx43 staining in untreated MAC-T cells (A) is lost upon 12 h 200 μM cAMP treatment. Cells were imaged under identical conditions. Bars = 10 μm.


A. Control

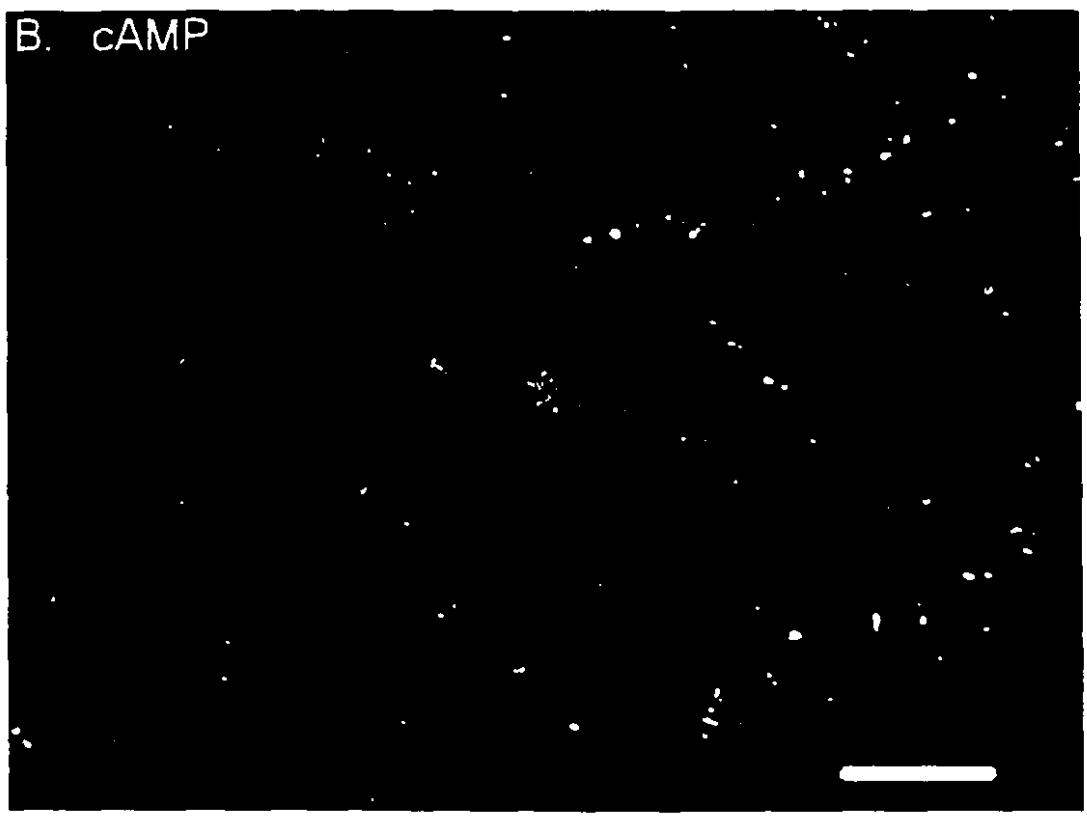

B. cAMP

Table 1. PhosphorImager analysis of Western blots (not shown) of all Cx43 species in bovine mammary epithelial cells (MAC-T) treated with TGF-β_1 (A) or dibutyrl cAMP (B). (A) TGF-β treatment (10 pM) resulted in an increase in phosphorylated and unphosphorylated expression of Cx43. (B) cAMP treatment also increased Cx43 expression, however, phosphorylated Cx43 species steadily increased as cAMP concentrations increased from 1 μM to 1mM. Lysates were adjusted to cell number.

