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**ROLE OF THE HUMAN CARCINOEMBRYONIC ANTIGEN
(CEA) FAMILY IN THE REGULATION OF CELL DIFFERENTIATION AND
APOPTOSIS**

By

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**A Thesis submitted to the Faculty of Graduate Studies and
Research in partial fulfillment of the requirements for the
degree of Doctor of Philosophy, Science**

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Thesis Abstract

Human carcinoembryonic antigen (CEA) is the prototypic member of a large family of highly related cell surface glycoproteins that includes CEACAM6 (formerly NCA) and CEACAM1 (formerly BGP). The extracellular domains of CEA/CEACAM6 are bound to the external surface of the plasma membrane through a glycosylphosphatidyl inositol (GPI) anchor and are over-expressed in more than 50% of all human cancers. In contrast, CEACAM1 contains extracellular, transmembrane and cytoplasmic domains, and its level of expression is down-regulated in human tumors of the colon and prostate. When over-expressed on the surface of various cell types in model systems, CEA/CEACAM6, but not CEACAM1, function as pan-inhibitors of cell differentiation and cell polarization and cause a distortion of tissue architecture. Anoikis is a quality control mechanism that must be inhibited in cancer cells for such a distortion to persist. This thesis presents data demonstrating that CEA/CEACAM6 over-expression on the surface of a variety of cell lines inhibited anoikis. The molecular basis for the inhibitory effects of CEA/CEACAM6 on both anoikis and differentiation is shown to be correlated with perturbation of the function of certain integrins. In contrast to CEA/CEACAM6, the expression of the CEACAM1 glycoprotein neither perturbed integrin function nor prevented anoikis and, consistent with this, inhibited tumor growth. As a conclusion, we propose that CEA/CEACAM6, but not CEACAM1, over-expression on the surface of cancer cells inhibits cell differentiation and anoikis through perturbation of integrin functions. These inhibitory effects could instrumentally contribute to tumor formation and progression.

Résumé de Thèse

L'antigène carcinoembryonnaire humain (CEA) est un membre d'une famille des glycoprotéines de surface cellulaire incluant CEACAM6 (anciennement NCA) et CEACAM1 (anciennement BGP). Les domaines extracellulaires de CEA/CEACAM6 se lient à la surface externe de la membrane par un ancrage aux glycosylphosphatidyl inositol (GPI) et sont surexprimés dans plus de 50% de tous les cancers humains. Contrairement à CEA et CEACAM6, CEACAM1 contient , en plus d'un domain extracellulaire, un domain transmembranaire et un domain cytoplasmique. Le niveau d'expression de CEACAM1 est diminué dans les tumeurs de colon et de prostate chez l'humain. Lorsqu'ils sont surexprimés sur la surface des cellules de plusieurs types cellulaires servant de systemes modèles, CEA/CEACAM6, contrairement à CEACAM1, agissent comme des inhibiteurs de la différenciation et de la polarisation cellulaire amenant ainsi une distorsion de l'architecture du tissu. L'anoikis est un mécanisme de contrôle de l'architecture d'un tissu qui est inhibé dans les cellules cancéreuses lorsqu' une telle distorsion persiste. Cette thèse présente les résultats démontrant que la surexpression de CEA/CEACAM6 à la surface de plusieurs lignées cellulaires inhibe l'anoikis. Le mécanisme moléculaire de cet effet inhibiteur de CEA/CEACAM6 sur l'anoikis semble être du à une perturbation de la fonction de certaines intégrines. Contrairement à CEA/CEACAM6, l'expression de CEACAM1 ne perturbe pas la fonction des intégrines, previent l'anoikis et inhibe la croissance tumorale. En conclusion, nous pouvons suggérer que la surexpression de CEA/CEACAM6, et non celle de CEACAM1, à la surface des cellules cancéreuses inhibe les fonctions des intégrines. Ces effets inhibiteurs contribuent de façon majeure à la formation et à la progression de tumeurs.

Preface

In accordance with the specifications outlined in the Faculty of Graduate Studies and Research "Guidelines for Thesis Preparations" (October 1997), the text of papers already published or submitted for publication formatted to follow the style of this thesis, have been incorporated. The relevant section of the "Guidelines for Thesis Preparations" states:

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2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.
3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: a table of contents; an abstract in English and French; an introduction which clearly states the rational and objectives of the research, a comprehensive review of the literature (in addition to that covered in the introduction to each paper); a final conclusion and summary; and, rather than individual reference lists after each chapter or paper, one comprehensive bibliography or reference list, at the end of the thesis, after the final conclusion and summary.
4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.
5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In

addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

Chapter 2 contains the complete text of Ordoñez, C., Screatton, R., Ilantzis, C. and C.P. Stanners. 2000. Human Carcinoembryonic Antigen functions as a general inhibitor of anoikis. *Cancer Research* 60, no. 13:3419-24 (reformatted to conform to the style of this thesis).

Chapter 3 contains the complete text of Ordoñez, C., Screatton, R., Ilantzis, C., Fan M., DeMarte L. and C.P. Stanners. 2000. Human Carcinoembryonic Antigen Inhibits Cell Differentiation and Apoptosis by Perturbing the Function of the $\alpha_5\beta_1$ Integrin Receptor. *Submitted to Journal of Cell Science* (reformatted to conform to the style of this thesis).

Chapter 4 contains the complete text of Kunath T., Ordoñez C., Turbide C. and N. Beauchemin. 1995. Inhibition of colonic tumor cell growth by biliary glycoprotein. *Oncogene* 11, 2375-82 (reformatted to conform to the style of this thesis).

Appendix-A contains the complete text of Ordoñez, C., Screatton, R. and C.P. Stanners. 2000. The Glycosylphosphatidyl inositol (GPI) anchor of the Human Carcinoembryonic Antigen is Required to Inhibit Anoikis. *Manuscript in preparation* (reformatted to conform to the style of this thesis).

Appendix-B contains the text of Ordoñez C., Screatton R., Ilantzis C., Edwards M. and C. P. Stanners 2000. Human Carcinoembryonic Antigen Inhibits Orthovanadate-Induced

Apoptosis of L6 Rat Myoblasts. *Manuscript in preparation* (reformatted to conform to the style of this thesis).

Contributions of Authors

All figures reported in Chapter 2 and Appendix-A represent my own work. Robert Screaton and Chris Ilantzis contributed myoblasts and colonocytes transfectants used in these studies. All eight figures reported in Chapter 3, with the exception of Fig.6 contributed by Mannie Fan, represent my work. Robert Screaton, Chris Ilantzis and Luisa DeMarte contributed transfected cell lines to these studies.

In Chapter 4, I contributed to figures 1A, 2B, 2C, 3B and Table 1. I derived single-cell transfected clones of BgpA and BgpD in CT51 colonocytes and evaluated the level of expression of BgpA/BgpD on the surface of these cells by FACS analysis. I found that BgpA and BgpD inhibited the growth in soft agar of CT51 transfected cells. I also found that BgpD inhibited tumor growth in syngeneic mice. Using retroviral-mediated infection, Tilo Kunath generated cell populations consisting of pooled clones of BgpA/BgpD expressing CT51 cells. He also demonstrated inhibition of tumor growth in syngeneic mice by BgpD but not BgpA expressing cells. He was also responsible for all statistical analysis. Claire Turbide performed growth curves (Fig. 2) and western blotting (Fig. 1B). A letter describing in detail each author's contribution to Chapter 4 signed by all authors is enclosed with the submission of this thesis.

In Appendix-B, all figures are the result of my work with the exception of the electrophoresis gel showing DNA laddering (Fig.) prepared by Robert Screaton. Marcel Edwards did many experiments with taxol (data not shown in this manuscript) facilitating the findings reported in this chapter.

Acknowledgements

I would like to dedicate this work to my daughter without whom I would never have felt the need to pursue my dreams; to my mother for nurturing my mind, my father for setting an example, my sister for her love and support, and to my little pollito Rebecca for all the rest. My unlimited gratitude goes to Chris and Rob for their invaluable contributions. I would like to thank all those that drank wine with me for their friendship and stimulating discussions (Luisa, Pilar, Carlos, Alex, Brigitte, Derek, Nabeel, Peter, Oren, Allison, Tilo, Patrick, Claire, Nicole and the list goes on, you know who you are...). Thanks to Sarita, and finally, I would like to acknowledge my supervisor Dr. Cliff Stanners for his guidance and faith.

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Chapter 1

Thesis Introduction

General Introduction

Aims and Scope of the Present Work

Human Carcinoembryonic Antigen (CEA) was discovered in a quest to identify cell surface proteins exclusively expressed in cancer cells (Gold and Freedman, 1965). It was found later that CEA is also expressed on the surface of normal human colonocytes (Fritsche and Mach, 1977; Shively et al., 1978), although expression in many human cancers is much greater. CEA remains today as one of the most reliable tumor markers in the diagnosis and prognosis of human cancer (Wang et al., 1994). CEA is over-expressed in more than 50% of all adenocarcinomas (Hammarström et al., 1998).

CEA functions, at least *in vitro*, as a homotypic intercellular adhesion molecule (Benchimol et al., 1989). Dr. Clifford P. Stanners proposed that CEA over-expression on the surface of cancer cells contribute instrumentally to the formation and progression of human tumors by disturbing normal tissue architecture through its intercellular adhesive function (Benchimol et al., 1989). This hypothesis is supported by findings indicating that CEA functions as a pan-inhibitor of cell differentiation and polarization (Screaton et al., 1997)(Ilantzis C., L. DeMarte, R. Screaton, C.P. Stanners, submitted for publication).

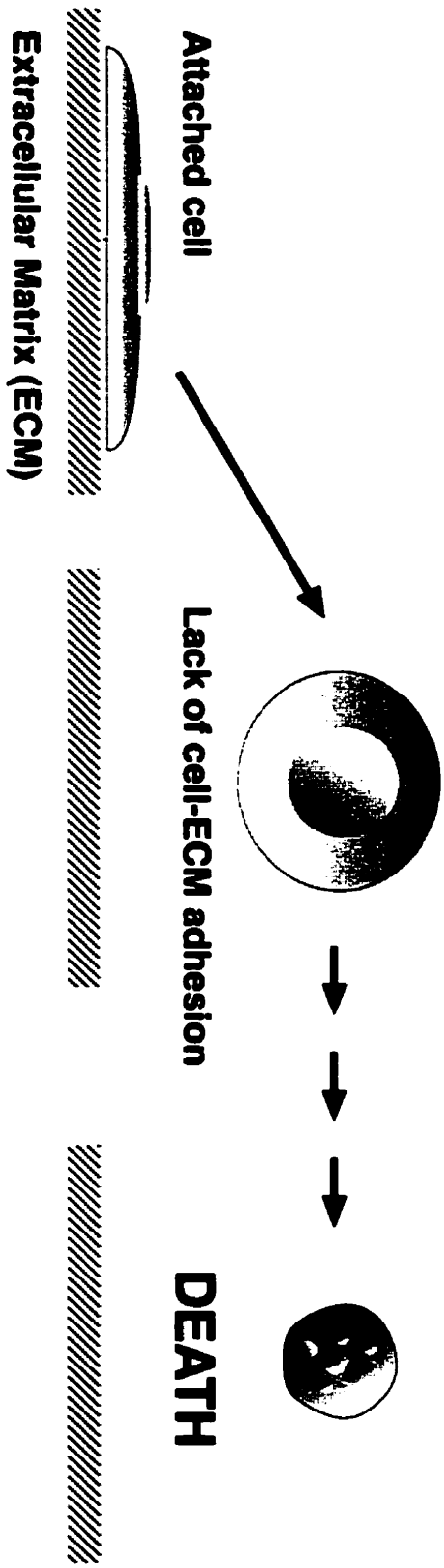
The homeostasis of adult epithelia is preserved, amongst other mechanisms, by the harmonic balance between cell proliferation, differentiation and apoptosis (Giancotti and Ruoslahti, 1999). The disruption of this balance can lead to malignant transformation. CEA over-expression in cancer cells does not seem to stimulate cell proliferation (Screaton et al., 1997) but does inhibit cell differentiation (Eidelman et al., 1993; Screaton et al., 1997). The possible role of CEA in the regulation of apoptosis in cancer cells was unexplored.

The main purpose of this thesis work was to find whether CEA could contribute to tumor formation by inhibiting apoptosis. *Anoikis* is an apoptotic program that functions as a quality control mechanism preserving normal tissue architecture (Frisch and Ruoslahti, 1997). Cells that lose contact with their extracellular matrix (ECM) die of anoikis (Fig. 1) (Frisch and Francis, 1994; Meredith et al., 1993), thus impeding the invasion and colonization of surrounding tissues by these cells (Frisch and Ruoslahti, 1997; Ruoslahti and Reed, 1994). The disruption of tissue architecture and dysplasia associated with malignant transformation requires the inhibition of quality control mechanisms such as anoikis.

Figure 1

Representation of a cell undergoing anoikis after losing contact with its ECM. Many cell types undergo a type of cell death known as anoikis (*Greek term for homelessness*) when they lose contact with their extracellular matrix (ECM). These cells require adhesion to their ECM in order to survive.

ANOIKIS



Our interest in anoikis was based on Dr. Stanners hypothesis suggesting that CEA over-expression disturbs normal tissue architecture contributing to tumor formation. In Chapter 2 of this thesis, we present compelling evidence indicating that CEA functions *in vitro* as a pan-inhibitor of anoikis. The inhibition of anoikis by CEA could promote tumor formation by increasing the survival of cancer cells that have lost contact with their ECM, thus allowing aberrant tissue architecture to persist.

CEA is the prototypic member of a family of cell surface glycoproteins. Here, we will be concerned with only two additional family members that also play a role in human cancer: CEACAM6 (formerly NCA) and CEACAM1-4L (formerly BGP_a). CEACAM6, like CEA, is over-expressed in human cancers and inhibits anoikis (Chapter 2). In contrast, CEACAM1 expression is down-regulated in early stages of prostate and colon cancer (Hammarström et al., 1998; Hsieh et al., 1995; Kleinerman et al., 1995a; Neumaier et al., 1993; Rosenberg et al., 1993). CEACAM1, unlike CEA and CEACAM6, does not inhibit anoikis (Chapter 2). The extracellular domain of both CEA and CEACAM6 is bound to the membrane through a glycosylphosphatidyl inositol (GPI) anchor (Hefta et al., 1990; Hefta et al., 1988). In contrast, CEACAM1 contains an extracellular domain followed by transmembrane and cytoplasmic domains (Hinoda et al., 1988).

CEA expression on the surface of L6 rat myoblasts inhibits myogenic differentiation (Eidelman et al., 1993; Screaton et al., 1997). Our group has recently shown that there are at least two structural requirements for such an inhibitory effect by CEA. A mutated version of CEA lacking 70 amino acids of the N-terminal domain was defective in intercellular adhesion (Zhou et al., 1993) and failed to inhibit myogenic differentiation (Eidelman et al., 1993). This suggests the requirement of an active adhesive extracellular domain for the effect. Secondly, the specific GPI anchor of CEA is crucial for the inhibition of myogenic differentiation (Screaton et al., 2000). In Appendix-

A of this thesis, we demonstrate that both the GPI anchor and extracellular domains of CEA play critical and specific roles in the inhibition of anoikis in L6 myoblasts.

Anoikis is regulated by the function of integrin receptors (Frisch and Ruoslahti, 1997). Integrins are the main cellular receptors that recognize ECM components (Hynes, 1992). We tested the possibility that CEA and CEACAM6 may inhibit anoikis through regulation of integrin functions. In chapter 3, we identify the integrin $\alpha_5\beta_1$ receptor as a molecular target of CEA/CEACAM6 effects.

The $\alpha_5\beta_1$ integrin is the main cellular receptor for fibronectin (Argraves et al., 1987; Ruoslahti, 1988). The ligation of this integrin to fibronectin triggers a signal that inhibits anoikis in many cell types (Frisch and Ruoslahti, 1997; O'Brien et al., 1996; Zhang et al., 1995). The integrin $\alpha_5\beta_1$ receptor plays an active role in the polymerization of fibronectin, a process known as matrix assembly (Christopher et al., 1997; Morla and Ruoslahti, 1992; Morla et al., 1994; Ruoslahti, 1996a). Polymerized fibronectin is the active isoform of this ECM component (Morla et al., 1994). The expression of CEA or CEACAM6 but not CEACAM1 on the surface of L6 myoblasts increased cellular binding to fibronectin and matrix assembly, presumably due to activation of the $\alpha_5\beta_1$ receptor. This activation might also be responsible for the inhibition of cell differentiation by CEA and CEACAM6. The myogenic differentiation of CEA/CEACAM6 expressing myoblasts is rescued by blocking $\alpha_5\beta_1$ -fibronectin interactions (Chapter 3). We propose that CEA/CEACAM6 over-expression on the surface of cancer cells inhibit anoikis and cell differentiation by modifying integrin functions.

In Appendix-B, we describe CEA and bcl-2 inhibitory effects on vanadate and taxol induced apoptosis. The bcl-2 oncogene is the prototypic member of a family of anti-apoptotic regulators (Adams and Cory, 1998; Zhang et al., 1995). These results confirm the notion that CEA inhibits certain apoptotic pathways. However, the mechanism of CEA-mediated resistance to drug-induced apoptosis remains unknown.

Chapter 4 contains compelling evidence supporting the hypothesis that CEACAM1 functions as a tumor suppressor protein. The expression of CEACAM1 in a colorectal cancer cell line decreased growth in soft agar and tumor formation when these cells were injected into syngeneic mice.

Review of the Literature

1) Organization of the CEA family

The Human Carcinoembryonic Antigen (CEA) family comprises a set of genes (about 29 genes, pseudogenes and gene-like sequences) clustered in human chromosome 19. These genes encode three subfamilies of glycoproteins: a) the CEA subfamily of cell surface molecules consisting of 12 genes; b) the Pregnancy Specific Glycoproteins (PSG), a subfamily of 11 genes, most of which encode proteins secreted to the extracellular milieu; and c) the CEA Gene Family Members (CGMs), a subfamily of 6 genes for which no functional cDNAs have yet been identified.

A distinctive structural feature of the CEA family is the presence in these glycoproteins of V-type and C2-type Immunoglobulin (Ig) domains. Based on this, the CEA family is classified as part of the Immunoglobulin (Ig) Superfamily (Paxton et al., 1987). Here, we will be concerned only with three members: CEA, CEACAM1-4L (formerly BGP_a) and CEACAM6 (formerly NCA), for which a role in human carcinogenesis has been proposed. As we will discuss in detail below, CEA and CEACAM6 are thought to have oncogenic potential, whereas CEACAM1 might function as a tumor suppressor.

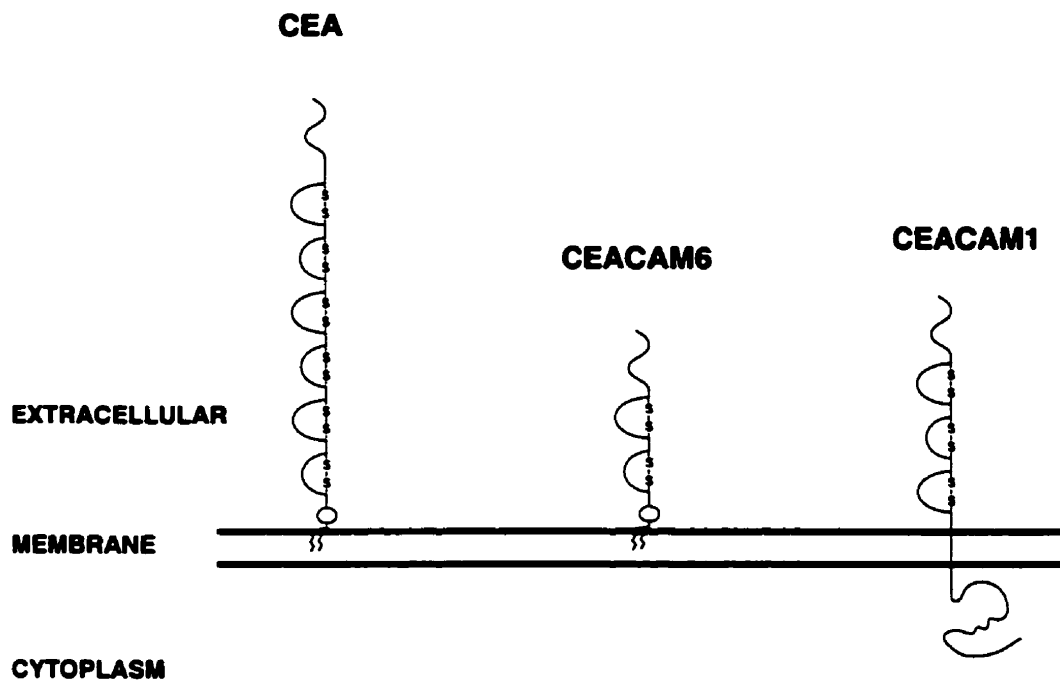
1.1) Structure and Evolution of the CEA family

The extracellular domains of CEA, CEACAM1, and CEACAM6 consist of an Ig variable-like N-terminal domain followed by six (A1B1, A2B2, A3B3), three (A1B1, A2), or two (A1B1) C2-type Ig domains, respectively (Fig. 2) (Beauchemin et al., 1987; Hefta et al., 1990; Hinoda et al., 1988; Zimmermann et al., 1987). CEA, CEACAM6 and CEACAM1 share striking homology at both the nucleotide and amino acid levels. Relative to CEA, CEACAM1/CEACAM6 are 80/90% homologous, respectively, at the

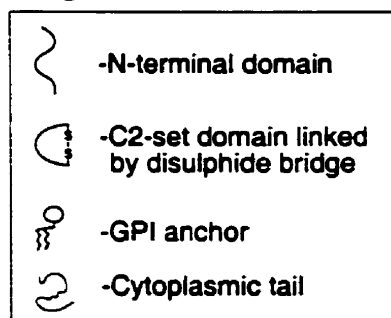
nucleotide level and 70/85% homologous, respectively, at the amino acid level. CEA and CEACAM6 are bound to the plasma membrane by glycosylphosphatidylinositol (GPI) anchors (Hefta et al., 1988). In contrast, CEACAM1 contains a transmembrane domain followed by a cytoplasmic tail (Fig. 2). This distinctive structural feature may explain the opposite roles in human cancer of CEACAM1 versus CEA/CEACAM6, as we will discuss later.

Figure 2

Structure of CEA, CEACAM6 and CEACAM1. The extracellular domains of CEA and CEACAM6 are bound to the external surface of the plasma membrane by glycosylphosphatidylinositol (GPI) anchors. In contrast, the extracellular domains of CEACAM1 are followed by a transmembrane domain and a cytoplasmic tail.



Legend



There are different spliced isoforms of CEACAM1 that differ in the length of their cytoplasmic tails and extracellular domains (Barnett et al., 1993). Here, we will be concerned with CEACAM1-4L (long tail) and CEACAM1-4S (short tail) that differ only in the length of their respective cytoplasmic domains. The corresponding extracellular and transmembrane domains of both isoforms, 4L and 4S, are identical to each other.

GPI anchored proteins

As mentioned above, CEA and CEACAM6 are bound to the plasma membrane through a GPI-anchor. Such anchors are synthesized in the endoplasmic reticulum (Kinoshita et al., 1997; Stevens, 1995). The eukaryotic GPI anchor consists of a backbone of N-acetyl-glucosamine, three mannose residues, and ethanolamine bound to a phosphoinositol molecule covalently linked to a glycerolipid moiety located within the outer leaflet of the plasma membrane. This type of linkage should limit the association of CEA/CEACAM6 to the exoplasmic leaflet of the plasma membrane bilayer. CEA/CEACAM6 are likely attached to the GPI anchor through an amide linkage between their C-terminus and the core phosphoethanolamine moiety of the anchor. There are variations of the core structure of the eukaryotic GPI anchor such as palmitoylation of the inositol ring, number and length of lipid chains attached to glycerol, and modifications of mannose residues (McConville and Ferguson, 1993). The specific modifications of the core structure of CEA/CEACAM6-GPI anchors are not known yet.

GPI anchored proteins, such as CEA and CEACAM6, are specifically targeted to apical membranes and excluded from the basolateral membranes of polarized epithelial cells (Rodriguez-Boulan and Nelson, 1989). GPI anchored proteins are found in specific rafts or microdomains of the plasma membrane known as detergent insoluble glycolipid domains (DIGs) (Friedrichson and Kurzchalia, 1998; Pande, 2000; Varma and Mayor, 1998). Several members of the Src-family of protein tyr kinases and GTP-binding

proteins involved in signal transduction pathways are often associated with the cytoplasmic side of DIGs (Lisanti et al., 1994; Pande, 2000).

Evolution of the CEA family

The genes that encode the GPI-bound glycoproteins CEA and CEACAM6 are exclusively found in the primate radiation, including humans (Hossani H, C.P. Stanners, submitted for publication)(Stanners et al., 1992). In contrast, human CEACAM1 has murine homologues (Zimmermann, 1998). The rodent CEA family contains three distinct genes that encode CEACAM1-like glycoproteins, whereas only one CEACAM1 gene has been found in the human CEA family. The most striking difference between the human and rodent CEA families is the absence of GPI-bound glycoproteins in rodents. The prevailing view is that the CEACAM1 gene is the ancestor of the CEA family and that other members evolved by gene duplication and exon-shuffling (Zimmermann, 1998). It has been suggested that the CEA family is still in transitional evolution (Stanners et al., 1992).

2) Expression of CEA Family Members in Normal Tissues

CEA is expressed on the apical surface of columnar epithelial cells and mucus secreting goblet cells of the human gastrointestinal tract, mainly in the upper one-third of the colonic crypts. In addition, CEA is expressed in squamous epithelial cells of the tongue and cervix, in sweat glands and epithelial cells of the prostate (Hammarström et al., 1998). CEACAM1 is expressed in epithelial cells of the colon, liver, gall bladder, kidney and urinary bladder. CEACAM1 is also expressed in haemopoietic cells such as lymphocytes and granulocytes, and in some endothelial cells as well. CEACAM6 is expressed in epithelial cells of the colon, lung and stomach, and in granulocytes and monocytes.

3) Functions of the CEA Family

3.1) Intercellular Adhesion

CEA, CEACAM1 and CEACAM6 function *in vitro*, at least, as intercellular adhesion molecules (Benchimol et al., 1989; Rojas et al., 1996; Stanners and Fuks, 1998). The interactions between these molecules keep cells attached together. There are many types of cell adhesion molecules, such as cadherins, selectins, integrins and other members of the Immunoglobulin Superfamily. For the purposes of this thesis, we will focus on the functions of the CEA family and integrin receptors (see below).

Our Laboratory has previously proposed the “Double Reciprocal Model” to explain the molecular mechanism of homotypic adhesion mediated by CEA (Stanners and Fuks, 1998; Zhou et al., 1993). Based on this model, the N-domain of the CEA molecule will bind the A3B3 domain of a second CEA molecule and vice-versa on apposed cell surfaces. This model is supported by data obtained with transfected LR-73 cells expressing on their surfaces chimeric proteins constructed by swapping extracellular domains of NCAM and CEA (Zhou et al., 1993). In this experimental system, LR-73 cells expressing chimeric proteins containing the N-terminal or A3B3 domains of CEA alone, did not self adhere but significantly adhered to each other. In contrast, the A1B1 and A2B2 domains did not participate in intercellular adhesion. The addition to CEA expressing LR-73 cells of peptides corresponding to the N and A3B3, but not A1B1 and A2B2, domains of CEA inhibited intercellular adhesion. Moreover, a mutated version of CEA lacking 70 amino acids of the N-terminal domain (Δ N-CEA) was defective in intercellular adhesion (Zhou et al., 1993).

CEA is also capable of heterotypic adhesive interactions with CEACAM6 and CEACAM1 (Stanners and Fuks, 1998). The molecular mechanisms of intercellular

adhesion (homotypic and heterotypic) for CEACAM1 and CEACAM6 are not as well understood as it is the case for CEA. Thus far, it is known that the N-domains of both molecules are crucial for their adhesive functions.

As we will discuss in detail below, the impact of intercellular adhesion in the functioning of an organism is not merely mechanical by bringing cells together, but also leads to a sophisticated signal transduction response triggered by self-binding of these adhesive receptors.

3.2) CEA and CEACAM6 function as general inhibitors of cell differentiation

CEA and CEACAM6, but not CEACAM1, function as pan-inhibitors of cellular differentiation both *in vitro* and *in vivo* (Eidelman et al., 1993; Rojas et al., 1996; Screaton et al., 1997; Stanners, 1998). The forced expression (cDNA transfection) of CEA and CEACAM6 inhibited myogenic differentiation of L6 rat myoblasts (Eidelman et al., 1993), neurogenic differentiation of P19 cells (Malette B., C.P. Stanners, Submitted for publication), adipogenic differentiation of C3HT101/2 and 3T3-L1 cells (DeMarte L, C.P. Stanners, unpublished observations), and colonic differentiation of SW1222 and Caco-2 human colorectal cancer cells (Ilantzis C., L. DeMarte, R. Screaton, C.P. Stanners, submitted for publication). These *in vitro* results were validated using *in vivo* experimental model systems. For instance, L6 rat myoblasts are capable of myogenic differentiation when injected intramuscularly into nude mice (Screaton et al., 1997). This process was also inhibited by the ectopic expression (cDNA transfection) of CEA (Screaton et al., 1997). In addition, high levels of expression of CEA on the cell surface of malignant colonocytes purified from human colorectal tumor specimens have been correlated with a poor degree of differentiation of these cells (Ilantzis et al., 1997).

The structural requirements for the inhibitory effect of CEA on myogenic differentiation are a fully functional intercellular adhesive domain and the specific CEA-

GPI anchor (Screaton et al., 2000). This is supported by the following experimental facts: 1) Δ N-CEA, a mutated version of CEA defective in intercellular adhesion, does not inhibit myogenic differentiation of L6 rat myoblasts (Eidelman et al., 1993); 2) The GPI anchor of CEA carries specific biological information leading to the inhibition of myogenic differentiation in L6 myoblasts (Screaton et al., 2000). This notion is supported by compelling experimental evidence obtained with chimeric proteins consisting of the extracellular domains of either CEACAM1 or NCAM fused to the CEA-GPI anchor (Screaton et al., 2000). These chimeric proteins, like CEA, inhibit myogenic differentiation when expressed on the surface of L6 myoblasts; 3) Neither transmembrane CEACAM1 nor NCAM bound by its own GPI-anchor inhibit L6 myogenic differentiation (Screaton et al., 2000); 4) The GPI-anchors of NCAM and CEA seem to be structurally and functionally different (Screaton et al., 2000).

3.3) Signal Transduction

The CEA family plays an active role in multiple signal transduction processes (Draber and Skubitz, 1998). Using transfected basophilic leukemia cells as a model, it has been demonstrated that GPI-bound CEA participates in a stimulatory signal transduction pathway. In this system, CEA colocalizes with Lck, a member of the Src-family of protein tyrosine kinases, in specific membrane microdomains (Draber P, C.P. Stanners, submitted for publication). The GPI anchor of CEA is required for its function in signal transduction. Transmembrane CEACAM1 is inactive in this system. In contrast, a chimeric protein (BC-2) consisting of CEA-GPI anchor fused to the extracellular domain of CEACAM1 was capable of signaling.

CEACAM1 plays an active role in signal transduction in many other systems (Obrink, 1997; Öbrink and Hunter, 1998). As mentioned above, there are two isoforms of CEACAM1 differing in the length of their cytoplasmic tails: CEACAM1-L (long tail)

and CEACAM1-S (short tail). The long cytoplasmic tail of CEACAM1-L contains two tyrosines within a specific sequence homologous to a domain known as “Immunoreceptor Tyrosine-based Inhibition Motif”(ITIM) (Huber et al., 1999). The biological significance of the presence of this sequence in CEACAM1-L is not fully understood yet. The SH2 domain of the phosphatase SHP-1 can bind to the phosphorylated ITIM domain of CEACAM1-L (Beauchemin et al., 1997; Huber et al., 1999). In addition, the treatment of human granulocytes with monoclonal antibodies that recognize CEACAM1 triggered tyrosine phosphorylation of the long cytoplasmic tail of CEACAM1-L (Skubitz et al., 1996; Stocks et al., 1996). Other studies demonstrated that CEACAM1-L can be phosphorylated by pp60 src (Brummer et al., 1995).

CEACAM1-L not only can bind to protein tyrosine phosphatases SHP1 and SHP2 (Huber et al., 1999) but also to calmodulin (Edlund and Obrink, 1993) and actin (Sadekova et al., 2000). Both CEACAM1-L and CEACAM1-S bind calmodulin under conditions of high intracellular concentrations of calcium, a process thought to regulate intercellular adhesion (Edlund and Obrink, 1993). The phosphorylation of ser 449 in CEACAM1-S by protein kinase C decreases the ability of CEACAM1-S to bind calmodulin (Edlund et al., 1998). CEACAM1-L also binds actin, a cytoskeletal protein, through a distal portion of its long cytoplasmic tail (Sadekova et al., 2000). Such interaction regulates intercellular adhesion by determining the localization of CEACAM-L to sites of cell-cell contact, a process regulated by the Rho-family of GTPases. In contrast, CEACAM1-S neither binds actin nor localizes to sites of cell-cell contact.

3.4) Angiogenesis

Angiogenesis consists of the proliferation of endothelial cells and the organization of these cells into three-dimensional structures known as blood vessels (Folkman and D'Amore, 1996; Hanahan and Folkman, 1996). The Vascular Endothelial Growth factor

(VEGF), when bound to its receptors (VEGF-R1 and VEGF-R2), triggers a signal required for angiogenesis (Klagsbrun and D'Amore, 1996). Recently, it has been found that CEACAM1 expression levels on the surface of endothelial cells is increased by the exposure of these cells to VEGF (Ergun et al., 2000). Moreover, CEACAM1 purified from granulocytes and endothelial cells stimulated angiogenesis in both *in vitro* and *in vivo* assays (Ergun et al., 2000). In this study, the addition of an anti-CEACAM1 monoclonal antibody inhibited VEGF-induced angiogenesis. CEACAM1 expressed in granulocytes and endothelial cells may stimulate angiogenesis by two mechanisms: 1) Transmembrane CEACAM1 may mediate adhesion of granulocytes to endothelial cells through E-selectin or stimulate morphogenesis of blood vessels by endothelial cells; 2) soluble CEACAM1 released from both granulocytes and endothelial cells may induce angiogenesis during inflammation (Ergun et al., 2000).

3.5) Other Functions of the CEA Family

Certain bacteria and viruses selectively bind to CEA-family members on the cell surface (Chen et al., 1997b; Dveksler et al., 1993a; Dveksler et al., 1993b; Hauck et al., 2000; Holmes et al., 1993; Skubitz et al., 1999; Thompson et al., 1991). For instance, *Neisseria gonorrhoeae* is a Gram-negative bacteria that binds to CEA family members (Bos et al., 1997; Chen et al., 1997b; Gray-Owen et al., 1997a; Gray-Owen et al., 1997b; Hauck et al., 2000). This binding may play a critical role in the infective cycle of the bacteria. Likewise, the murine hepatitis virus binds to the murine homolog of CEACAM1 (Holmes et al., 1993). The biological significance of these binding events is not clearly understood. It has been hypothesized that CEA-family members act as a primordial defense mechanism against infection (Hammarström et al., 1998).

4) Role of the CEA family in Cancer

As mentioned above, CEA and CEACAM6 are overexpressed in more than 50% of all human adenocarcinomas, including major sites as colon (Nollau p 1997), lung and breast (Hammarström et al., 1998). Other CEA/CEACAM6 over-expressing tumors are gastric, pancreas, skin, ovary and cervix cancers (Albers et al., 1988; Athanassiadou et al., 1994; Banks and Cooper, 1991; Boucher et al., 1989; Cournoyer et al., 1988; Sanders et al., 1993; Sheahan et al., 1990; Sheibani et al., 1986; Zimmermann et al., 1988). In contrast, CEACAM1 is down-regulated in early stages of adenocarcinomas of the colon (Nollau 1997), breast (Riethdorf 1997) and prostate (Kleinerman et al., 1995a). CEA, CEACAM1 and CEACAM6 are mostly expressed on the apical surface of the human colonic epithelium, but are also expressed in other cell types (Hammarström et al., 1998; Ilantzis et al., 1997). This normal pattern of expression is dramatically disrupted upon malignant transformation, when CEA/CEACAM6 are over-expressed over the entire surface of the malignant cells whereas CEACAM1 is largely absent (Ilantzis et al., 1997). Based on these observations, the prevailing hypothesis is that CEA/CEACAM6 over-expression plays an oncogenic role whereas CEACAM1 functions as a tumor suppressor protein. This notion is supported by the fact that the injection of L6 myoblasts (Screaton et al., 1997) and Caco-2 human colorectal cancer cells (Ilantzis C., DeMarte L., Screaton R., C.P. Stanners, submitted for publication) over-expressing CEA and CEACAM6 on their surfaces increased tumorigenicity in nude mice. In contrast, the forced expression of CEACAM1 in CT51 murine colorectal cancer cells and prostate cancer cells causes a decrease in tumor formation when these cells were injected subcutaneously in syngeneic mice (Chapter 4) and nude mice, respectively (Hsieh et al., 1995; Kleinerman et al., 1995b).

Recently, the molecular requirements for the tumor suppressor effect of CEACAM1-L (long tail) has been identified (Izzi et al., 1999). A point mutation in tyr

488 of CEACAM1-L abrogated the tumor suppressor effect suggesting that the ITIM domain is required. Furthermore, mutations in the C-terminal portion of CEACAM1 also abrogated the tumor suppressor effect. This C-terminal region regulates CEACAM1 binding to phosphatases SHP-1 and SHP-2. In contrast, the removal of the N-terminal domain required for intercellular adhesion had no effect.

The question of the potential mechanism of CEA/CEACAM6 effects arises. There are at least three key physiological processes that function in harmony to maintain the homeostasis of a normal human epithelium: cell proliferation, cell differentiation and apoptosis. The disturbance of these processes, such an increase in cell proliferation or inhibition of cell differentiation and apoptosis, or a combination of these events, could contribute to malignant transformation of the epithelial cells. The forced over-expression of CEA/CEACAM6 on the cell surface of several cell lines inhibited cell differentiation without increasing the rate of cell proliferation (see above) In addition, this thesis will deal with the possible role of CEA/CEACAM6 over-expression in apoptosis.

To explain the oncogenic mechanism of CEA/CEACAM over-expression in cancer cells, Dr. Stanners had proposed a tissue architecture model based on two main functions of CEA/CEACAM6: intercellular adhesion and inhibition of cell differentiation (see below).

4.1) Tissue Architecture Model

Human and murine colonic crypts consist of a monolayered epithelium organized in a longitudinal morphogenetic pattern with a proliferative zone at the bottom of the crypts and a differentiation zone at the top (Stappenbeck et al., 1998). Human colonocytes migrate along this morphogenetic pattern. Human colonocytes with mitotic potential or stem cells multiply in the proliferative zone. The daughter cells migrate along the longitudinal morphogenetic gradient towards the differentiation zone. There,

colonocytes reach a terminal differentiation stage and are unable to divide. CEA/CEACAM6 are normally expressed on the apical surface of the upper third of the colonic crypts, mostly in the differentiation zone. In this context, it is unlikely that CEA/CEACAM6 are involved in intercellular adhesion and do not promote tumorigenesis.

The scenario changes dramatically upon malignant transformation of colonocytes. Malignant colonocytes over-express CEA/CEACAM6 over their entire cell surfaces, including their basolateral membranes, and are distributed along the entire colonic crypts including the proliferative zone (Benchimol et al., 1989; Ilantzis et al., 1997). Furthermore, malignant colonocytes form a multilayered epithelium, an architectural pattern found in embryonic colonic epithelia but not in the normal adult colon (Benchimol et al., 1989). In this context, the intercellular adhesion function of CEA/CEACAM6 may play an active role promoting tumorigenesis.

A model was proposed suggesting that CEA over-expression in colonocytes with mitotic potential disrupts the normal tissue architecture of human colonic crypts (Benchimol et al., 1989). According to this model, when CEA is over-expressed over the entire cell surface, CEA-CEA homotypic adhesive interactions could promote the formation of cellular aggregates arranged as a multilayered epithelium, as it is observed in embryonic colonic epithelia and in early stages of human colorectal adenocarcinomas. This aberrant-type of tissue architecture will inhibit cell differentiation allowing the accumulation of genetic lesions that eventually lead to tumor formation and progression.

Such effects of CEA/CEACAM6 disturbing normal tissue architecture might be expected to require the inhibition of quality control mechanisms in order to persist. One such mechanism is anoikis (see below), an apoptotic program involved in the maintenance of normal tissue architecture (Frisch and Francis, 1994; Frisch and Ruoslahti, 1997). Hypothetically, the “tissue architecture model” explained above will

become more attractive if CEA/CEACAM6 could inhibit anoikis. The potential role of CEA/CEACAM6 over-expression in the inhibition of anoikis is the subject of Chapters 2 and 3 of this thesis. A modification of the initial tissue architecture model, including CEA/CEACAM6 inhibitory effects on anoikis, is presented in Chapter 2 of the thesis.

5) Role of Anoikis in the maintenance of Tissue Architecture

As mentioned above, anoikis (*Greek term for homelessness*) is an apoptotic program triggered when certain cell types lose contact with their ECM. Many cell types such as epithelial and endothelial cells (Frisch and Ruoslahti, 1997; Meredith et al., 1993; Park et al., 1999; Yawata et al., 1998), neurons (Bozzo et al., 1997; Rozzo et al., 1997)[Chen, 1997 #961, melanocytes (Scott et al., 1997), osteoclasts (Sakai et al., 2000), myelocytic cells (Nakamura et al., 1998) and skeletal muscle cells (Montanaro et al., 1999; Mukasa et al., 1999) require adhesion to their ECM for cell survival. Anoikis has been demonstrated *in vivo* in hippocampal neurons (Chen and Strickland, 1997), in colonocytes of human colonic crypts (Grossmann et al., 1998; Ikeda et al., 1998), in gastric epithelium (von Herbay and Rudi, 2000), in the involution of mammary glands (Alexander et al., 1996; Lund et al., 1996), and during cavitation of the mouse embryo (Coucouvanis and Martin, 1995).

The time taken by a suspended cell to enter anoikis after becoming detached from its ECM depends on the cell type. For instance, colonocytes freshly extracted from human colon undergo anoikis as early as 2 hours after removal from their ECM (Strater et al., 1996), whereas L6 rat myoblasts require at least 12 hours (Chapter 2). Most cells are able to survive if they reattach to their ECM before anoikis is triggered (Frisch and Ruoslahti, 1997).