A

	Transforming growth factor- β1				
Concentration	0	1 pM	10 pM	100 pM	1 nM
44-46 kD	1.0	1.0	2.6	2.1	2.0
42-43 kD	1.0	1.1	1.5	1.5	2.0

B

	dibutyrl cAMP				
Concentration	0	1 μM	10 μM	100 μM	1 mM
44-46 kD	1.0	1.3	1.8	2.3	2.8
42-43 kD	1.0	1.4	1.6	1.6	1.5

SUMMARY 5

Gap junctional proteins, connexins, and gap junctional intercellular communication (GJIC) were examined in chapter 5. All the fibroblast and epithelial cell lines were positive for connexin43 protein as determined by Western blot and immunocytochemical analyses. These bovine mammary cells included the MAC-T cell line and fibroblast and epithelial cell lines and primary cell populations described in chapter 1. Western blot analysis revealed that Cx43 expression was not significantly different between non-transfected and SV-40 large-T-antigen transfected epithelial cells. Additionally, Cx43 expression was similar in primary cell populations when compared to cell lines. Also, fibroblast and epithelial cells expressed similar levels of Cx43 protein when measured by Western blot and quantified by PhosphoImager analysis. One major change in Cx43 protein expression occurred when fibroblasts were transfected with the SV-40 Large-T-antigen and displayed a transformed phenotype. These cells had much lower Cx43 protein levels, especially mature Cx43 species that are involved in GJIC at the cell surface. Similarly, these cells had no immunodectable Cx43 at the cell surface, but still had minimal cytoplasmic Cx43 immunoreactivity as evidenced by immunocytochemistry. Functional analysis of these transformed fibroblasts revealed no dye transfer, when microinjected with Lucifer Yellow. Thus, transformation of cells, but not SV-40 Large-T-antigen transfection inhibited Cx43 expression and GJIC.

All other cell lines, MAC-T, MEB-E (FbE) and MFB-3 transferred Lucifer Yellow to at least third order cells from the microinjected cell. Dye transfer in these non-transformed epithelial and fibroblast cell lines appeared to be limited only by dilution of the dye caused by transfer. Since Cx43 protein levels and dye transfer was similar in all non-transformed bovine mammary epithelial and fibroblast cells, we sought to determine if these proteins had similar immunolocalization patterns. Staining with a Cx43 primary antibody and immunofluorescent secondary antibodies and visualization by immunofluorescent confocal microscopy revealed minimal differences in Cx43 protein expression within a cell type (i.e. epithelial or fibroblast). Epithelial cells had well defined Cx43 punctate staining at the cell surface (plaques). Most intracellular Cx43 staining in epithelial cells was perinuclear. Fibroblasts, had punctate staining at the cell surface. However, fibroblasts also had intracellular punctate staining and cell surface punctate Cx43 staining outside of intercellular contact areas. Thus, we have shown that Cx43 is present in stromal and parenchymal bovine mammary cells and that transformation of these cells may lead to diminished Cx43 protein expression and loss of GJIC. These data prompted us to examine if growth inhibitors discussed in chapters 3 and 4 could regulate this abundant protein.

TGF-β1 (chapter 3), retinoic acid (chapter 4) and the second messenger cyclic AMP have been shown by ourselves and others to inhibit MAC-T cell proliferation. By Western blot analysis, all of these growth inhibitors enhanced total Cx43 expression by 1.5-3 fold in MAC-T cells. Additionally, cAMP caused a preferential increase in the

phosphorylated forms of Cx43. Cyclic AMP and TGF-β were not toxic (chapter 3), but RA caused cytotoxicity at 10 μM or higher (chapter 4). Interestingly, at 10 or 100 μM RA inhibited Cx43 expression and preferentially inhibited its phosphorylated forms. Immunocytochemistry revealed no major Cx43 alterations by RA or TGF-β treatment, though cAMP treated MAC-T cells showed decreased perinuclear Cx43 staining and increased cell surface Cx43 staining. Cytotoxic concentrations of RA eliminated cell surface Cx43. No difference was observed in dye transfer between control, RA, TGF-β, or cAMP treated cells whether cells were cultured in serum free media or 1% FBS supplemented media. Retinoic acid (10 μM) eliminated Lucifer Yellow dye transfer in MAC-T cells.