Anoikis is thought to function *in vivo* as a surveillance mechanism preserving normal tissue architecture (Frisch and Ruoslahti, 1997). Anoikis prevents the detached

cells from colonizing foreign tissues thus preventing dysplasia. Moreover, anoikis is triggered not only in cells that have detached from their ECM, but also in those cells bound to ECM components that are distinct from their proper ECM (Boudreau et al., 1995). Therefore, detached cells can die of anoikis when they are in suspension or trying to attach to foreign ECM. In addition, the disruption of the cellular cytoskeleton can also trigger anoikis (Rosen et al., 2000). In contrast, most cancer cells are thought to be resistant to anoikis and capable of forming metastasis in foreign tissues after detaching from their primary tumor.

5.1) Molecular signals regulating Anoikis

Some of the molecular signals that regulate anoikis might be cell type specific (Frisch and Ruoslahti, 1997). However, a hypothetical general model based on compiled data from different cellular systems can be constructed to understand the principles of anoikis regulation. This model consists of a network of stimulatory and inhibitory molecular signals determining the cell's decision to survive or commit suicide.

The induction of anoikis is accompanied by both the generation of stimulatory signals and the suppression of other signals of inhibitory nature. Examples of stimulatory signals are: 1) a cytosolic increase in reactive oxygen species (ROS) (Li et al., 1999a), 2) the activation of cellular caspases (Frisch, 1999), 3) the activation of protein kinase C (PKC- α) (Okuda et al., 1999), 4) the activation of Bak (Rosen et al., 2000), 5) the translocation of Bax from the cytosol to the mitochondria (Gilmore et al., 2000), 6) the activation of the c-Jun N-terminal kinase (JNK) (Cardone et al., 1997), and 7) the presence of functionally intact wild type tumor suppressor proteins such as p53 (Park et al., 1999; Vitale et al., 1999) and hypophosphorylated pRb (Boudreau et al., 1996; Frisch and Ruoslahti, 1997). As mentioned above, the execution of anoikis also requires the suppression of inhibitory signals such as: 1) down-regulation of the expression levels of

Bcl-2 family members such as Bcl-X(L) (Rosen et al., 2000) and, 2) inhibition of NF-kappaB activity (Park et al., 1999).

Caspases are key regulators of anoikis. Caspases constitute a family of proteolytic enzymes synthesized by the cell as zymogens or pro-enzymes. Caspases, as other zymogens, undergo activation by specific proteolytic cleavages (Thornberry and Lazebnik, 1998). Thus far, about 13 caspases have been identified. They share similar amino acid sequences, structure and substrate specificity. Some of the substrates of caspases are cellular proteins that inhibit apoptosis. For instance, I^{CAD}/DFF45, an inhibitor of the nuclease responsible for apoptosis-associated DNA fragmentation and Bcl-2 proteins (Adams and Cory, 1998) are targets of proteolytic degradation by caspases.

Caspases participate in the initiation and execution of many types of apoptosis including anoikis. The induction of anoikis triggers activation of both caspases 3 and 8 ((Park et al., 1999; Rytomaa et al., 1999). The activation of caspase-8 and its substrate BID seems to be one of the initiating events in anoikis (Frisch, 1999; Rytomaa et al., 1999) and it is inhibited by Bcl-2 and Bcl-X(L) (Rytomaa et al., 1999).

Caspase activation eventually leads to the proteolytic activation of the c-Jun N-terminal kinase or stress activated protein kinase (SAPK) (Cardone et al., 1997). There is controversy about the role of SAPK in anoikis. Some authors believe SAPK is definitely required for anoikis, whereas others claimed that SAPK activation is an epi-phenomenon (Krestow et al., 1999).

The involvement of death receptors or death domain containing proteins in triggering anoikis is suggested by the inhibition of anoikis in MDCK cells by proteins that block the death domains (Frisch, 1999; Rytomaa et al., 1999). Both silencer of death domains (SOD) and a dominant negative form of the FAS-associated death domain protein (FADD) inhibited anoikis of MDCK cells. The effect of SOD required regulation

of the chaperone activity of hsc70, a heat shock protein. The CD95, DR4 and DR5 death receptors do not seem to affect anoikis.

Bcl-X(L), Bax and Bak are members of the Bcl-2 family of proteins involved in the regulation of apoptosis (Adams and Cory, 1998). The Bcl-2 family plays both positive and negative roles in the cell's decision to live or die. For instance, Bcl-2 is the prototypic member of this family and functions as a general inhibitor of apoptosis (Yawata et al., 1998). Bcl-X(L), like Bcl-2, inhibits anoikis. In contrast, Bak and Bax function as pro-apoptotic proteins.

Anoikis is inhibited by the ligation of integrin receptors to their specific ECM ligands (Bozzo et al., 1997; Frisch and Ruoslahti, 1997; Vitale et al., 1998). Cell-ECM adhesion results in cell spreading, changes in cell shape and cytoskeleton rearrangements generating a survival signal(s) that blocks anoikis (Chen et al., 1997a). The activation of the focal adhesion kinase (FAK) and phosphatidylinositol 3-kinase (PI3-K) upon integrin receptor occupancy lead to the activation of Akt, a ser/thr kinase that phosphorylates and inhibits the functions of Bad and Caspase-9, two pro-apoptotic proteins (Giancotti and Ruoslahti, 1999; Ruoslahti, 1999). The phosphorylation of Bad by Akt prevents the binding of Bad to Bcl-XL, an anti-apoptotic protein (Page et al., 2000).

In addition to the activation of the FAK/PI3-K/Akt pathway, cell-ECM adhesion induces a translocation of activated MAPK from the cytosol to the nucleus (Danilkovitch et al., 2000). Another molecular signal triggered by integrin receptor occupancy is the coupling of the adaptor proteins p130 Crk-associated substrate (CAS) and c-CrkII (Crk). CAS has the ability to bind to FAK, thus participating in integrin-dependent signal transduction pathways. The molecular coupling of CAS and Crk leads to inhibition of anoikis, an effect that requires the activation of the small GTPase Rac. In contrast, either the uncoupling of CAS from Crk (Cho and Klemke, 2000) or the cleavage of CAS by caspase-3 induces anoikis (Kook et al., 2000).

Like integrin receptors, inhibitors of metalloproteinases (TIMPs) are also capable of activating FAK and inhibiting anoikis (Li et al., 1999b). TIMPs are natural inhibitors of a family of proteolytic enzymes known as matrix metalloproteinases (MMPs). MMPs are capable of degrading ECM and induce anoikis of breast epithelial cells during mammary involution (Lund et al., 1996). In contrast, TIMP-1 over-expression in MCF10A breast cancer cells inhibited anoikis (Li et al., 1999b). This effect depends on the activation of FAK and it seems to be independent of TIMP-mediated stabilization of cell-ECM adhesion.

The loss of function of certain tumor suppressor proteins such as p53, pRb and PTEN can cause resistance to anoikis in cancer cells (Davies et al., 1998; Frisch and Ruoslahti, 1997; Lu et al., 1999; Vitale et al., 1999). For instance, PTEN over-expression induced anoikis of breast (Lu et al., 1999) and ovarian (Minaguchi et al., 1999) cancer cells *in vitro*. The PTEN gene encodes a multifunctional protein phosphatase that dephosphorylates the same substrates phosphorylated by the phosphatidylinositol 3'-kinase (PI3-K), an enzyme capable of inhibiting anoikis (see above). Such phosphatase activity is required for PTEN-mediated induction of anoikis. In fact, a phosphatase-defective mutant of PTEN has no effect on anoikis. Furthermore, PTEN forced expression in human glioma cells inhibited phosphorylation and activation of Akt (Davies et al., 1998), a ser/thr kinase involved in the inhibition of anoikis (see above).

Another mechanism capable of inhibiting anoikis in cancer cells is the over-expression or activation of certain oncogenes such as bcl-2, H-ras, K-ras and src. Most of the oncogenic proteins encoded by these oncogenes participate directly or indirectly in integrin signaling (Giancotti and Ruoslahti, 1999). For example, Bcl-2 inhibits anoikis (see above) and its level of expression in cancer cells may be regulated by certain integrins (Frisch and Ruoslahti, 1997; Zhang et al., 1995). Another oncogene responsible for anoikis resistance in cancer cells is ras (Khwaja et al., 1997). Both activated K-Ras

and H-Ras, oncoproteins with GTPase activity, induced a down-regulation of Bak, a pro-apoptotic member of the Bcl-2 family (see above), and rescued the expression of Bcl-X(L), an anti-apoptotic member of the Bcl-2 family (see above), in transformed intestinal epithelial cells (Rosen et al., 1998; Rosen et al., 2000). The enforced down-regulation of Bcl-X(L) expression in these cells induced anoikis and reduced their tumorigenic potential. In addition, Ras activates the PI3-K/Akt pathway involved in the inhibition of anoikis (Khawaja et al., 1997). The transformation of embryonic fibroblasts with Src, an oncogenic protein with tyr kinase activity, renders these cells resistant to anoikis (McGill et al., 1997).

The over-expression of the oncogene ILK, a ser/thr kinase that binds β_1 , β_2 and β_3 integrin subunits, inhibits anoikis in rat intestinal epithelial cells (Delcommenne et al., 1998). ILK functions upstream of PI3-K and AKT in the inhibition of anoikis (Delcommenne et al., 1998). Like ILK, the over-expression of β -Catenin, an oncogene mutated in a variety of human cancers, in normal epithelial cells inhibited anoikis and induced the malignant transformation of these cells (Orford et al., 1999).

Another potential oncogenic mechanism causing inhibition of anoikis consists of the action of certain growth factors. For instance, insulin-like growth factor-1 (IGF-1) inhibits anoikis presumably by activating FAK and it is thought to promote tumor formation (Prisco et al., 1999; Valentinis et al., 1999; Valentinis et al., 1998). Colony stimulating factor-1 (CSF-1), another growth factor, inhibits anoikis of purified rabbit osteoclasts (Sakai et al., 2000). Integrin-induced phosphorylation and activation of the epidermal growth factor (EGF-R) results in inhibition of anoikis in human primary skin fibroblasts and ECV304 endothelial cells (Moro et al., 1998). Furthermore, the macrophage-stimulating protein (MSP), a growth and motility factor for epithelial cells, inhibited anoikis of epithelial cells by stimulating the PI3-Kinase/Akt pathway (Danilkovitch et al., 2000).

A new approach to prevent and treat human neoplasia is to develop drugs capable of inducing anoikis of cancer cells. For instance, sulindac sulfide, a non-steroid anti-inflammatory drug (NSAID), induces anoikis of human colon carcinoma cells presumably by decreasing tyr phosphorylation of FAK and rearrangement of the cytoskeleton (Weyant et al., 2000). Etoposide, an anti-cancer drug that inhibits the enzyme topoisomerase-II, induces anoikis of Rat-1 cells by a process involving the cleavage of CAS by caspase-3 (Kook et al., 2000).

The binding of integrin receptors to ECM components trigger signals regulating not only anoikis but also other cellular functions such as cell proliferation, cell migration, cell polarization and cell differentiation (Giancotti and Ruoslahti, 1999). As mentioned above, CEA/CEACAM6 over-expression inhibits cell differentiation. The next section is dedicated to a potential mechanism responsible for such CEA/CEACAM6 effects.

6) Role of the ECM in the regulation of Cell Differentiation

A priori, it would seem very difficult to envisage how CEA/CEACAM6 could inhibit such distinct differentiation processes (myogenic, adipogenic, neurogenic, and colonic differentiation), that are regulated by specific and distinctive signal transduction pathways and transcription factors. One hypothesis was that CEA/CEACAM6 modify a general molecular mechanism common to all these differentiation pathways. One such mechanism is cell adhesion to the extracellular matrix (ECM), known to regulate many types of cell differentiation including the same types inhibited by CEA/CEACAM6 (see Introduction to Chapter 3). The extracellular matrix (ECM) is a network composed of glycoproteins (collagens, fibronectin, laminin, vitronectin, etc) and glycosylaminoglycans that surrounds many eukaryotic cell types, and provides signals that regulate cell proliferation, survival, migration and cell differentiation (Lukashev and Werb, 1998).

Here, we focus only on the role of the ECM on cell differentiation. It is the prevailing view that cell differentiation is controlled in pluricellular organisms through a combination of soluble and insoluble regulatory cues (Adams and Watt, 1993; Lelievre et al., 1996). The first are represented by growth factors and hormones that either circulate in the bloodstream or act in an autocrine or paracrine fashion on the target cells (Bischof et al., 2000; Foster et al., 1998). The insoluble cues are the components of the ECM (Lelievre et al., 1996). The recognition of positive and negative signals provided by the ECM will result in the commitment of the cell to progress through the cell cycle, to undergo cell differentiation, or to enter an apoptotic program (Adams and Watt, 1993; Aharoni et al., 1997).

The regulatory effects of the ECM on cell differentiation start very early during the development of pluricellular organisms and persists later into adult stages (Gumbiner, 1996). For instance, fibronectin is required at morula stages in the *Xenopus* embryo as demonstrated by the inhibition of embryo development by the addition of anti-fibronectin monoclonal antibodies and RGD peptides (Adams and Watt, 1993). Moreover, the knockout of the mouse fibronectin gene causes malformations of the heart and other organs indicating a role of fibronectin in the regulation of cell differentiation during embryonic development (George et al., 1997). Similarly, the knockout of the laminin gene causes malformations of different organs (Ryan et al., 1996).

Fibronectin has an inhibitory effect on myogenic, adipogenic and colonic differentiation but stimulates the differentiation of red blood cells (Adams and Watt, 1993). Myogenic differentiation terminates with the fusion of mononucleated myoblasts into multinucleated giant cells known as myotubes (Wakelam, 1985). The addition of fibronectin to mouse C2C12 and rat L6 myoblasts *in vitro* abrogates myogenic differentiation (Podleski et al., 1979; von der Mark and Ocalan, 1989). Furthermore, the addition of an anti-fibronectin polyclonal antibody to L6 rat myoblasts in culture

accelerates myogenic differentiation of these cells, thus suggesting that the antibody is interfering with an inhibitory signal provided by fibronectin (Podleski et al., 1979). The differentiation of cells into adipocytes is inhibited in the presence of fibronectin (Castro-Munozledo et al., 1987). The epithelial differentiation of Caco-2 human colorectal cancer cells is similarly accompanied by a decrease in fibronectin mRNA synthesis (Levy et al., 1994; Vachon et al., 1995).

In contrast to fibronectin, laminin has a stimulatory effect on epithelial, myogenic and neurogenic differentiation. The differentiation of breast epithelial cells requires the presence of laminin and other ECM components (Lochter and Bissell, 1995). The addition of laminin to mouse myoblasts accelerates the formation of myotubes (von der Mark and Ocalan, 1989). Likewise, the differentiation of neuronal precursor cell lines requires attachment to a laminin substratum (Luckenbill-Edds, 1997; Matsuzawa et al., 1996; Nurcombe, 1992).

7) Structure and Function of Integrin Receptors

The regulatory signals provided by the ECM are recognized by cell surface glycoproteins called integrins, the main cellular receptors for ECM components (Hynes, 1992). Integrins are heterodimeric receptors composed of α and β subunits. There are about 22 integrin receptors that consist of different combinations of α and β subunits (Bazzoni and Hemler, 1998). Each integrin subunit contains an extracellular domain followed by a transmembrane region and a cytoplasmic tail.

Integrins are ubiquitously expressed in the organism. However, the set of integrin receptors expressed on the cell surface is specific for each cell type. Epithelial cells and other cell types adhere to the ECM through their specific set of integrin receptors expressed on their surface. The recognition of ECM components by integrin receptors is a very complex process. One integrin receptor could recognize several ECM components

($\alpha_v\beta_3$ recognizes fibronectin, vitronectin, thrombospondin, etc), and in some instances, one ECM component can be recognized by several integrin receptors (fibronectin is recognized by the $\alpha_5\beta_1$, $\alpha_v\beta_1$, and $\alpha_v\beta_3$ integrin receptors) (Hynes and Lander, 1992).

Integrin functions play a crucial role in the regulation of cell proliferation, differentiation, motility and anoikis (Giancotti and Ruoslahti, 1999). Cancer cells often acquire a variably aberrant set of integrin receptors (Dedhar, 1995; Mizejewski, 1999; Varner and Cheresch, 1996) allowing them to invade and colonize other tissues in the body, a process known as metastasis (Fidler, 1999).

7.1) Integrin Signaling

Unlike many growth factor receptors, integrins lack enzymatic activity and therefore, need the recruitment of other adaptor proteins that link them to enzymes like src and ras, that are involved in signal transduction (Dedhar and Hannigan, 1996; Giancotti and Ruoslahti, 1999; Lafrenie and Yamada, 1996; Lelievre et al., 1996). The term integrin stands for integration of the ECM with the cytoskeleton (Giancotti and Ruoslahti, 1999). Integrin receptors create physical continuity between the extracellular protein network that constitutes the ECM and the intracellular protein network that constitutes the cytoskeleton (Lelievre et al., 1996; Yamada and Miyamoto, 1995). Based on this, Donald Inberg has proposed a "tensegrity" model where a mechanical signal transduction system regulates cell behaviour (Chen et al., 1997a). Integrin receptors couple the ECM with the cytoskeleton generating mechanical tension that results into the stretching of cytoskeletal fibers. This triggers signals that control cell proliferation and survival. The precise molecular nature of these signals is not well understood. The prevailing view is that integrins function by triggering a combination of mechanical (stretching of the cytoskeleton) and chemical signals (tyr and ser phosphorylation) that translates into the control of genetic expression.

The cytoplasmic tail of integrin receptors physically interacts with the focal adhesion kinase (FAK) and cytoskeletal proteins like paxillin and α -actinin (Giancotti and Ruoslahti, 1999). The ligation of an integrin receptor by an ECM component triggers a cascade of protein-protein interactions that cause the recruitment of many other cytoskeletal proteins like vinculin, actin, tensin, and many other signal transducer proteins like ras and src (Dedhar and Hannigan, 1996; Giancotti and Ruoslahti, 1999; Hannigan and Dedhar, 1997; Hynes, 1992; Miyamoto et al., 1995; Yamada and Miyamoto, 1995). This leads to the formation of focal adhesion contacts, a special type of structure localized in the plasma membrane at the sites of cell adhesion to the ECM (Hynes, 1992). FAK is a key component of the focal adhesion contacts. This tyr kinase plays a critical role in the transduction of signals by certain ligated integrin receptors (Frisch and Ruoslahti, 1997; Giancotti and Ruoslahti, 1999).

Recently, the $\beta 1$ integrin subfamily has been subdivided into two classes: those integrin receptors that signal through Shc ($\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$), and those that signal through FAK ($\alpha_2\beta_1$, $\alpha_6\beta_1$) (Giancotti and Ruoslahti, 1999). These two subfamilies of integrins are coupled to distinct signal transduction pathways and are specialized in regulating different cellular events.

7.2) The Fyn/Shc/Ras Pathway

Upon ligation to fibronectin, some integrin receptors such as $\alpha_5\beta_1$, $\alpha_1\beta_1$ and $\alpha_v\beta_3$ activate Fyn, a member of the Src-family of protein tyr kinases, that becomes activated and recruits Shc (Giancotti and Ruoslahti, 1999). The recruitment of Shc could be triggered by Lck, Yes, or Src in cells that do not express Fyn. It has been suggested that the activation of Fyn could be due to the action of an integrin-dependent protein tyr phosphatase that removes the phosphate group from the autoinhibitory tyr of Fyn. The $\alpha_5\beta_1$ integrin binds Fyn through the membrane adapter protein caveolin-1 (Pande, 2000;

Wei et al., 1996). The SH3 domain of activated Fyn recognizes the proline-rich domain of Shc (Giancotti and Ruoslahti, 1999). The recruitment of Shc by Fyn allows the binding of Shc to adapter proteins Grb2 and Sos, thus activating Ras, a small GTPase protein that links integrins to the ERK pathway. The latter stimulates the transcription of the Cyclin D gene that leads to progression through the cell cycle, thus inhibiting cell differentiation.

7.3) The FAK pathway

Other integrin receptors such as $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_6\beta_1$ activate FAK upon ligand binding (Giancotti and Ruoslahti, 1999). The activated FAK binds to the β_1 cytoplasmic tail directly or through the cytoskeletal proteins talin and paxillin. The activation of FAK induces its autophosphorylation in tyr 327 that is recognized by the SH2 domain of Src, which then phosphorylates the tyr 925 of FAK. Src and FAK phosphorylates many cytoskeletal proteins such as paxillin, talin and tensin that have been recruited to the focal adhesion sites upon integrin binding to its ligand (Hynes, 1992; Otey and Burridge, 1990; Yamada and Miyamoto, 1995). The phosphorylation and activation of FAK triggers the recruitment of Grb-2/Sos and subsequent activation of Ras (Giancotti and Ruoslahti, 1999). As mentioned earlier (see Section 5.1- "Molecular Signals Regulating Anoikis"), this pathway leads to the activation of PI-3 kinase and Akt, a ser/thr kinase known to inhibit the functions of Bad and Caspase-9, thus inhibiting anoikis.

7.4) Outside in and inside out signaling

Integrin function could be regulated by outside in or inside out signaling (Dedhar and Hannigan, 1996; Faull et al., 1993; Hynes, 1992). The first refers to the changes that occur after ligation of the integrin receptor to an ECM component. The Fyn/Shc/Ras and FAK pathways discussed above are examples of outside in signaling. The inside out signaling refers to protein-protein interactions that occur between integrins and

intracellular proteins which cause a change in the affinity of the integrin receptor for the ligand or activation of the integrin receptor by its clustering on the cell surface (Bazzoni and Hemler, 1998). The integrin linked kinase (ILK) is a ser/thr kinase that binds and phosphorylates the cytoplasmic tail of the β_1 integrin subunit (Hannigan et al., 1996; Huang and Wu, 1999). This modification causes a functional activation of the $\alpha_5\beta_1$ integrin due to increased cell surface clustering of this receptor (Wu et al., 1998). The overexpression of ILK *in vitro* leads to malignant transformation (Hannigan et al., 1996; Radeva et al., 1997).

7.5) Evolution of integrin receptors

Unlike CEA, exclusively found in humans and other primates, integrin homologues are expressed in almost all pluricellular organisms from sponges to humans (Brown, 2000a; Brown, 2000b; Brown et al., 2000). For example, *C. elegans* express an integrin homolog composed of two subunits that plays a key role in the morphogenesis of the worm (Brown, 2000a). There are two positional specific antigens in *Drosophila melanogaster*, PS1 and PS2, that are heterodimeric homologs of the mammalian integrin receptors (Brown et al., 2000). PS1 is composed of α_1 and β subunits and PS2 is comprised of α_2 and β subunits. Both PS1 and PS2 are crucial for the morphogenesis of the wings and other structures of the morphogenetic program of the fly (Brown et al., 2000). The study of the corresponding integrin homologues in simpler organisms that allow easier genetic manipulation, like *C. elegans* and *Drosophila melanogaster*, will certainly improve the current understanding of the functional principles of these receptors.

8) The $\alpha_5\beta_1$ Integrin Receptor plays a negative role in myogenic differentiation

The inhibitory role of the $\alpha_5\beta_1$ integrin receptor on myogenic differentiation is very well documented. The introduction of the $\alpha_5\beta_1$ cDNA into primary quail skeletal muscle cells

blocks myogenic differentiation (Sastry et al., 1996; Sastry et al., 1999). The cell surface level of expression of the $\alpha_5\beta_1$ integrin receptor decreases upon myogenic differentiation of chicken myoblasts (Boettiger et al., 1995).

Fibronectin, the ligand of the $\alpha_5\beta_1$ integrin receptor, provides an inhibitory signal for myogenic differentiation. The addition of soluble fibronectin to C2C12 myoblasts inhibits myogenic differentiation (von der Mark and Ocalan, 1989). The addition of an anti-fibronectin polyclonal antibody to L6 rat myoblasts accelerates myogenic differentiation, probably due to interference with the negative signal associated with fibronectin (Podleski et al., 1979). Moreover, the proteolytic degradation of fibronectin is required for the myogenic differentiation of mouse C2C12 myoblasts (Dourdin et al., 1997).

Altogether, these observations support the notion that the engagement of the $\alpha_5\beta_1$ integrin receptor to fibronectin triggers a negative signal that blocks myogenic differentiation. The precise nature of this signal is not known. However, one pathway potentially involved in this type of regulation is the Fyn/Shc/Ras pathway described above.

9) Inhibition of Anoikis by the $\alpha_5\beta_1$ Integrin Receptor

Anoikis is inhibited in many cell types by the activation or overexpression of the $\alpha_5\beta_1$ integrin receptor (Frisch and Ruoslahti, 1997). The forced over-expression of the $\alpha_5\beta_1$ integrin in HT29 intestinal epithelial cells inhibits anoikis *in vitro* in the absence of growth factors (serum free culture medium) (O'Brien et al., 1996). The over-expression of the $\alpha_5\beta_1$ integrin on the cell surface induces an increase in the level of expression of the Bcl-2 protein, a well known inhibitor of anoikis (Zhang et al., 1995). Conversely, adhesion to fibronectin, the ligand of the $\alpha_5\beta_1$ integrin, triggers a survival signal in many cell types (Frisch and Ruoslahti, 1997; Scott et al., 1997; Vitale et al., 1998). The

presence of fibronectin in the serum of most tissue culture media is one of the key survival factors contributing to the viability of cell cultures (Di Matola et al., 2000). The over-expression of ILK in rat intestinal cell lines inhibits anoikis by inducing clustering and activation of the $\alpha_5\beta_1$ integrin receptor (Huang and Wu, 1999; Wu et al., 1998). The binding of the $\alpha_v\beta_3$ integrin receptor to vitronectin inhibits anoikis of endothelial, glioma (Uhm et al., 1999) and melanoma cells (Petitclerc et al., 1999). Unlike the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ receptors, other integrins (like $\alpha_v\beta_1$ and $\alpha_2\beta_1$) do not provide survival signals (Frisch and Ruoslahti, 1997; Giancotti and Ruoslahti, 1999).

One of the most promising new strategies to develop anti-cancer therapies is to inhibit tumor angiogenesis (Hanahan and Folkman, 1996). Amongst many other targets for angiogenesis inhibitors, the $\alpha_v\beta_3$ integrin receptor is a potential target for drugs designed to induce anoikis of endothelial cells within the tumor (Cheresh, 1998).

10) Fibronectin Polymerization. Role of the $\alpha_5\beta_1$ Integrin Receptor

Fibronectin is a dimeric glycoprotein that exists in two isoforms: soluble fibronectin that circulates in the plasma and insoluble fibronectin that polymerizes on the cell surface and becomes incorporated in the ECM (Sakai et al., 1996; Schwarzbauer and Sechler, 1999; Wu, 1997). Polymerized fibronectin is the biologically active isoform that provides signals for the regulation of cell proliferation, differentiation and anoikis (Hocking et al., 1998). The assembly of fibronectin into a matrix requires the active participation of the cell surface (Christopher et al., 1997). Fibronectin matrix assembly does not occur in a cell free environment. Two cell surface receptors are involved in fibronectin matrix assembly (Hocking et al., 1998). One of these receptors has not been cloned yet, but there is compelling biochemical evidence for its contribution (Hocking et al., 1998). This receptor is known to bind the N-terminal portion of fibronectin and is thought to be involved in the initialization of the polymerization reaction (Christopher et al., 1999;

Hocking et al., 1998). The second receptor is the $\alpha_5\beta_1$ integrin receptor that plays a crucial role in the progression of the matrix assembly process (Christopher et al., 1997; Fogerty et al., 1990; Pankov et al., 2000; Pickering et al., 2000; Sakai et al., 1996; Wu, 1997; Zhang et al., 1993). The $\alpha_5\beta_1$ integrin recognizes the RGD sequence in the central domain of fibronectin (Fogerty et al., 1990; Hocking et al., 1996; Ruoslahti, 1996b). Matrix assembly is inhibited in the presence of blocking mAbs that recognize the α_5 and β_1 subunits of this receptor (Fogerty et al., 1990; Pickering et al., 2000).

This Thesis shows data supporting the notion that the over-expression of CEA/CEACAM6 on the surface of cancer cells instrumentally contributes to tumor formation and progression by inhibiting cell differentiation and anoikis. The molecular mechanism of these inhibitory effects seems to be related to perturbation of the functions of the $\alpha_5\beta_1$ integrin receptor. In contrast, CEACAM1 cell surface expression inhibited tumor formation and correspondingly, neither perturbed integrin functions nor inhibited anoikis.

In Chapter 2, the authors demonstrate that CEA/CEACAM6, but not CEACAM1, over-expression on the surface of distinct cell lines inhibit anoikis. Chapter 3 deals with the molecular mechanism of such inhibition. Finally, Chapter 4 shows how the forced expression of a murine homologue of CEACAM1 on the surface of CT51 mouse colonic carcinoma cells inhibited tumor formation when these cells were injected into syngeneic mice.

Chapter 2

Human Carcinoembryonic Antigen functions as a General Inhibitor of Anoikis

ABSTRACT

Human Carcinoembryonic Antigen (CEA), a widely used tumor marker, and CEACAM6 (NCA) are upregulated in many types of human cancers while family member CEACAM1 (BGP) is usually downregulated. Deregulated overexpression of CEA/CEACAM6 but not CEACAM1 can inhibit the differentiation, and disrupt the polarization and tissue architecture of many different types of cells. In this report we show that CEA and CEACAM6, but not CEACAM1, markedly inhibit the apoptosis of cells when deprived of their anchorage to the extracellular matrix, a process known as anoikis. By blocking this tissue architecture surveillance mechanism, the architectural perturbation initiated by CEA/CEACAM6 can thus be maintained.

Introduction

CEA and highly related CEA family member, CEACAM6 (formerly NCA), members of the Immunoglobulin Superfamily (IgSF), show deregulated cell surface overexpression in about 50% of human cancers (Chevinsky, 1991); CEACAM1 (formerly BGP), on the other hand, differing from CEA/CEACAM6 chiefly by its mode of cell membrane anchorage (transmembrane vs glycosphosphatidyl inositol), is usually downregulated (Neumaier et al., 1993). These CEA family members function *in vitro*, at least, as intercellular adhesion molecules (Benchimol et al., 1989; Oikawa et al., 1989; Stanners and Fuks, 1998). A common view that CEA represents a “differentiation marker”, merely reflecting the differentiation status of tumors that express it, has been challenged recently by evidence that CEA (and CEACAM6, but not CEACAM1) could play an instrumental role in tumorigenesis by the disruption of cell polarity and tissue architecture and the inhibition of cell differentiation of many different types of cells (Eidelman et al., 1993; Screaton et al., 1997; Stanners and Fuks, 1998). If, indeed, deregulated CEA/CEACAM6 overexpression in colonocytes at the base of colonic crypts can cause the aberrations in tissue architecture observed in human colonic carcinomas, CEA/CEACAM6 overexpression must also overcome the control mechanisms that normally preserve the tissue architecture of the crypts. We have obtained evidence that the function of specific integrins is perturbed by CEA/CEACAM6 over-expression, thus affecting cell-extracellular matrix (ECM) interactions that are necessary for the establishment of tissue architecture and the correct deployment of differentiation programs. It has been proposed that anoikis, a type of apoptotic program triggered when cells lose contact with their ECM (Frisch and Francis, 1994; Frisch and Ruoslahti, 1997; Meredith et al., 1993), functions *in vivo* as a surveillance mechanism which prevents dysplasia and preserves normal tissue architecture by destroying any cells that attempt to deviate from their

normally operative spatial constraints (Frisch and Ruoslahti, 1997). Our model (Fig. 4) would require that this process be inhibited, if aberrant multilayered tissue architecture initiated by CEA/CEACAM6 overexpression were to be maintained. We demonstrate here that forced overexpression of CEA/CEACAM6 but not CEACAM1 on the surface of L6 rat myoblasts, MDCK epithelial cells, and human SW1222 and Caco-2 colorectal cancer cells, inhibited their anoikis *in vitro*.

Materials and Methods

Cell Lines

L6 rat myoblasts, SW1222 and Caco-2 human colorectal carcinoma cells, and Madin Darby Canine Kidney (MDCK) epithelial cells were grown as monolayer cultures in DMEM (L6, MDCK) or α -MEM (SW1222, Caco-2) containing 10% fetal bovine serum (growth medium, GM; GIBCO BRL, Gaithersburg, MD) supplemented with 100 μ g/ml streptomycin and 100 U/ml penicillin (GIBCO BRL) at 37°C in a humidified atmosphere with 5% CO₂. All cell lines were subcultured before confluence and seeded at a density of 1×10^4 cells/cm².

cDNA Transfections and Infections

Transfection procedures and transfected cell lines used in this study have been previously described (Eidelman et al., 1993; Screaton et al., 1997). Briefly, stable transfectants of rat L6 myoblasts were obtained by the calcium phosphate precipitation method using the p91023B expression vector (courtesy of R. Kaufman, Genetics Institute, Boston) containing full length cDNAs encoding cell adhesion proteins: CEA, CEACAM6, CEACAM1-4L (formerly splice variant BGP α), CEA deletion mutant Δ NCEA (lacking the last 75 amino acids of the N domain) (Eidelman et al., 1993; Zhou et al., 1993), NCAM-125 (GPI-linked NCAM splice variant with muscle specific domain) (Dickson et

al., 1987) and pSV2neo plasmid as a dominant selectable marker. SW1222 and Caco-2 transfectants were obtained with the Zn^{2+} -inducible episomal expression vector pML1 containing the hygromycin gene plus full length cDNAs encoding either CEACAM1, CEA, Δ NCEA or a cDNA containing just the entire coding region of CEACAM6. SW1222-Hygro and Caco-2-Hygro (vector alone) controls were obtained with pML1 containing the hygromycin gene. Pooled clones of stably transfected cells were selected with 400 μ g/ml of Neomycin (G418) (L6) or 200 μ g/ml of Hygromycin-B (SW1222, Caco-2). L6 myoblasts expressing comparably high levels (FACS mean value: 150-225) of the proteins encoded by the transfected cDNAs were selected by FACS using specific monoclonal antibodies. The SW1222-CEACAM6 transfectant and the Caco-2-CEA/CEACAM6 doubly transfectant cells expressed, after promoter induction with Zn^{2+} , about 9 and 20 fold higher cell surface levels of CEACAM6 or CEA/CEACAM6, respectively, than control cells transfected with the vector alone. Although G418 (L6) and Hygromycin-B (SW1222, Caco-2) were removed from the culture media during functional assays, no loss of cell surface expression of the transfected cDNAs was observed (not shown).

L6 and MDCK cells in the exponential phase of growth, were infected with replication-defective recombinant retrovirus containing either pBabe(human bcl-2)puro (L6) (Screaton et al., 1997) or pLXSN(CEA cDNA)neo (MDCK) or the vector alone as a control. Stably transfected MDCK cells were selected with 400 μ g/ml of G418 and cells expressing high levels of CEA on the cell surface were selected by FACS using specific anti-CEA monoclonal antibodies.

In order to avoid phenotypic perturbations due to clonal variation, all transfected cell lines used in this study were prepared as pooled (total) populations consisting of multiple clones selected with G418 or Hygromycin. The polyclonality of the CEA expressing L6 cell population was demonstrated by Southern blot (Screaton et al., 1997).

Apoptotic Assays

In order to induce over-expression of CEA/CEACAM6, SW1222 and Caco-2 transfected cells were cultured in GM supplemented with 0.1 mM ZnSO₄ for 24 hours prior to the experiment. To measure anoikis of L6, MDCK, and Zn²⁺ induced SW1222 and Caco-2 (control and transfected cells), 0.2x10⁶ cells/ml of each cell line were suspended in PolyHEMA (Poly 2-hydroxyethylmethacrylate, Aldrich Chemicals, Milwaukee, WI) coated 6-well tissue culture plates for a period of 12 to 72 hours in the presence (GM) or absence (serum free DMEM) of growth factors. The percentage of apoptotic cells was estimated by staining the nuclei with DAPI (Boehringer Mannheim, Roche Diagnostics, Laval, Canada), or using the TUNEL assay (ONCOR, S7100-KIT, Intergen, Boston, MA) following instructions from the manufacturer. Briefly, to stain with DAPI the cells were fixed on ProbeOn™ Plus microscope slides (FisherBiotech, USA) with 4% paraformaldehyde for 20 minutes, washed, permeabilized for 5 minutes with 0.1% Triton X-100 (T-8787, SIGMA, St Louis, MO), and stained with 10 µg/ml of DAPI in PBS. Cells with fragmented (DAPI) or stained (TUNEL) nuclei were scored as apoptotic cells. The apoptotic index was calculated by scoring no less than 1000 cells. All observations were reproduced at least twice by independent experiments. HMα5-1 (anti-rat α₅ integrin subunit), Ha2/5 (anti-rat β₁ integrin subunit), and Ha1/29 (anti-rat α₂ integrin subunit) mAbs (PharMingen, Mississauga, ON) were added to the suspended cells in the POLYHEMA-coated wells at a final concentration of 1 µg/ml each in GM, and incubated for 48 hours before DAPI staining.

All cell lines used in the anoikis assays formed cellular aggregates in suspension over PolyHEMA-treated surfaces. The average size of these aggregates was determined by dividing the total number of cells per sample by the total number of aggregates, determined in triplicate. To determine the total number of cells per sample, the

aggregates were dissociated at 37°C for 30 minutes with a collagenase enzyme cocktail that contained 690 units of crude collagenase/ml (Worthington Biochemical Corp., Freehold, NJ; code CLS-1) in PBS. The cell concentration in the resulting single cell suspension was measured using a particle counter (Coulter Electronics Inc., Hialeah, FL). The average aggregate size was 30% less for the parental L6 cells than all of the transfectant populations, which had average aggregate sizes of within 5% of each other.

Statistical Analysis

Differences between groups (CEA/CEACAM6 expressing cells vs control cells) were analyzed by Student's *t* test. *P* < 0.05 was considered significant.

Results

Effects of CEA and CEACAM6 expression on anoikis of rat L6 myoblasts

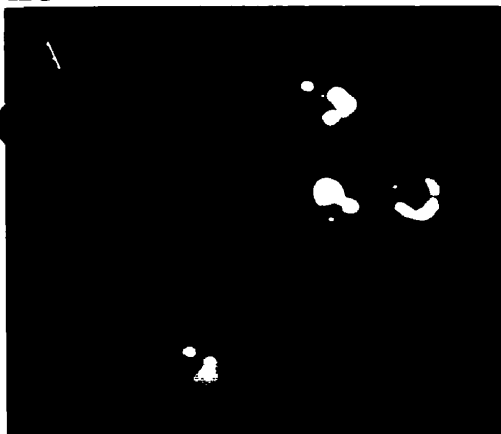
To test whether CEA and CEACAM6 overexpression on the cell surface inhibits anoikis *in vitro*, we used pooled stably transfected L6 rat myoblast clones as an ectopic model. These transfectants, together with appropriate controls, were cultured in suspension in serum free DMEM or GM in tissue culture dishes coated with PolyHEMA to prevent cell attachment to the substratum. Under these conditions, parental L6 myoblasts underwent anoikis due to the lack of survival signals provided by the ECM or substratum, showing characteristic fragmentation of their nuclei (Fig. 1A) and positive staining by the TUNEL assay (Fig. 2B). L6 myoblasts expressing CEA or CEACAM6 on their surfaces, on the other hand, were much less prone to undergo anoikis both in GM (*P*=0.001 and *P*=0.004 respectively) (Fig. 1 E, F, &G) or serum-free DMEM (*P*=0.003 and *P*<0.001

respectively) (Fig. 2 D & E); control L6 myoblasts expressing CEACAM1 (Fig. 1B; Fig. 2 C&E) or cells transfected with the vector alone (not shown) showed about the same level of anoikis as the parental cells.

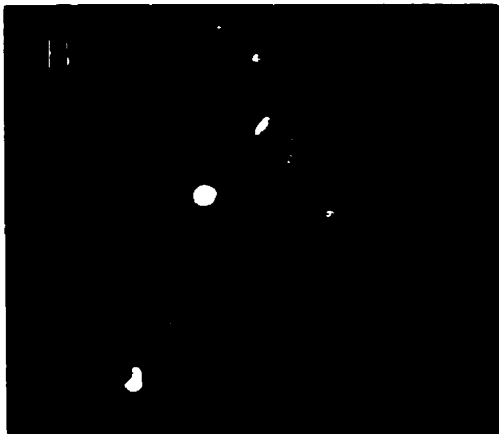
Figure 1. A-F)

DAPI staining of L6 parental and transfected myoblasts suspended in GM for 48 hours on PolyHEMA coated surfaces. Apoptotic cells showed characteristic fragmented nuclei while survivors showed intact nuclear morphology. **A)** L6 parental cells, **B)** L6 myoblasts expressing human CEACAM1, **C)** human Bcl-2 protein, **D)** the human GPI-linked isoform of NCAM (NCAM-125), **E)** CEA, **F)** CEACAM6. **G)** The apoptotic index (see graph) was calculated by scoring the percentage of apoptotic cells in three independent samples of 500-1000 cells each. The statistical average and standard deviation of three independent experiments are indicated. Both CEA and CEACAM6 expression on the surface of L6 myoblasts significantly inhibited anoikis ($P=0.005$ and $P=0.004$ respectively). A second independent pooled population of stably transfected L6 myoblasts (L6-CEA-2) showed similar results ($P=0.001$) to L6-CEA transfected cells. These results were confirmed using the TUNEL assay in two separate experiments (data not shown).