Therefore, both stromal and epithelial mammary cells express Cx43 protein. Transformation of cells or cellular cytotoxicity causes downregulation of Cx43 and decreased GJIC. Additionally, growth inhibitors increase Cx43 protein, especially phosphorylated species. MAC-T cells, however, are well coupled without growth inhibitor treatment, even in serum-free conditions. Thus, these increases in Cx43 expression did not lead to increased GJIC as measured by transfer of Lucifer Yellow dye. Therefore, growth inhibitors may not increase GJIC in previously well coupled (by gap junctions) MAC-T cells, but may effect GJIC in virgin or involuting mammary glands, when connexin expression (in mice) is reportedly very low or nonexistant.

CONCLUSION

Several conclusions can be drawn from this thesis. Mammary cells, whether primary populations, clonal lines, or clonal immortalized (by the SV-40 Large-T-antigen) cell lines respond similarly to mitogens, growth inhibitors and have similar gap junctional protein expression and GJIC. However, when these cells exhibit a transformed phenotype, cellular proliferation, morphology, connexin protein expression and GJIC are all effected. Interestingly, fibroblasts were preferentially transformed by SV-40 Large-T-antigen transfection when compared to epithelial cells.

Bovine mammary epithelial cells proliferate in response to insulin and IGFs, and undefined components in serum and serum albumin. However, many mammogens that are well characterized in vivo or in other species do not affect isolated bovine mammary epithelial cell proliferation. Most notably is the lack of responsiveness to EGF. This was especially intriguing since EGF is a major mitogen to mouse and human mammary epithelium and has often been considered a potent mammogen in all species.

After a comprehensive investigation of bovine epithelial mitogens, we sought to determine what factors inhibit proliferation of these cells. Autoregulated TGF-β receptors were identified in both epithelial and fibroblast cells, making them a likely candidate. TGF-β1 and TGF-β2 both arrested MAC-T cell proliferation at very low concentrations (4 pM), but only inhibited fibroblast proliferation by 50% at much higher

concentrations (1.28 nM). No differences in inhibition of cellular proliferation by TGF-β were noted between SV-40 Large-T-antigen transfected fibroblasts and non-transfected fibroblasts. TGF-βs effects were not cytotoxic and were reversible. Thus, TGF-β is the first physiological growth inhibitor identified that completely and reversibly arrests bovine mammary epithelial cell proliferation.

Retinoids were next investigated, and retinoic acid was found to inhibit cellular proliferation of MAC-T cells. Retinoic acid was cytotoxic at concentrations 10-fold higher than concentrations that maximally inhibit cellular proliferation. Additionally, RA inhibition of cellular proliferation occurred following increased IGFBP-2 protein expression and decreased IGFBP-3 protein expression into the media and at the cell surface. This did not occur after TGF-β treatment. Additionally, when MAC-T cells were growth stimulated by IGF-I analogues that do not bind IGFBPs, RA does not inhibit cellular proliferation. Therefore, RA appeared to inhibit cellular proliferation by an IGFBP dependent pathway. TGF-β inhibited cellular proliferation independent of IGFBP expression. Moreover, RA induced growth inhibition did not persist for more than 4 d, whereas TGF-β arrest of cellular proliferation did not decrease even by 7 d. Therefore, TGF-β and retinoic acid inhibited cellular proliferation of bovine mammary epithelial cells, but function through different pathways.

Finally, I sought to determine if growth inhibition was correlated with increased connexin expression and enhanced GJIC as others have postulated. Since 1) cAMP has been

shown to enhance connexin expression and GJIC in many cells, and 2) others have demonstrated this second messenger inhibits MAC-T cell proliferation, cAMP was used in these trials in addition to TGF-β and retinoic acid. First, Cx43 expression was demonstrated in bovine mammary epithelial and fibroblast cells, regardless of whether they were transfected with a plasmid bearing the SV-40 Large-T-antigen. When cells were transformed by the SV-40 Large-T-antigen, Cx43 expression was decreased and GJIC eliminated. However, MAC-T cells (which are transfected but not transformed by the SV-40 Large-T-antigen transfection) had similar Cx43 protein quantities and GJIC as non-transfected cells.