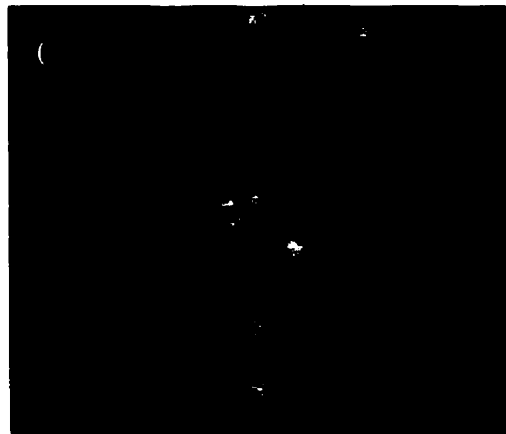
L6



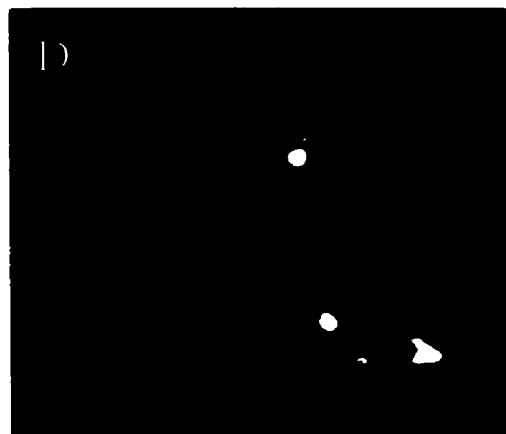
L6-CEACAM1



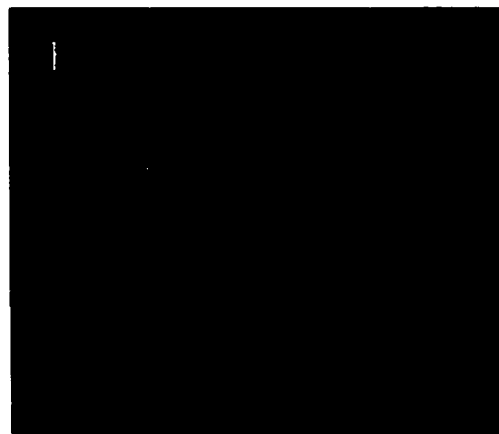
L6-bcl-2



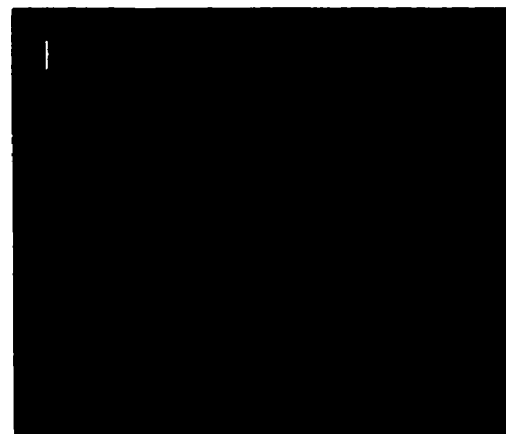
L6-NCAM



L6-CEA



L6-CEACAM6



G

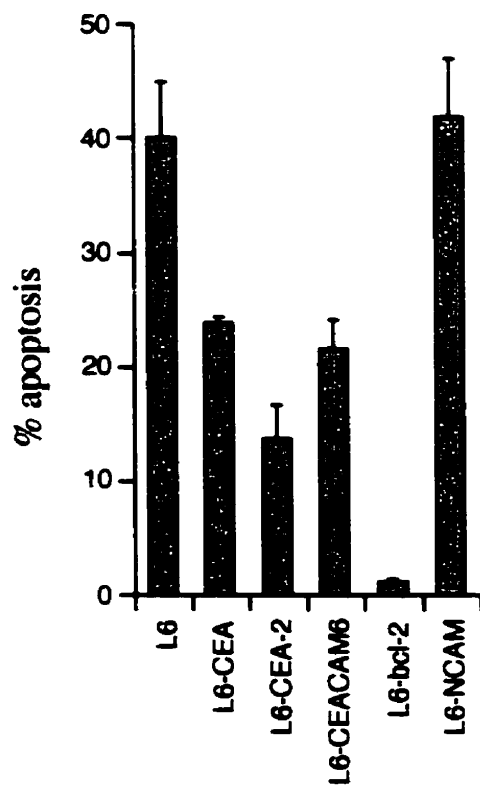
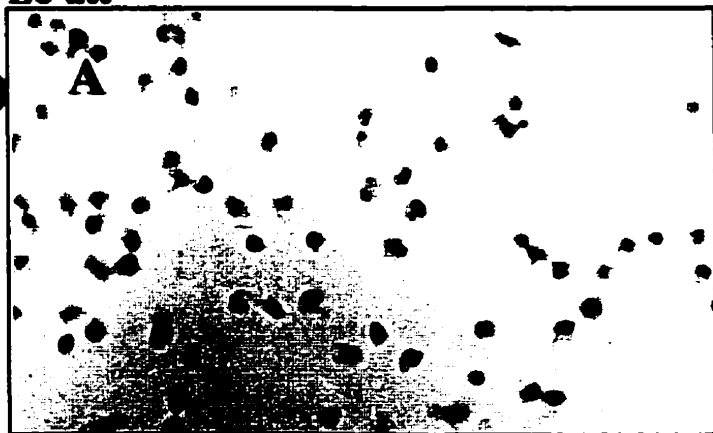
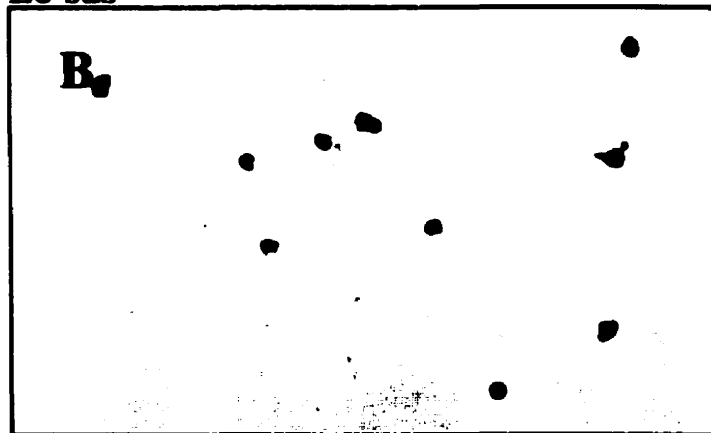
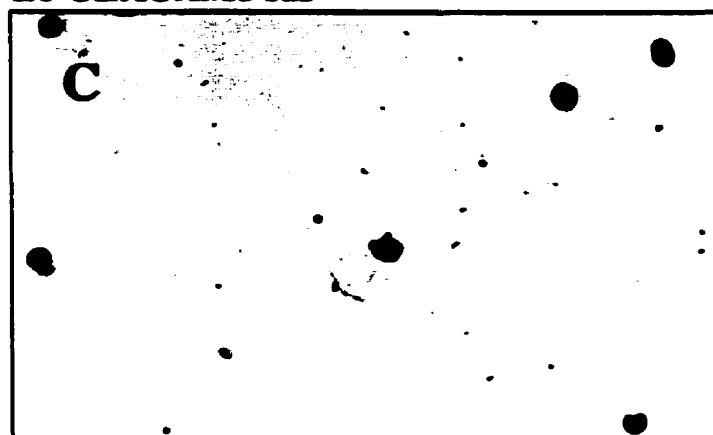
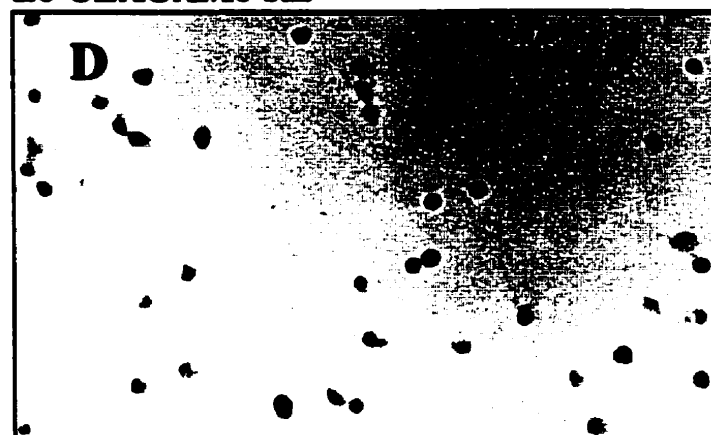
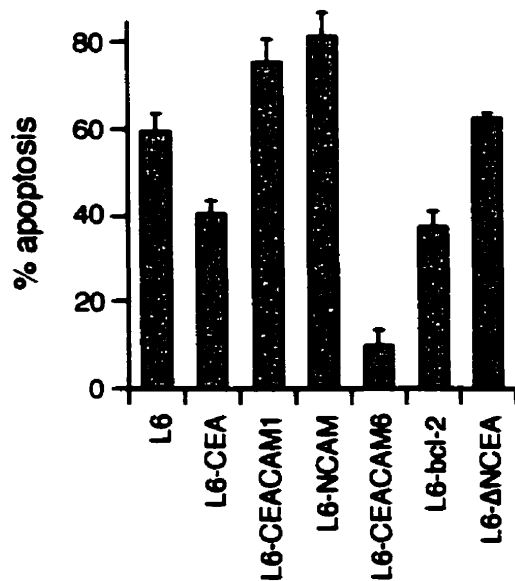
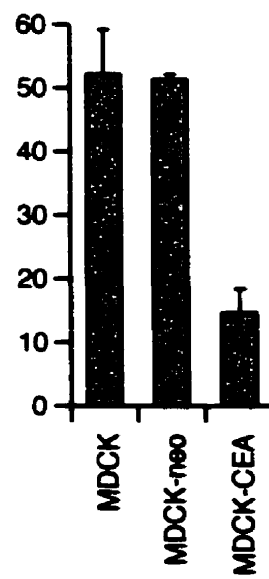


Figure 2.

Apoptotic cells detected by positive staining using the TUNEL assay. **A)** control attached L6 parental myoblasts, indicated here as L6-att, were cultured attached to the tissue culture plastic surface, and did not undergo apoptosis. **B-D)** parental and transfected L6 myoblasts suspended in serum free DMEM on PolyHEMA coated surfaces for 24 hours, indicated as "cell line-sus". **B)** L6 parental myoblasts, **C)** CEACAM1 and **D)** CEACAM6 expressing L6 myoblasts. **E)** The apoptotic index was calculated as indicated above. These results were corroborated by DAPI staining in four independent experiments. **F)** Quantitation of anoikis of transfected MDCK cells suspended in GM on PolyHEMA coated surfaces for 24 hours. The average and standard deviations of two independent experiments is presented here.

L6-att**L6-sus****L6-CEACAM1-sus****L6-CEACAM6-sus****E****F**

To test for non-specific effects due to intercellular adhesion, L6 transfected myoblasts expressing high levels of the NCAM isoform NCAM-125, another GPI-anchored intercellular adhesion molecule of the IgSF, were used as a control. NCAM-125, despite being expressed at higher levels than CEA (FACS mean value: 225 for NCAM-125 versus 190 for CEA) did not inhibit anoikis (Fig 1D,1G&2E). This result indicates that the inhibitory effects of CEA and CEACAM6 are not due to indirect effects of their intercellular adhesion function or GPI anchorage *per se*. As a further test of specificity, L6 myoblasts expressing Δ NCEA, a deletion mutant lacking the last 2/3 of the N-terminal domain (Eidelman et al., 1993; Zhou et al., 1993) were tested for anoikis. These transfectants were as prone to undergo anoikis as L6 parental cells (Fig 2E). The deleted portion of the N domain has been shown to be required for both the intercellular adhesion (Zhou et al., 1993) and the differentiation inhibitory effects of CEA (Eidelman et al., 1993).

Suspended parental L6 myoblasts in the absence of growth factors (serum free DMEM) showed apoptotic features as early as 12 hours (data not shown). However, in the presence of growth factors (GM-growth medium), apoptosis of suspended L6 myoblasts was only 5% after 24 hours (data not shown) and increased to 40% after 48 hours (Fig. 1G). As mentioned above, CEA and CEACAM6 expression inhibited anoikis of L6 myoblasts both in the presence or absence of growth factors. Suspended L6 myoblasts expressing human Bcl-2, a known inhibitor of anoikis (Frisch and Ruoslahti, 1997; Meredith et al., 1993), were less prone to undergo cell death ($P<0.001$) than L6 parental myoblasts (Fig. 1 C, G; Fig. 2E). Interestingly, Bcl-2-mediated inhibition of anoikis was more prominent in the presence (Fig. 1G) than in the absence of growth factors (Fig. 2E). The ectopic expression of CEA on the surface of MDCK cells inhibited anoikis ($P=0.001$) when these cells were suspended in PolyHEMA-coated dishes (Fig. 2F), thus confirming the effect.

Effect of CEA and CEACAM6 over-expression on anoikis of human colonocytes

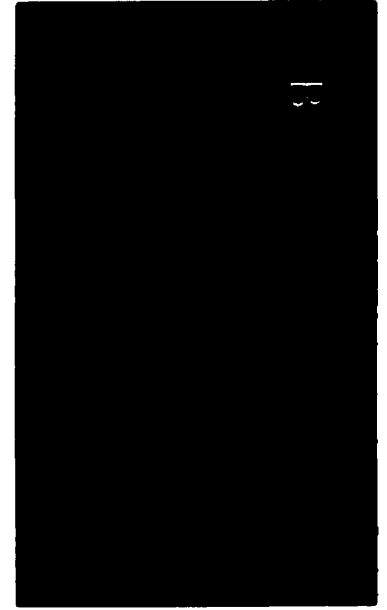
CEA and CEACAM6 are not endogenously expressed by either skeletal muscle cells or MDCK cells. To test CEA/CEACAM6 inhibitory effects in a medically relevant model system, anoikis of stably transfected human colorectal carcinoma cell lines, SW1222 and Caco-2, both capable of colonic epithelial differentiation (Pignatelli and Bodmer, 1988; Pinto et al., 1983) was measured. Parental SW1222 and Caco-2 cells express relatively low levels of endogenous CEA and CEACAM6 (Hauck and Stanners, 1991). The SW1222-CEACAM6 transfectant and the Caco-2-CEA/CEACAM6 doubly transfectant cells from subconfluent growing cultures expressed, after promoter induction with Zn^{2+} , about 9 and 20 fold higher cell surface levels of CEACAM6 and CEA/CEACAM6, respectively, than control cells transfected with the vector alone. These levels of CEA/CEACAM6 expression correspond to those observed *in vivo* in human colonic tumors (Ilantzis et al., 1997). SW1222 cells over-expressing CEACAM6 were found to be unable to form glandular-like spheroids of polarized cells in collagen gels; Caco-2 cells over-expressing CEA/CEACAM6 lost their ability to form monolayers of polarized cells and instead formed stratified layers of disorganized cells, closely resembling dysplastic colorectal carcinomas⁵.

CEACAM6 over-expressing SW1222 and CEA/CEACAM6 over-expressing Caco2 cells were 2 and 4-fold less prone to undergo anoikis, respectively, than transfectants expressing CEACAM1, ΔN -CEA or cells transfected with the vector alone ($P<0.01$) (Fig. 3). CEACAM6 alone also inhibited anoikis ($P=0.01$) of transfected Caco-2 cells (Fig. 3F). Transfected Caco-2 cells over-expressing only CEA could not be tested because of a pronounced tendency to lose cell surface CEA expression during culture (data not shown). These results show that deregulated over-expression of CEA and

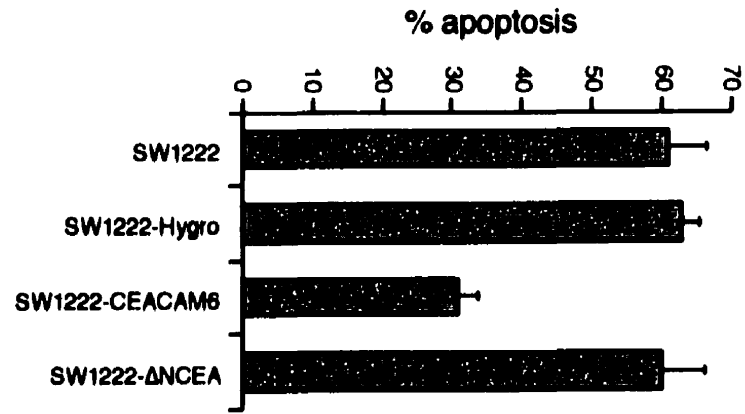
CEACAM6, but not CEACAM1, can not only disrupt cellular polarization and tissue architecture of human epithelial colonocytes⁵ but can also inhibit their architectural quality control mechanism, anoikis.

Figure 3. A-D)

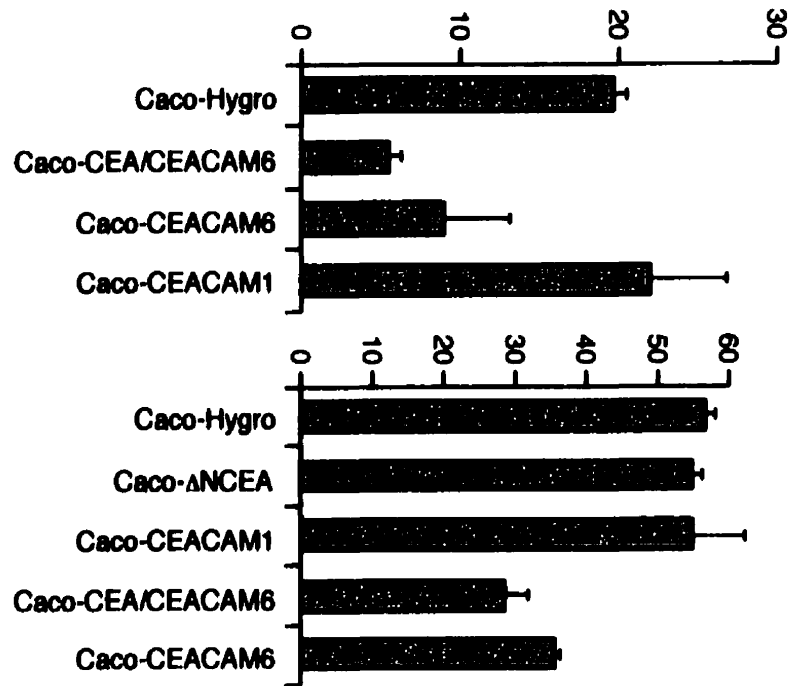
DAPI staining of SW1222 parental and transfected cells. **A)** control SW1222 parental cells attached to uncoated substratum. **B)** SW1222 parental, **C)** SW1222-CEACAM6 and **D)** SW1222-Hygro (vector alone) control cells, cultured on PolyHEMA-coated dishes for 24 hours in GM, **E)** Quantitation of anoikis of transfected SW1222 (24 hours) and **F)** Quantitation of anoikis of Caco-2 human colorectal cancer cells suspended in GM on PolyHEMA-coated dishes for 48 hours (left) and 72 hours (right). The data represents the average of two typical experiments of 6 independent experiments with similar results.



F



F



Discussion

The observed inhibition of a quality control mechanism preserving tissue architecture by CEA/CEACAM6 cell surface over-expression has important implications for the role of these molecules in human cancer, especially in the light of the fact that so many cancers show over-expression of these CEA family members. The question of mechanism arises. L6, MDCK, SW1222 and Caco-2 cells formed aggregates when cultured in suspension on PolyHEMA coated surfaces (not shown). Apoptotic cells were detected in these cellular aggregates but most of the apoptotic cells detached from them. In contrast, most of the cells that survived anoikis were found in the aggregates, suggesting that cell-cell interactions could provide a survival signal. This phenomenon has been observed before in 3T3 cells that become resistant to anoikis in the presence of insulin-like growth factor-I (Valentinis et al., 1998). This raises the question as to whether the intercellular adhesion activity of molecules such as CEA and CEACAM6 could indirectly induce resistance to anoikis as a consequence of increasing the number of cells in the aggregates. This is not the case, however, since CEACAM1 and NCAM-125 are also intercellular adhesion molecules and cells expressing them showed the same number and size of cellular aggregates as CEA/CEACAM6-expressing cells (see Materials and Methods) but no inhibition of anoikis. We suggest that intercellular interactions could nevertheless play a role in inhibiting anoikis by fostering clustering of CEA/CEACAM6 molecules by both anti-parallel and parallel binding on the cell surface, thus amplifying a CEA/CEACAM6-mediated anti-apoptotic signal. In support of this suggestion, the deletion mutant, Δ NCEA, that is defective in mediating both intercellular adhesion (Zhou et al., 1993) and the myogenic differentiation block (Eidelman et al., 1993) is also defective in inhibiting anoikis.

CEACAM1 over-expression in L6 and Caco-2 transfected cells did not inhibit anoikis. These results are consonant with the observation that CEA and CEACAM6 cell surface expression is upregulated in many cancers, while CEACAM1 expression is downregulated (see Introduction). L6 myoblasts expressing GPI-anchored NCAM-125 also underwent anoikis as readily as parental and CEACAM1 expressing L6 myoblasts. This result suggests that the CEA/CEACAM6 inhibitory function is specific and not an effect of GPI anchorage *per se*. CEA/CEACAM6 inhibition of anoikis seems to be specific for this type of apoptosis since CEA expression on the surface of L6 myoblasts does not inhibit v-myc-induced apoptosis (Screaton et al., 1997).

The $\alpha_5\beta_1$ integrin receptor, when bound to fibronectin, triggers a survival signal in many tissue culture cell lines (Frisch and Ruoslahti, 1997). In the absence of the $\alpha_5\beta_1$ integrin/fibronectin interaction, many cell types undergo anoikis. We have recently demonstrated that CEA/CEACAM6 expression on the surface of L6 myoblasts modifies cell adhesion to fibronectin due to a change in the functional status of the $\alpha_5\beta_1$ integrin receptor⁶. The anti-apoptotic intracellular signals generated by CEA/CEACAM6-mediated activation of the $\alpha_5\beta_1$ integrin receptor are currently under investigation.

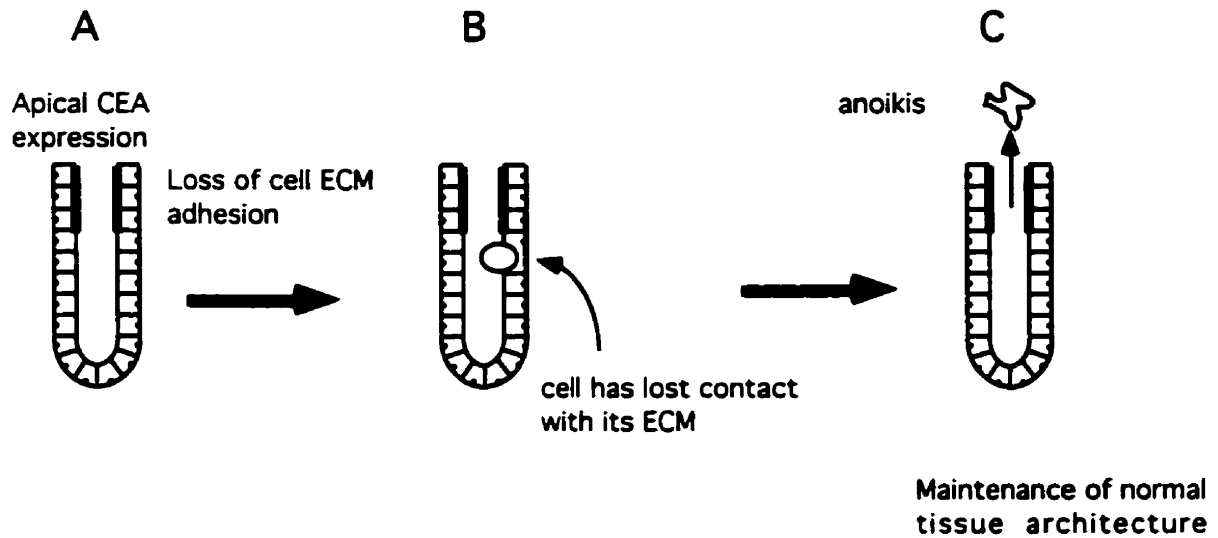
We hypothesize that the CEA/CEACAM6-mediated inhibition of anoikis could contribute to the disruption of normal tissue architecture that accompanies malignant transformation (Fig. 4). We propose that the CEA/CEACAM6 inhibitory effect on anoikis only occurs when these glycoproteins are overexpressed as in dysplastic cells, over the entire colonocyte surface (Fig 4D&E), but not when CEA is restricted to the apical surface of normal colonocytes (Fig 4A), where interference with integrin (localized on the basal membrane) functions is unlikely. CEA/CEACAM6-mediated inhibition of anoikis could allow malignant colonocytes to survive out of the plane of the monolayer in the absence of cell-basement membrane adhesion, a condition that would cause death of

normal colonocytes (Strater et al., 1996), thus permitting the persistence of aberrant tissue architecture (Fig. 4F). This disruption of colonic tissue architecture would also inhibit cellular differentiation, further contributing to malignant progression. Considering the fact that CEA and/or CEACAM6 over-expression is observed at other major sites, such as breast and lung, the inhibitory effects of these molecules on anoikis could be of major significance to the understanding of human cancer.

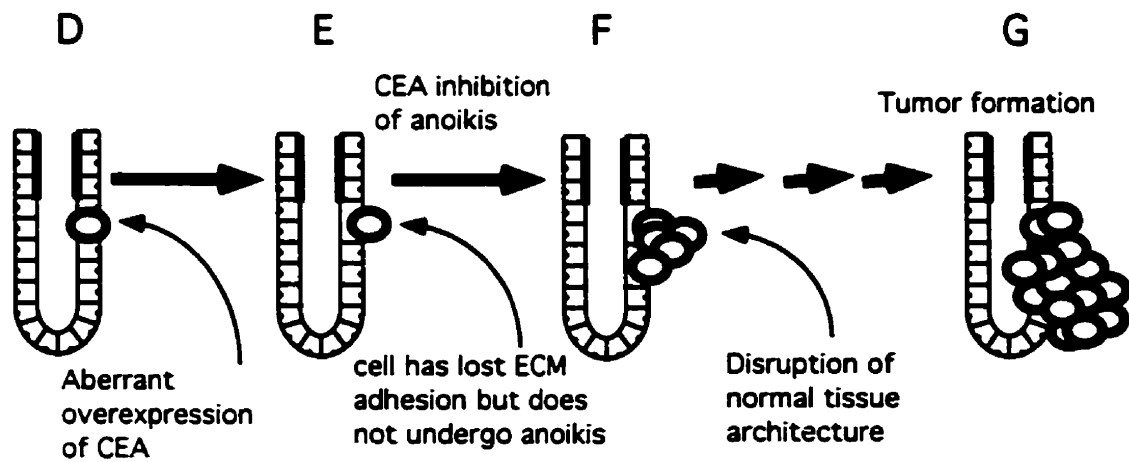
Figure 4.

Tissue architecture model **A-C)** Anoikis functions as a surveillance mechanism preserving the normal architecture of human colonic crypts. **D-G)** CEA/CEACAM6-mediated inhibition of anoikis disrupts tissue architecture. **A)** CEA (denoted as **||**, thick dark lines) and CEACAM6 are apically expressed in the top third of normal human colonic crypts (Ilantzis et al., 1997). Only CEA is indicated, to simplify the model. **B)** In normal crypts, colonocytes that have lost contact with the basement membrane (ECM) undergo anoikis and are unable to survive out of the plane of the epithelial monolayer, thus **C)** tissue architecture is preserved. **D)** Unlike the expression pattern in normal colonic crypts, CEA and CEACAM6 are over-expressed over the entire surface of malignant colonocytes (thick dark circles **●**). **E)** A CEA/CEACAM6 over-expressing cell that has lost cell-ECM adhesion has longer survival capacity and proliferates **(F)** out of the plane of the single columnar epithelium, disrupting normal tissue architecture and inhibiting cellular differentiation. The latter event, together with other genetic lesions, will contribute to tumor formation and progression **(G)**

NORMAL COLONIC CRYPT



MALIGNANT COLONIC CRYPT



In Chapter 2, we demonstrated that the over-expression of CEA/CEACAM6 on the surface of distinct cell lines inhibit anoikis. The next logical step was to search for the molecular mechanism of such inhibition. To tackle this problem, we have employed two distinct approaches:

- To determine the structural features of CEA required for the inhibition of anoikis. This work is still ongoing (see Appendix-A).
- To test whether CEA interferes with integrin functions. Integrins are the main cellular receptors for ECM components and play a crucial role in the regulation of anoikis.

In the next chapter, we present evidence of a molecular mechanism involving integrins that could be responsible for CEA inhibitory effects on anoikis and cell differentiation.

Chapter 3

Human Carcinoembryonic Antigen Inhibits Cell Differentiation and Apoptosis by Perturbing the Function of the $\alpha_5\beta_1$ Integrin Receptor

SUMMARY

Human Carcinoembryonic Antigen (CEA) and CEACAM6 (NCA) are intercellular adhesion molecules that are upregulated in a wide variety of human cancers, including colon, breast and lung. When over-expressed by cDNA transfection in various cell lines *in vitro*, CEA and CEACAM6 function as pan-inhibitors of cell differentiation and anoikis, thereby increasing tumorigenicity. A molecular mechanism that could explain these pleiotrophic effects was sought. The ectopic expression of CEA/CEACAM6 on the surface of transfected rat L6 myoblasts and the deregulated over-expression of these molecules on human Caco-2 colonocytes in both cases, caused aberrant cell adhesion to the extracellular matrix (ECM) due to an activation of the $\alpha_5\beta_1$ integrin receptor. As a result, the cells entrap fibronectin on their surface to a greater extent and become encased in a "cocoon" of polymerized fibronectin, thus rendering them less able to bind to their ECM. Evidence implicating the $\alpha_5\beta_1$ integrin includes a demonstration that myogenic differentiation of CEA- or CEACAM6-transfected L6 cells and anoikis of both the L6 transfectants and CEA/CEACAM6-transfected Caco-2 cells could be rescued by adding mAbs against fibronectin (myogenic differentiation and anoikis) and the α_5 integrin subunit (anoikis). These findings suggest that CEA/CEACAM6-induced perturbation of the $\alpha_5\beta_1$ integrin-fibronectin interaction is responsible for their inhibitory effects on cell differentiation and anoikis.

INTRODUCTION

Human Carcinoembryonic Antigen, the prototypic member of a family of cell surface glycoproteins, is aberrantly expressed in a wide variety of human cancers at many sites, including colon, breast, lung, pancreas, cervix, stomach and skin (Chevinsky, 1991; Hammarström et al., 1998; Ilantzis et al., 1997); this has led to its development as an important tumor marker of wide clinical application in the management of human malignancies. CEA family members function *in vitro*, at least, as intercellular adhesion molecules (Benchimol et al., 1989; Öbrink, 1997; Oikawa et al., 1989; Stanners and Fuks, 1998). Family member CEACAM6 (formerly NCA) is also upregulated in many human tumors, whereas CEACAM1 (formerly BGP), is either downregulated or shows much less change in expression (Ilantzis et al., 1997; Nollau et al., 1997; Rosenberg et al., 1993; Stanners, 1998). Structurally, all CEA family members fall into the Immunoglobulin Superfamily (IgSF) with CEACAM1 differing from CEA and CEACAM6 mainly in its mode of membrane linkage, i.e., transmembrane for CEACAM1 vs glycosylphosphatidylinositol (GPI) anchorage for CEA/CEACAM6. The homotypic and heterotypic interactions required for cell adhesion occurs between their extracellular domains which, for CEA, CEACAM6 and CEACAM1-4L (the most common splice variant of CEACAM1), consist of an N-terminal V-type immunoglobulin (Ig)-like domain followed by six, two, and three C2-type Ig-like domains, respectively (Buck, 1992).

We have previously demonstrated, using cell lines with differentiation potential, that CEA and CEACAM6 function as general inhibitors of cell differentiation (Stanners, 1998). The forced expression by cDNA transfection of CEA and/or CEACAM6, but not CEACAM1, in rat L6 and mouse C2C12 myoblasts, P19 teratocarcinoma cells, C3H10T1/2 and 3T3-L1 fibroblasts, and Caco-2 human colorectal adenocarcinoma cells

markedly inhibited terminal myogenic (Eidelman et al., 1993; Screaton et al., 1997), neurogenic (Malette B. & C.P. Stanners, submitted for publication), adipogenic (DeMarte L. & C.P. Stanners, unpublished observations), and colonic differentiation (Ilantzis C., L. DeMarte, R. Screaton, C.P. Stanners, submitted for publication), respectively, of these cell lines *in vitro*. Human Caco-2 colonocytes express relatively low levels of endogenous CEA and CEACAM6 on their apical membranes as they differentiate into a “palisade” monolayer of colonocytes *in vitro* (Hauck and Stanners, 1991), resembling the polarized pattern of expression of CEA/CEACAM6 on the apical membrane of normal differentiated colonocytes. In contrast, singly or doubly transfected Caco-2 cells expressing relatively high levels of CEA/CEACAM6 over their entire surfaces throughout the growth cycle, resembling the pattern and levels of expression of CEA/CEACAM6 seen in human colorectal carcinomas (Ilantzis et al., 1997), were unable to differentiate on collagen-coated filters and were arranged in an unpolarized, multi-layered configuration (Ilantzis C., L. DeMarte, R. Screaton and C.P. Stanners, submitted for publication). Consistent with these *in vitro* results, cytofluorometric analysis of purified colonocytes from freshly excised human colonic carcinomas showed a negative correlation between the cell surface levels of CEA and CEACAM6 and the degree of differentiation of the tumors (Ilantzis et al., 1997).

The pan-inhibition of differentiation and distortion of cell and tissue architecture by CEA/CEACAM6 might be expected to be tumorigenic. In fact, ectopic expression of CEA/CEACAM6 on the surface of L6 rat myoblasts (Screaton et al., 1997) and deregulated over-expression of these molecules on the surface of Caco-2 human colorectal cancer cells (Ilantzis C., L. DeMarte, R. Screaton and C.P. Stanners, submitted for publication) increased tumorigenicity when these cells were injected into nude mice. CEACAM1 over-expression, on the other hand, was not tumorigenic. In fact, its murine homologues inhibited tumor formation by transfected colonic or prostatic cell lines

(Hsieh et al., 1995; Kunath et al., 1995; Turbide et al., 1997). CEACAM1 has therefore been suggested to function *in vivo* as a tumor suppressor (Kleinerman et al., 1995a).

As mentioned above, CEA and CEACAM6 are bound to the plasma membrane by a (GPI) anchor (Hefta et al., 1990; Hefta et al., 1988; Takami et al., 1988), a structural feature in the CEA family which, interestingly, is found exclusively in the primate radiation (Naghibalhossaini F. and C.P. Stanners, submitted for publication) whereas CEACAM1 is anchored to the cell surface by a transmembrane domain followed by a cytoplasmic tail (Hinoda et al., 1988). Recent results indicate that it is the mode of membrane anchorage that determines the tumorigenic properties of CEA family members in that these can be switched between CEA and CEACAM1 by exchanging their anchor-determining carboxy-terminal domains (Screaton, R.A., L. DeMarte and C.P. Stanners, submitted for publication).

The inhibitory effects of CEA/CEACAM6 expression on cell differentiation have been demonstrated with several examples of both epithelial and mesenchymal cell lines. This led us to search for a molecular process common to and necessary for all these molecularly disparate differentiation programs in different cell types. One such process is the regulation of cell adhesion to the ECM. Cell-matrix adhesion plays a crucial role in the regulation of cell differentiation in pluricellular organisms (Adams and Watt, 1993; Aharoni et al., 1997; Lukashev and Werb, 1998). The ECM provides environmental cues that guide epithelial and mesenchymal cells into specific and distinct differentiation programs during embryogenesis and in many adult tissues (Adams and Watt, 1993; Lukashev and Werb, 1998). The myogenic (von der Mark and Ocalan, 1989), colonic (Basson et al., 1996), neurogenic (Luckenbill-Edds, 1997) and adipogenic (Castro-Munozledo et al., 1987) differentiation programs inhibited by CEA expression *in vitro*, and many others, are highly dependent upon cell-ECM interactions. Cell-ECM adhesion

is regulated by multiple cellular integrin receptors for ECM components, which are transmembrane heterodimeric proteins composed of α and β subunits (Hynes, 1992).

CEA/CEACAM6 expression on the surface of L6 myoblasts and Caco-2 colonocytes not only inhibits cell differentiation but also induces resistance to anoikis *in vitro* (Ordoñez C., R. Screaton, C. Ilantzis and C.P. Stanners, submitted for publication), an apoptotic program triggered in many different types of cells when they lose contact with the ECM (Frisch and Francis, 1994; Frisch and Ruoslahti, 1997; Meredith et al., 1993; Ruoslahti and Reed, 1994). Inhibition of apoptosis has also generally been assumed to contribute to malignant transformation. We show here that CEA and CEACAM6, but not CEACAM1, expression in L6 rat myoblasts and over-expression in Caco-2 human colonic epithelial cells, modifies cell-ECM adhesion by inducing the functional activation of the $\alpha_5\beta_1$ integrin receptor without affecting its cell surface expression level. Due to this activation, cell adhesion to fibronectin-coated surfaces was increased in cultures of CEA- or CEACAM6-transfected myoblasts in the exponential phase of growth, but decreased in the stationary growth phase when differentiation normally occurs. We present evidence that this change during the growth cycle is due to the more tenacious binding of the activated $\alpha_5\beta_1$ integrin receptors to polymerized fibronectin on the plasma membrane, thus causing an increase in fibronectin matrix assembly and a progressive decrease in cell adhesion to fibronectin-coated surfaces. We therefore contend that the mechanism of the general inhibition of cell differentiation and anoikis by CEA and CEACAM6 is through regulation of cell-matrix interactions via the functional activity of specific integrins.

MATERIAL AND METHODS

Reagents and Antibodies

PolyHEMA (Poly 2-hydroxyethylmethacrylate) was obtained from Aldrich Chemicals, Milwaukee, WI. NHS-Biotin (N-Hydroxysuccinimide-Biotin) was obtained from Pierce, Rockford, IL. Triton X-100 (T-8787) was purchased from SIGMA, St Louis, MO. Insulin (Product number I-6634, SIGMA, St. Louis, MI) was dissolved in Tris-HCl pH 8.0 at 10 mg/ml. Rat fibronectin, rat laminin, and RGD-containing linear GRGDSP and control GRGESP peptides were obtained from GIBCO BRL, Burlington, ON. Alamar blue was purchased from BioSource International, Camarillo, CA. BSA (bovine serum albumin, fraction V) used to block exposed plastic surfaces in adhesion assays, was from SIGMA, St. Louis, MI. Monoclonal antibody (mAb) BF-G6 (Regeneron Pharmaceuticals, NY) recognizes the heavy chain of myosin; anti-fibronectin mAb clone-10 (Transduction Laboratories, Mississauga, ON) recognizes human fibronectin and cross-reacts with rat fibronectin; mAb 36.3 was a generous gift of Dr. A. Fuks (McGill Cancer Centre, Montreal, PQ, Canada) and recognizes rat histocompatibility antigen 36.3 (Engel et al., 1982). The mAbs used in cell adhesion and apoptotic assays were HM α 5-1, Ha1/29, Ha2/5, IIA1 and F11 from Pharmingen, Mississauga, ON. MAb HM α 5-1 recognizes the rat α_5 integrin subunit and inhibits cell adhesion to fibronectin; mAbs Ha1/29, Ha2/5, IIA1 and F11 recognize the rat α_2 integrin subunit, the rat β_1 integrin subunit, the human α_5 integrin subunit and the mouse β_3 integrin subunit, respectively. Abs used in FACS analysis to check for stability of CEA/CEACAM6 expression on the surface of transfected cells were rabbit and goat polyclonal antibodies against CEA. These polyclonal antibodies crossreact with CEACAM1 and CEACAM6. MAb D14.6.43 (E-Z-EM Inc., NY), 9A6FR (gift from Dr. F. Grunert, Univ. of Freiburg, Germany) and TEC-11 (gift from P. Draber, Institute of Molecular Genetics, Prague, Czech Republic)

specifically recognize CEA, CEACAM6 and CEACAM1, respectively, and do not cross-react with other CEA family members.

Cell Lines

L6 rat myoblasts (Yaffe, 1968), Caco-2 human colorectal carcinoma cells (obtained from the American Type Culture Collection ATCC, Rockville, MD), CHO-derived LR-73 (Pollard and Stanners, 1979) and TR-3 cells (a more transformed revertant of LR-73), and Madin Darby Canine Kidney (MDCK) (also from ATCC) epithelial cells were grown as monolayer cultures in DMEM (L6, MDCK) or α -MEM (Caco-2, LR-73, TR-3) containing 10% fetal bovine serum (growth medium, GM; GIBCO BRL, Gaithersburg, MD) supplemented with 100 μ g/ml streptomycin and 100 U/ml penicillin (GIBCO BRL) at 37°C in a humidified atmosphere with 5% CO₂. All cell lines were subcultured before confluence and seeded at a density of 1×10^4 cells/cm². Studies with early (2 days, about 50% confluent) and late (4 days, 100% confluent) cultures of L6 rat myoblasts were performed by seeding these cells at a density of 1×10^4 cells/cm² and incubating for different times in GM before use in experiments.

cDNA Transfections and Infections

Our transfection procedures and transfected cell lines used in this study have been previously described (Eidelman et al., 1993; Screaton et al., 1997). Briefly, stable transfectants of cell lines L6, LR-73, TR-3 and Caco-2 were obtained by the calcium phosphate precipitation method using the p91023B expression vector (courtesy of R. Kaufman, Genetics Institute, Boston) containing full length cDNAs encoding cell adhesion proteins: CEA, CEACAM6, CEACAM1-4L (formerly splice variant BGPa), CEA deletion mutant Δ NCEA (lacking the last 75 amino acids of the N domain), human NCAM-125 [GPI-linked NCAM splice variant with muscle specific domain (Dickson et

al., 1987)] and pSV2neo plasmid as a dominant selectable marker (L6, LR-73, TR-3). Caco-2 single transfectants were obtained using the Zn²⁺-inducible episomal expression vector pML1 containing the mouse metallothionein promoter (mMT1) and the hygromycin-B resistance gene (Lukashev et al., 1994), alone (Caco-Hygro population), and containing full length cDNAs encoding CEA (pML1-CEA), CEACAM1 (pML1-CEACAM1) and a cDNA containing the entire coding region of CEACAM6 (pML1-CEACAM6). CEA/CEACAM6 over-expressing doubly transfected Caco-2 cells were obtained using equimolar amounts of both pML1-CEA and pML1-CEACAM6.

Pooled clones of stably transfected cells were selected with 400 µg/ml of Neomycin (G418) (L6, LR-73, TR-3) or 200 µg/ml of Hygromycin-B (Caco-2). L6 myoblasts expressing high surface levels of the proteins encoded by the transfected cDNAs were selected by FACS using specific monoclonal antibodies (see reagents and antibodies). The transfectant populations (L6 and LR-73) were enriched for more stably expressing cells by culturing without G418 for ~20 doublings, followed by FACS re-selection for high expressors (see expression levels in Table I). The polyclonal composition of stably transfected L6-CEA cells was shown by multiple bands obtained by Southern blot analysis of their genomic DNA (Screaton et al., 1997). A second CEA-expressing independent pooled population of transfected L6 myoblasts (L6-CEA-2) was obtained using the same transfection procedures as for the L6-CEA pooled population.

Hundreds of clones of Caco-2 transfectants expressing both CEA and CEACAM6 were pooled together constituting a stable total population that expressed 20 fold higher levels of CEA and CEACAM6 (see Table I) than Caco-Hygro or untransfected Caco-2 parental cells. Although G418 (L6) and Hygromycin-B (Caco-2) were removed from the culture media for all functional assays, no loss of cell surface expression of the transfected cDNAs was observed (data not shown).