All growth inhibitors stimulated total Cx43 protein expression. Cyclic AMP increased phosphorylated species of Cx43 more than unphosphorylated species. Cyclic AMP also caused a shift in cellular localization of Cx43 from a perinuclear pool to the cell surface. No noticeable changes in immunolocalization of Cx43 were observed following RA or TGF-β treatment. RA at cytotoxic concentrations inhibited Cx43 total protein, preferentially inhibited phosphorylated Cx43 species, eliminated cell surface localization of Cx43, and abolished transfer of Lucifer Yellow dye. RA at non-toxic concentrations, TGF-β or cAMP did not affect dye transfer.

Thus, growth inhibition is correlated with an increase in Cx43 protein. However, when growth inhibitors are at or near cytotoxic concentrations, Cx43 protein expression and GJIC are inhibited. Also, SV-40 Large-T-antigen induced transformation inhibits Cx43

protein expression and GJIC. MAC-T cells do not have decreased GJIC when cells are serum-starved and subsequently growth inhibited. Likewise, although RA, TGF-β and cAMP increase Cx43 protein expression; these increases do not translate into enhanced GJIC. Thus, a correlation exists between growth inhibitors and connexin expression in the mammary gland. This thesis provides compelling in vitro evidence to these emerging fields of research. Previous to the research presented in this thesis, studies concerning 1) GJIC or 2) the effects of RA, TGF-β, or mitogens on bovine mammary epithelial cells and fibroblast cells were absent. However, there is now an emergence of interest in bovine mammary cell culture and the action of retinoids and TGF-β on bovine mammary epithelial cells.

Contributions to Original Knowledge

The results presented in this thesis represent the following contributions to original knowledge.

Chapter 1

This is the first report describing characteristics of isolated or clonal fibroblasts from the bovine mammary gland. Additionally, cellular transformation was induced by transfection with the SV-40 Large-T-antigen only in mammary fibroblasts and not mammary epithelial cells.

Chapter 2

This is the only existing report of the identification of direct acting mitogens on a clonal bovine mammary epithelial cell line. The data presented in this chapter describes the direct role of EGF in the bovine mammary gland. Previously, EGF had only been postulated to effect bovine mammary epithelial cell proliferation. Furthermore, it is demonstrated that mEGF or hEGF is ineffective in altering proliferation of clonal or primary bovine mammary epithelial cells.

Chapter 3

The potent growth inhibitory effects of TGF-β1 and TGF-β2 on bovine mammary cell proliferation were demonstrated. There are no previous reports describing any aspect

of TGF-β action in the bovine mammary gland and its cellular constituents in vivo or in vitro. This report also identifies TGF-β receptors in the bovine mammary gland for the first time.

Chapter 4

Retinoic acid was demonstrated to transiently inhibit bovine mammary epithelial cell proliferation in this study. No reports have examined how retinoids affect mammary cell proliferation in any species previously. Additionally, this study has established the involvement of a novel IGFBP pathway in retinoic acid induced mammary epithelial cell growth inhibition.

Chapter 5

Connexin43 expression was identified in bovine mammary epithelial cells and fibroblasts. Gap junctional communication also occurred in all non-transformed cell types. Transformation of fibroblasts induced by SV-40 Large-T-antigen transfection inhibited connexin43 expression, preferentially decreased mature (phosphorylated) connexin43 species and eliminated GJC in these cells. Connexin43 protein was increased by all growth inhibitors, though GJC was not altered by any growth inhibitors. Previously, no studies regarding any aspect of GJC in the bovine mammary had been reported. Also, this is the first study to correlate growth inhibition (induced by several independently acting growth inhibitors) and connexin expression.