L6 and MDCK cells in the exponential phase of growth were infected with replication-defective recombinant retrovirus containing either pBabe(human bcl-2)puro [L6] (Screaton et al., 1997) or pLXSN(CEA cDNA)neo [MDCK] or the vector alone [MDCK-neo] as a control. Stably transfected MDCK cells were selected with 400 µg/ml of G418 and cells expressing high levels of CEA on the cell surface were selected by FACS using anti-CEA mAb B18 (see Table I).

Pooled populations of transfectant clones were used in this study to exclude clonal variation as a factor determining phenotypic properties of the transfected cells. The only exception is TR-3-CEA4, which is a clone. All cultures of stably transfected L6, Caco-2, LR-73, TR-3 and MDCK cells used in experiments were obtained from early passages of frozen stocks.

Table I

CEA family members and NCAM-125 cell surface expression levels in various transfectant populations (effects on cell differentiation and anoikis are indicated)

Cell line	FACS mean value*	Cell differentiation [‡]	Anoikis [§]		
L6	3	normal	normal		
L6-CEA	186	blocked	inhibited		
L6-CEACAM6	205	blocked	inhibited		
L6-CEACAM1	56	normal	normal		
L6-ΔNCEA	235	normal	normal		
L6-NCAM-125	300	normal	normal		
MDCK-neo	3.2	n.d.	normal		
MDCK-CEA	247	n.d.	inhibited		
LR-neo	2.6	n.a. [¶]	n.a.		
LR-CEA	474	n.a.	n.a.		
TR-neo	1.8	n.a.	n.a.		
TR-CEA	338	n.a.	n.a.		
	**	<u>CEA</u>	<u>CC6</u>	<u>CC1</u>	
Caco-2-Hygro	13	98	26	normal	normal
Caco-2-CEA/CC6	290	2124	n.d.	inhibited	inhibited
Caco-2-CEACAM6	n.d.	1628	n.d.	n.d.	inhibited
Caco-2-CEACAM1	n.d.	n.d.	93	n.d.	normal
Caco-2-ΔNCEA	823	n.d.	n.d.	n.d.	normal

- * FACS mean values in arbitrary units were obtained using anti-CEA mAb J22 (L6 parental and transfected myoblasts, and LR-73 transfected cells), mAb ERIC-1 (L6-NCAM), anti-CEA mAb B18 (MDCK transfected cells), rabbit anti CEA polyclonal Ab (TR-3 transfected cells). Levels of CEA family members in Caco-2 transfected cells, cultured in the presence of 0.1 mM ZnSO₄, were measured with mAbs D-14 (CEA), 9A6 (CEACAM6) and A-20 (CEACAM1)
- ‡ Quantitation of CEA/CEACAM6 inhibitory effects on cell differentiation has been reported (Eidelman et al., 1993; Ilantzis C., L. DeMarte, R. Screaton, C.P. Stanners, submitted for publication)
- § Quantitation of CEA/CEACAM6 inhibitory effects on anoikis has been reported (Ordoñez C., R. Screaton, C. Ilantzis, C.P. Stanners, submitted for publication)
- || Not done
- ¶ Not applicable
- ** CC6=CEACAM6, CC1=CEACAM1

Purification of ECM and Cell Adhesion Assays

The ECM secreted by L6 and Caco-2 parental cell lines was extracted as described previously (Knudsen et al., 1987). Briefly, 1×10^4 cells/cm² were seeded in 24 well tissue culture plates and cultured for 7 days. Confluent cultures were subsequently washed twice with cold PBS, incubated for 10 minutes with 0.5% Triton X-100 in PBS on ice to remove cell membranes, and another 10 minutes with 0.25mM ammonium acetate to remove remaining nuclei and cytoskeleton. The remaining ECM was washed twice with ice cold PBS and incubated overnight with a solution of 1% BSA at 4°C to cover exposed plastic surfaces. The ECM was washed once with serum free DMEM before use in the adhesion assays. In order to measure cell-ECM adhesion, L6 and Caco-2 transfected cell lines were seeded at 1×10^4 cells/cm² in GM and incubated for a period of 4 days to reach confluency, collected by light trypsinization, incubated at 37°C in GM for 30-60 minutes, washed three times with serum free DMEM, and suspended at a concentration of 4×10^5 cells/ml in serum free DMEM. 0.25 ml/well of each cellular suspension was added to purified ECM. In the case of parental and transfected L6 rat myoblasts, both early and late cultures (defined above) were prepared for cell adhesion. Cells were allowed to adhere to the ECM-coated wells for one hour, after which the wells were washed twice with PBS to remove unattached cells. The remaining attached cells were removed with trypsin and their number determined using a particle counter (Coulter Electronics Inc., Hialeah, FL).

Adhesion to fibronectin and laminin was measured using 24 well plates coated with 10 µg/ml of fibronectin or laminin (0.25 ml/well) by overnight incubation at 4°C, followed by incubation for a minimum of 8 hours at 4°C with 1% BSA, then washing with serum-free DMEM. The number of L6 cells that adhered to fibronectin or laminin-coated wells was determined as described above for adhesion to purified ECM.

Myogenic Differentiation Assay

L6 myogenic differentiation was assessed as described previously (Eidelman et al., 1993; Screaton et al., 1997). Briefly, L6 rat myoblasts were seeded at 0.7×10^3 cells/cm² in GM. After 3 days incubation, the culture medium was changed to DMEM supplemented with 2% horse serum, denoted differentiation medium (DM). On day 7, myotube formation was estimated morphologically by phase contrast microscopy, and biochemically by immunochemical staining of the myosin heavy chain using monoclonal antibody, BF-G6. Insulin (SIGMA, St. Louis, MO) was added at 10 µg/ml in DM at one dose per day for 3 days. Anti-fibronectin and anti-MHC (36.3) antibodies were added at 0.25 µg/ml in DM every other day for 7 days. The fusion index (number of nuclei within myotubes containing three or more nuclei divided by the total number of nuclei counted) was determined after fixation of the cells with 2.5% glutaraldehyde and staining with hematoxylin. At least 500 nuclei in five randomly selected microscopic fields were scored.

Cell Detachment Assays

Detachment of cells from their substratum with RGD-containing and control peptides was carried out as described previously (Hayman et al., 1985). In this case, L6 rat myoblasts, Caco-2 human colonic epithelial cells and MDCK cells were grown as monolayers in GM for 12 hours, washed three times with serum-free DMEM, and treated for 3 (L6) and 7 (Caco-2, MDCK) hours with the peptides at 0.2 mg/ml (L6) or 1 mg/ml (Caco-2, MDCK) in serum-free DMEM. Detachment of the cells was assessed by phase contrast microscopy every 30 min.

Indirect Immunofluorescence and Matrix Assembly Assays

L6 myoblasts were cultured in either GM or DM (as described above) in multiwell chamber slides (Nunc; Nunc, Inc., Naperville, IL). Cells were fixed in 1:1 methanol/acetone at -20°C for 10 min. Slides were incubated with mAb BF-G6 for 30 min at room temperature or overnight at 4°C with anti-fibronectin mAb clone-10, followed by FITC-conjugated goat anti-mouse secondary antibody for another 30 min at room temperature. Control slides were stained with secondary antibody only. Fibronectin matrix assembly was assessed by immunofluorescence as described above or by using added biotinylated rat fibronectin. Rat fibronectin (GIBCO) was labeled with NHS-biotin following kit instructions (Pierce, Rockford, IL). L6 monolayers were incubated with biotinylated rat fibronectin for 24 hours at 37°C . Cells were fixed with 4% paraformaldehyde, washed with PBS and incubated with FITC-streptavidin conjugate (SIGMA) for 10 minutes in the dark. Stained cell monolayers were observed using a Nikon Eclipse E800 epifluorescence microscope and representative fields were photographed using a Nikon FDX-35 camera with fixed exposure times of 6 seconds. Matrix assembly was quantitated by counting the numbers of fibrils per microscopic field, scoring 10 randomly selected microscopic fields per sample.

Cell Adhesion to Antibody-Coated Substrates

Adhesion of L6 myoblasts to antibody-coated substrates was measured as previously described (Chen et al., 1997a) with some modifications. 96 well plates were first incubated with secondary antibodies (25 $\mu\text{g}/\text{ml}$ in PBS), followed by 1% BSA and then primary antibodies (3 $\mu\text{g}/\text{ml}$ in PBS) for 24 hrs at 4°C . Antibody-coated wells were washed once with serum-free DMEM before use. L6 cells were allowed to adhere to the mAb-coated surfaces for 15, 30, 45, and 90 min at 37°C . Unattached cells were removed by washing with PBS and the number of attached cells determined by alamar blue

(BioSource International, Camarillo, CA) staining following instructions from the manufacturer. Optical density was measured at 595nm and 570nm using a Model 550 Microplate Reader (Bio-Rad, CA, USA).

Apoptotic Assays

In order to induce higher expression of CEA/CEACAM6, Caco-2 transfected cells were cultured in GM supplemented with 0.1 mM ZnSO₄ for 24 hours prior to the experiment. To measure anoikis of L6 and Zn²⁺ induced Caco-2 (control and transfected cells), 0.2x10⁶ cells/ml of each cell line were suspended in polyHEMA coated 6-well tissue culture plates for a period of 12 to 72 hours at 37°C in the presence (GM) or absence (serum free DMEM) of growth factors. The percentage of apoptotic cells was estimated by staining the nuclei with DAPI or using the TUNEL assay (ONCOR kit), following the manufacturer's instructions. Briefly, to stain with DAPI the cells were fixed on ProbeOn™ Plus microscope slides (FisherBiotech, USA) with 4% paraformaldehyde for 20 minutes, washed, permeabilized for 5 minutes with 0.1% Triton X-100, and stained with 10 µg/ml of DAPI in PBS. Cells with fragmented (DAPI) or stained (TUNEL) nuclei were scored as apoptotic cells. HMα5-1 (anti-rat α5 integrin subunit), IIA1 (anti-human α5 integrin subunit), Ha1/29 (anti-rat α2 integrin subunit), clone-10 (anti-rat fibronectin) and 36.3 (anti-MHC) mAbs were added to the suspended cells in the polyHEMA-coated wells at a final concentration of 1 µg/ml. The apoptotic index was calculated by scoring no less than 1000 cells. All observations were reproduced at least twice by independent experiments.

RESULTS

Deregulated over-expression of CEA and/or CEACAM6 in many different types of cells markedly inhibits differentiation and anoikis *in vitro* (see Introduction and Table I). A cell/molecular process necessary for many differentiation programs and anoikis is cell-ECM adhesion. We will address two main questions: firstly, does CEA/CEACAM6 over-expression modify cell-ECM adhesion *in vitro*? And secondly, if so, is this the cause of the inhibitory effects of CEA/CEACAM6 expression on cell differentiation and anoikis?

CEA and CEACAM6 Expression Decreases Cell-ECM Adhesion

In order to investigate L6 myoblast cell-ECM adhesion *in vitro*, a cell binding assay was performed using the ECM synthesized by L6 parental myoblasts (L6-ECM). CEA- and CEACAM6-transfected L6 cells from confluent cultures reproducibly bound about 35% and 65% less to L6-ECM, respectively, than L6 parental cells. This decrease in cell-ECM adhesion was not observed with L6 myoblasts transfected with CEACAM1 or GPI-linked NCAM-125 (Fig 1A). Both CEACAM1 and GPI-linked NCAM-125, like CEA/CEACAM6, function *in vitro* as intercellular adhesion molecules but, unlike CEA/CEACAM6, do not block myogenic differentiation when ectopically expressed in L6 myoblasts (Rojas et al., 1996; Screaton R., L. DeMarte, C.P. Stanners, submitted for publication). The failure of the GPI-anchored isoform of NCAM to modify cell-ECM adhesion and inhibit myogenic differentiation of L6 myoblasts indicates that the effects of CEA/CEACAM6 are specific and not due to adventitious effects of GPI-anchorage *per se*. A deletion mutant of CEA (Δ N-CEA) that lacks one of the binding domains necessary for mediating intercellular adhesion (Zhou et al., 1993) and that does not block myogenic differentiation (Eidelman et al., 1993) was used as a further control. L6 cells transfected with this mutant bound L6-ECM as well as L6 parental cells (Fig 1A).

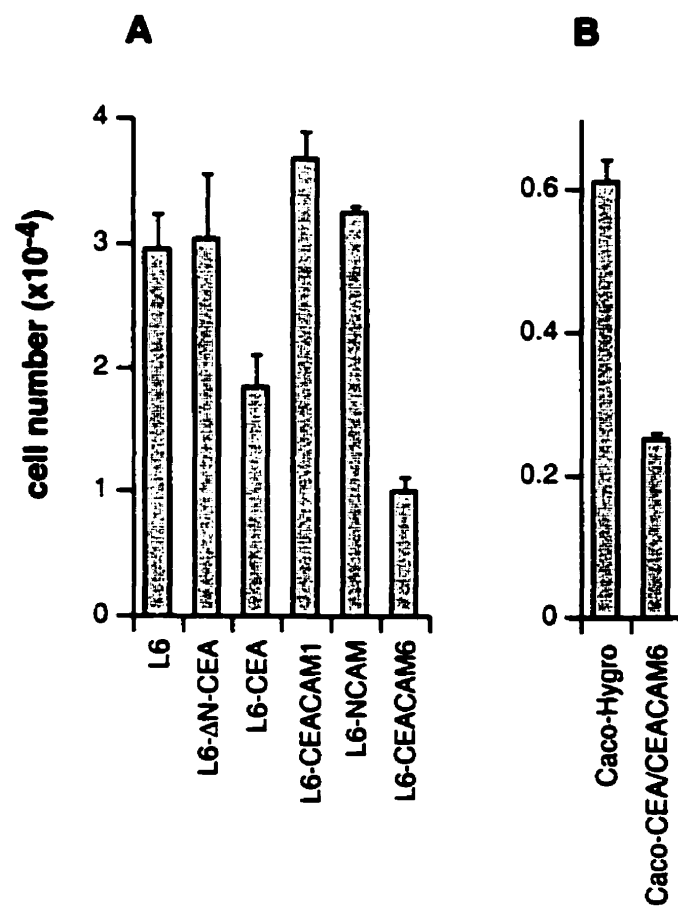
The decrease of cell-ECM adhesion observed for CEA/CEACAM6 transfected L6 cells was confirmed with ECM extracted not only from parental cells but also from CEA, Δ N-CEA, CEACAM1, and NCAM-125 transfected L6 cells (data not shown). This result suggests that the diminished cell-ECM adhesion was more likely due to interference with the function of an integrin receptor than to modification of the composition of their secreted ECM, although the latter was not directly investigated.

To validate the observations obtained with the L6 system, we investigated whether CEA/CEACAM6 also inhibits cell-ECM adhesion when over-expressed on the surface of Caco-2 human colonic epithelial cells. CEA/CEACAM6 doubly transfected Caco-2 cells from confluent cultures also bound Caco-ECM less than Caco-Hygro control transfected cells (Fig 1B). These results lead to the hypothesis that aberrant cell-ECM adhesion could be responsible for the CEA-mediated block of differentiation.

Early sub-confluent cultures of L6 myoblasts expressing CEA and CEACAM6 actually bound to extracted L6-ECM more than L6 parental cells (data not shown). We present evidence below that both the CEA/CEACAM6-mediated decrease in ECM adhesion by cells from late cultures observed here and the increase for early cultures is due to activation of an integrin receptor.

Figure 1

CEA and CEACAM6 over-expression on the cell surface of L6 rat myoblasts and Caco-2 human colonic epithelial cells inhibited cell-ECM adhesion. (A) L6 parental and transfected cells. (B) Caco-2 transfected cells. These experiments have been repeated 9 times (L6) and 3 times (Caco-2), with similar results. The averages of three independent experiments with standard deviations are shown.



Insulin-Mediated Rescue of Myogenic Differentiation Correlates with Modulation of Cell-ECM Adhesion

Insulin is a well known positive regulator of L6 myogenic differentiation (Pinset and Whalen, 1985). We were interested in determining first, whether the CEA/CEACAM6-block of L6 myogenic differentiation could be released by insulin treatment and second, if so, whether a correlation between such release and modulation of cell-ECM adhesion could be demonstrated.

L6 transfectants were induced to differentiate in DM containing 10 µg/ml insulin. After three days of insulin treatment, the fusion index of L6 parental cells was not significantly affected, although the morphology of the myotubes was different (Fig 2A). Myogenic differentiation of L6-CEACAM6 myoblasts was re-established (fusion index 0.39) by insulin treatment. L6-CEA cells, however, did not differentiate in the presence of insulin (Fig 2A), although sporadic formation of myotubes was observed (not shown).

To test whether the insulin effect on the myogenic differentiation of L6-CEACAM6 myoblasts was due to modulation of cell-ECM adhesion, the binding to L6-ECM was measured in the presence of insulin. The addition of insulin to suspended cells from late cultures increased the adhesion of L6-CEACAM6 myoblasts to L6-ECM by two fold, but did not affect cell-ECM adhesion of L6 parental or L6-CEA myoblasts (Fig 2B). These results indicated a direct correlation for insulin treated cultures between cell-ECM adhesion and myogenic differentiation.

Figure 2

Rescue of myogenic differentiation of CEACAM6-transfected myoblasts by insulin treatment. (A) L6 parental and transfected cells were cultured for three days in growth medium and then for another three days in DM containing 10 $\mu\text{g/ml}$ insulin. L6-CEACAM6 myoblasts treated with insulin underwent myogenic differentiation. The fusion index is indicated below each panel. This experiment was repeated three times with similar results. (B) Insulin treatment rescues cell-ECM adhesion of L6-CEACAM6 transfected myoblasts specifically. Suspended L6 parental, L6-CEA and L6-CEACAM6 transfectants were treated (indicated +i in the figure) or not (control) with 10 $\mu\text{g/ml}$ of insulin in serum-free DMEM for 30 minutes at 37°C, and then seeded over of purified L6-ECM. Attached cells were counted as described in Material and Methods (M&M). The average of two independent experiments and standard deviation are shown.

A

L6

CONTROL



0.76

INSULIN



0.81

L6-CEA



0.00



0.00

L6-CEACAM6

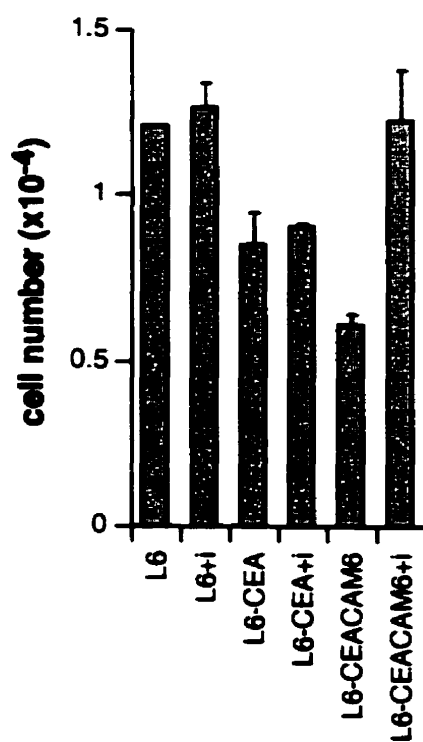


0.00



0.39

B



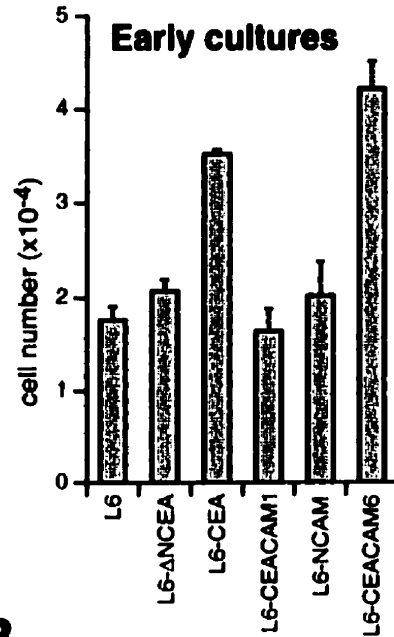
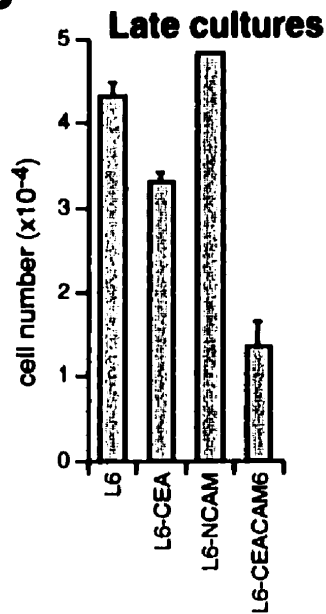
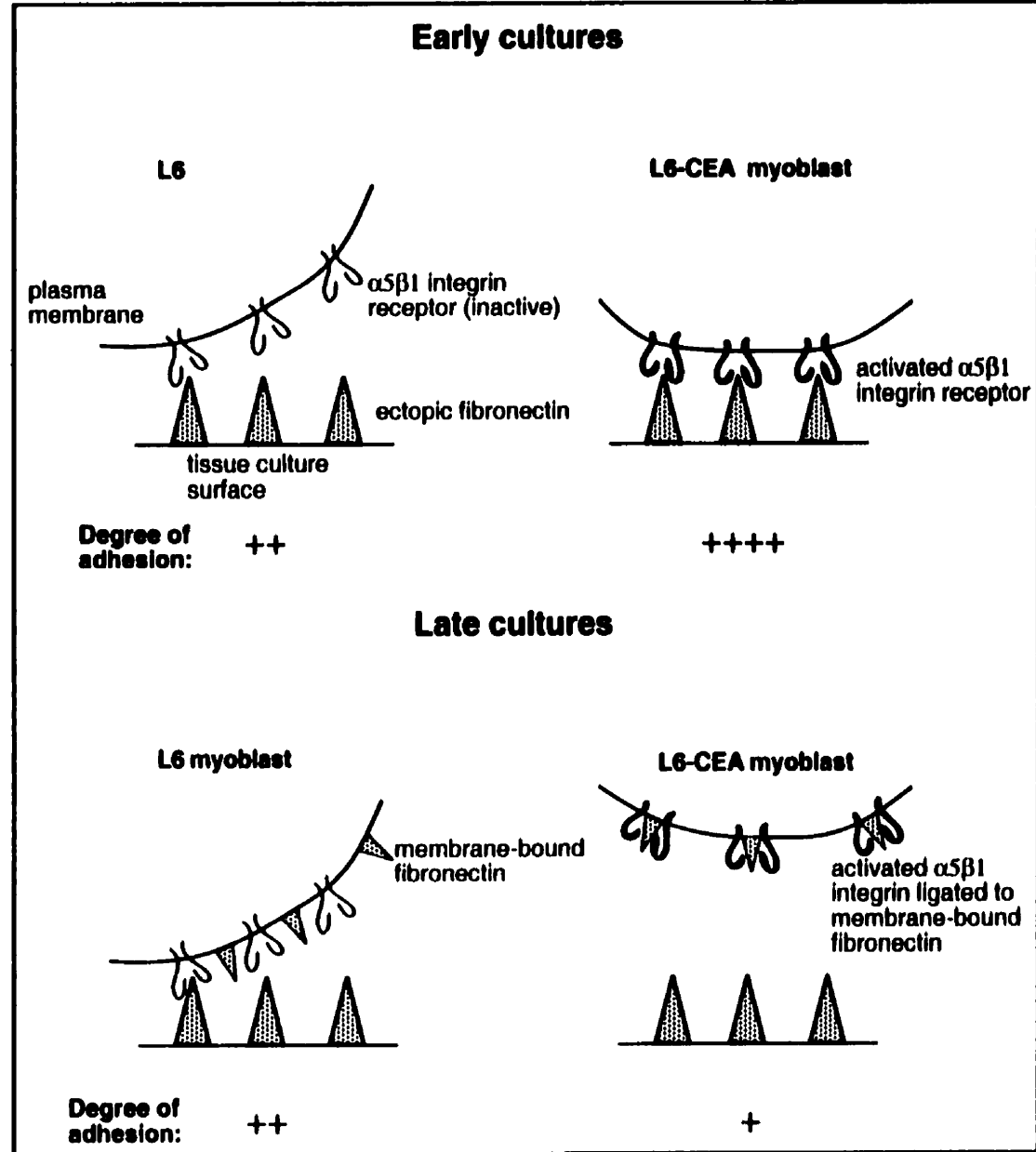
CEA and CEACAM6 Modify Cell Adhesion to Fibronectin

The effects of CEA/CEACAM6 expression on cell-ECM adhesion led us to investigate the role of these molecules in cell adhesion to specific ECM components. Cell adhesion to fibronectin was tested first, due to the key role of this ECM component in myogenic differentiation (von der Mark and Ocalan, 1989). Cells from both early and late cultures were used because endogenous fibronectin synthesis and fibronectin matrix assembly is known to be very low in early cultures of L6 myoblasts but to increase dramatically in late cultures (Podleski et al., 1979). We have confirmed this result by immunocytofluorescence and FACS analysis of cell surface fibronectin (data not shown).

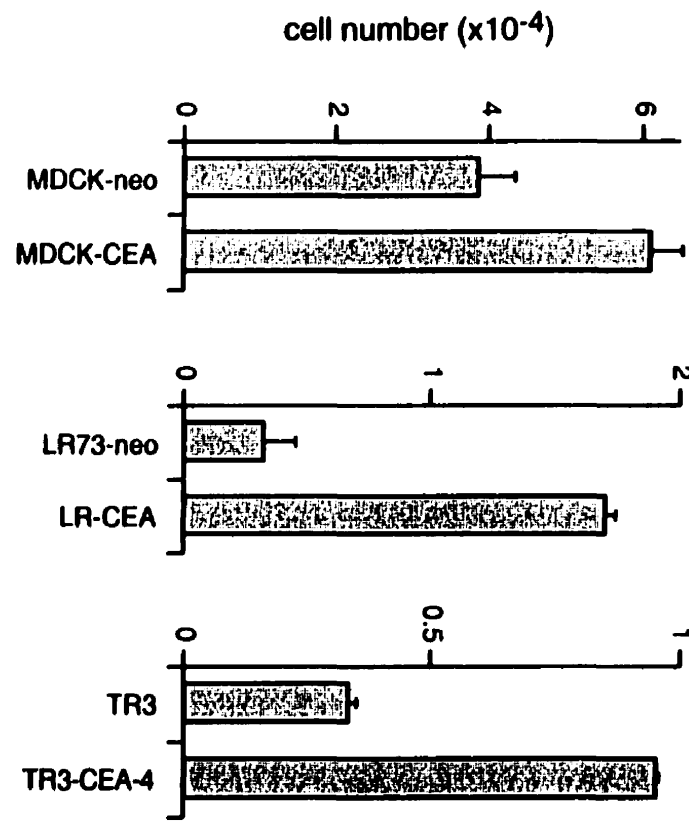
When cells from subconfluent early cultures were used in the fibronectin-adhesion assay, CEA- and CEACAM6-expressing L6 myoblasts bound more to fibronectin than L6 parental, Δ N-CEA, CEACAM1, or NCAM-125 control transfectant myoblasts (Fig 3A). This result suggests that CEA and CEACAM6 could induce an activation of an integrin receptor for fibronectin. In contrast, when late cultures of transfected L6 myoblasts were used, CEA- and CEACAM6-expressing myoblasts bound less than control myoblasts (Fig. 3B), as observed for binding to purified ECM (Fig. 1). This increase/decrease in cell adhesion to fibronectin could have been caused by the activation of an integrin receptor that progressively becomes engaged with membrane-deposited fibronectin, thus preventing cell adhesion to fibronectin-coated surfaces (Fig 3C). The reduction of cell adhesion to fibronectin-coated surfaces due to activation of the $\alpha_5\beta_1$ integrin receptor has been observed previously (Faull et al., 1993; Wu et al., 1998). The effects of CEA/CEACAM6 on cell adhesion to fibronectin seem to be specific as no changes in cell adhesion to laminin were observed in either early or late cultures of CEA transfectant L6 myoblasts (data not shown).

Figure 3

CEA/CEACAM6 expression on the surface of L6 myoblasts perturbs cell adhesion to fibronectin. Early (A) and late (B) cultures of L6-CEA and L6-CEACAM6 cells bound to fibronectin significantly more and less, respectively, than L6 parental and L6-NCAM-125 control cells. The averages of three independent experiments with standard deviations are indicated. (C) model to explain the behaviour of early and late cultures of CEA/CEACAM6 expressing L6 myoblasts in fibronectin-adhesion assays. Both early and late cultures of L6-CEA (or L6-CEACAM6, not shown) but not L6 parental myoblasts express an activated form of the $\alpha_5\beta_1$ integrin receptor on their cell surfaces. Early cultures of L6-CEA transfected cells, with activated $\alpha_5\beta_1$, bind ectopic fibronectin more efficiently than early cultures of L6 parental myoblasts. Both late cultures of L6-CEA and L6 parental myoblasts accumulate fibronectin on their cellular surfaces but only L6-CEA cells, expressing an activated form of the $\alpha_5\beta_1$, bind and polymerize membrane-deposited fibronectin, thereby interfering with the recognition of ectopic fibronectin coated on tissue culture surfaces. The plus signs below each panel indicate the respective degree of cell adhesion to fibronectin. (D) cells from early cultures of control and CEA transfectants (MDCK, LR-73 and TR-3) show increased binding to fibronectin-coated plates.

A**B****C**

D



These effects of CEA expression on cellular binding to fibronectin were extended to other cell types. As in the case of early cultures of L6 myoblasts, the binding of non-confluent cells from the canine kidney epithelial line, MDCK, from the CHO-derived quasi-normal line, LR-73, and from the more transformed sibling of LR-73, TR-3, was in every case increased by CEA expression (Fig 3D), demonstrating the generality of the effect.

An Anti-fibronectin Antibody Rescues Myogenic Differentiation of CEA- and CEACAM6-transfected L6 myoblasts

Fibronectin plays an important role in skeletal muscle differentiation (von der Mark and Ocalan, 1989). The synthesis of fibronectin by L6 rat myoblasts is required in early stages of their myogenic differentiation program (Podleski et al., 1979) but, paradoxically, fibronectin also plays an inhibitory role in late stages of myogenic differentiation of L6 myoblasts and other skeletal muscle cell lines (Podleski et al., 1979; von der Mark and Ocalan, 1989). CEA/CEACAM6-mediated inhibition of L6 differentiation could therefore be due to aberrant fibronectin binding through a perturbation of the function of cellular fibronectin receptors. To directly test this hypothesis, L6 transfectants were grown in DM in the presence of the anti-fibronectin mAb or an anti-rat MHC mAb as a control. Although myogenic differentiation of L6 parental cells was not affected by the presence of either mAb (Fig 4a,b,c), the anti-fibronectin mAb (Fig 4f,i), but not the control mAb (Fig 4e,h), produced remarkable myotube formation and myosin heavy chain expression in L6-CEA and L6-CEACAM6 cells, although myogenic differentiation was not restored to the same level as L6 parental cells. These observations are consistent with the suggestion that CEA/CEACAM6 effects on cell-fibronectin adhesion are the cause of the inhibition of myogenic differentiation.

Figure 4

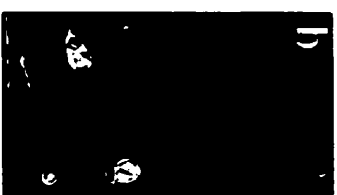
CEA/CEACAM6-induced block of L6 myogenic differentiation is rescued with the addition of the anti-fibronectin mAb clone-10. L6 transfected cells were cultured for 3 days in GM and then for another 7 days in DM plus 0.25 $\mu\text{g/ml}$ of anti-fibronectin or anti-rat MHC (36.3) monoclonal antibodies. The left half of each panel shows myoblast fusion by phase contrast microscopy (morphological differentiation) and the right half shows immunohistochemical staining with the anti-myosin antibody BF-G6 (biochemical differentiation). (a,d,g), untreated L6, L6-CEA and L6-CEACAM6 cells, (b,e,h) L6, L6-CEA and L6-CEACAM6 cells treated with antibody 36.3 (control); (c,f,i) L6, L6-CEA and L6-CEACAM6 cells treated with anti-fibronectin antibody. Significant formation of myotubes and positive staining for myosin by insulin treated L6-CEA and L6-CEACAM6 myoblasts can be seen (f,i). This experiment was repeated twice with identical results.

control

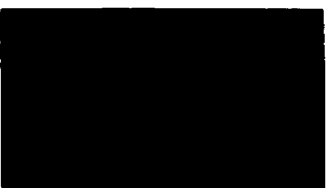
mAb 36.3

anti-Fn mAb

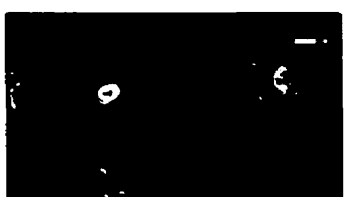
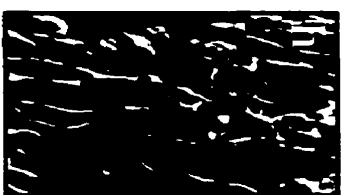
L6



L6-CEA



L6-CEACAM6



CEA and CEACAM6 Activate the $\alpha_5\beta_1$ receptor in Transfected L6 Myoblasts

The $\alpha_5\beta_1$ integrin receptor, the main cellular receptor for fibronectin, binds specifically to the RGD-motif present in fibronectin (Mould et al., 1997; Pierschbacher et al., 1985; Ruoslahti, 1996b), and plays a crucial role in the regulation of cell proliferation and differentiation of skeletal muscle cells (Sastry et al., 1996). Overexpression of the α_5 integrin subunit in primary cultures of quail skeletal muscle cells stimulated cell proliferation and inhibited differentiation of these cells (Sastry et al., 1996). Precedence therefore suggests that perturbation of the function of the $\alpha_5\beta_1$ integrin receptor, naturally implicated in the change in binding of CEA/CEACAM6-expressing cells to fibronectin, could underlie the block in L6 myogenic differentiation.

To investigate this hypothesis, early subconfluent cultures of CEA/CEACAM6 transfected cells and controls were allowed to attach to tissue culture plastic surfaces for 12 hours and then treated with the hexapeptide GRGDSP. CEA/CEACAM6-expressing L6, Caco-2 and MDCK transfected cells rounded up and detached first (within 30 min) from the substratum relative to control transfectants and, even after longer treatment, dramatic detachment was observed only in the CEA and/or CEACAM6 transfectants (Fig. 5A,B &C). The control peptide, GRGESP, had no such effect. One interpretation of these results is that CEA/CEACAM6 over-expressing cells have a higher affinity for the GRGDSP peptide, due perhaps to an increase in the level of expression or affinity of the $\alpha_5\beta_1$ integrin, or of some other RGD-specific integrin receptor.

Figure 5

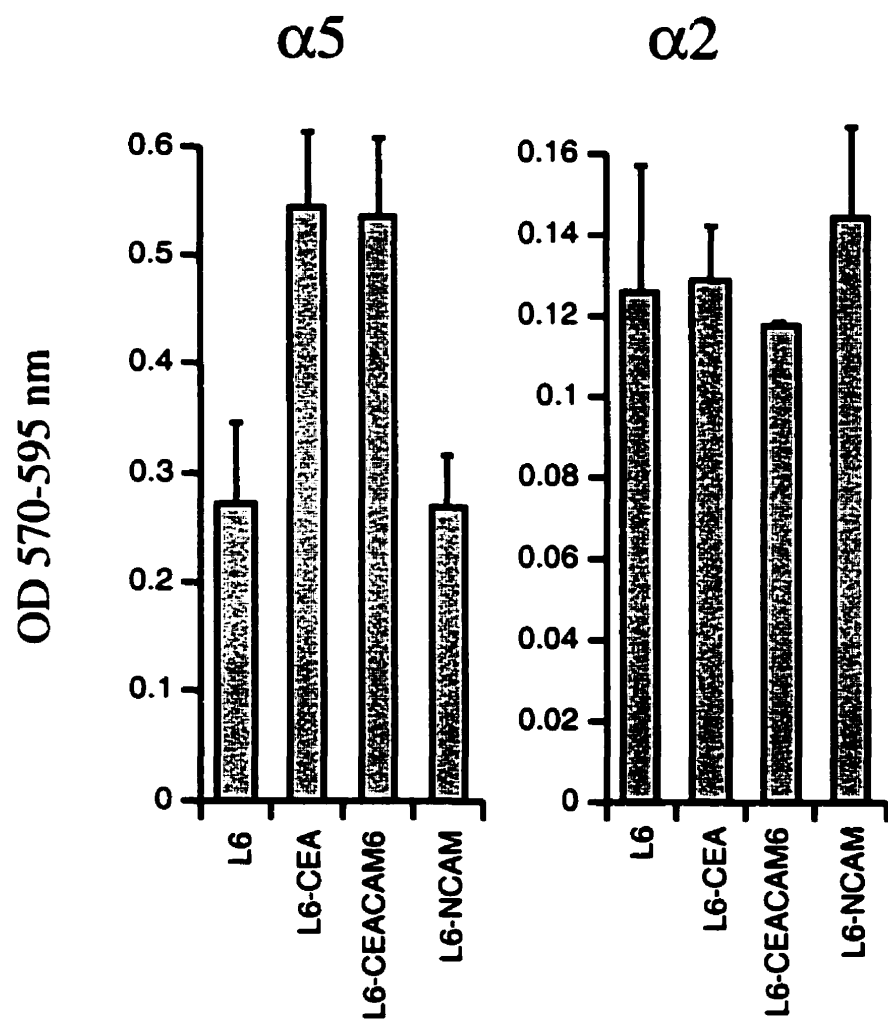
Increased sensitivity to treatment with RGD peptides of L6-CEA and L6-CEACAM6 myoblasts, MDCK-CEA and doubly transfected Caco-2-CEA/CEACAM6 colonic epithelial cells. (A) L6 parental, L6-CEA and L6-CEACAM6 cultures; (B) Caco-2 parental, vector-alone Caco-2-Hygro and Caco-2-CEA/CEACAM6 cultures; (C) MDCK parental and MDCK-CEA cultures. This experiment was repeated three times with identical results.

To directly test this hypothesis, we first measured cell surface levels of the $\alpha_5\beta_1$ integrin in CEA/CEACAM6 over-expressing cells using FACS analysis with mAbs that recognize the α_5 and β_1 subunits. The expression of CEA or CEACAM6 did not affect cell surface levels of either integrin subunit in either early or late cultures of L6 myoblasts (data not shown). Similarly, no significant differences in the level of cell surface expression of the α_v (related to α_5) (Yang et al., 1996) or α_2 integrin subunits could be detected (data not shown).

The functional status of the $\alpha_5\beta_1$ integrin receptor was next assessed using an assay that measures cellular adhesion to a specific anti- $\alpha_5\beta_1$ mAb (see Materials and Methods); in this assay, activation of the $\alpha_5\beta_1$ integrin will result in an increased binding. The expression of CEA or CEACAM6, but not NCAM-125, markedly increased the binding of early cultures of L6 myoblasts to immobilized anti- α_5 mAb but not to anti- α_2 mAb (Fig. 6). These results suggest that CEA/CEACAM6 expression on the cell surface induces a specific change in the configuration of the $\alpha_5\beta_1$ integrin receptor, thus predicting an increase in the avidity of CEA/CEACAM6-expressing L6 myoblasts for fibronectin, as observed (Fig. 3A).

Figure 6

CEA/CEACAM6 expression on the surface of L6 myoblasts increased cell adhesion to immobilized HM α 5-1 mAb (against the α_5 integrin subunit) but not to immobilized Ha1/29 mAb (against the α_2 integrin subunit). L6-CEA and L6-CEACAM6 cells bound to HM α 5-1 more than L6 parental and L6-NCAM-125 control cells. No significant cellular binding to substrates coated with secondary antibodies alone was observed (not shown). The graph represents the average of three independent experiments (standard deviation is indicated for each cell line).

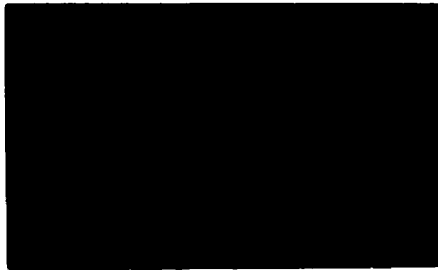
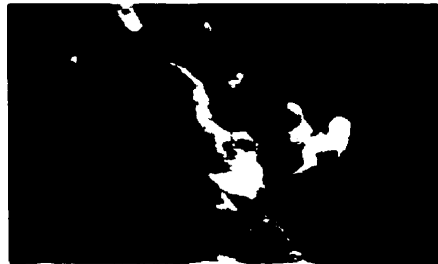
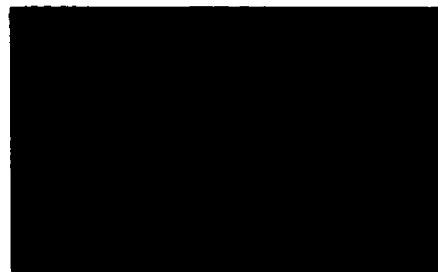
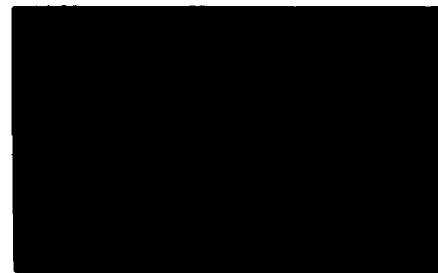
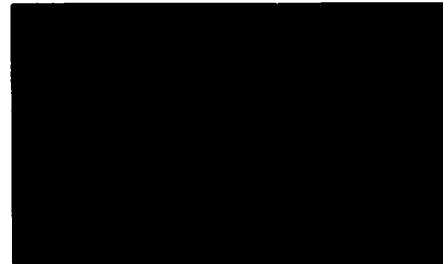
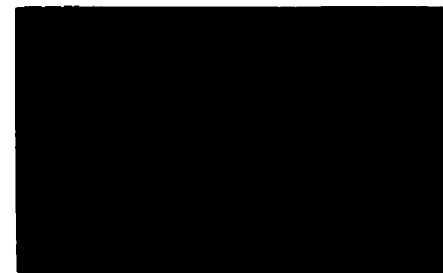
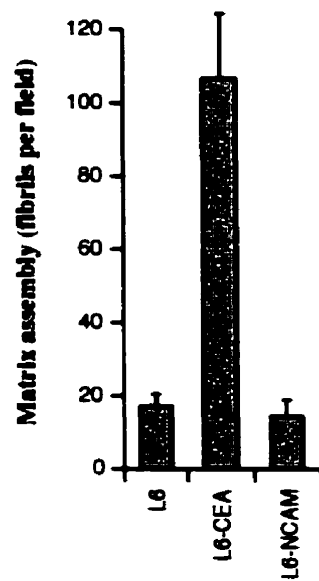


CEA/CEACAM6 Expression in L6 Myoblasts Increases Fibronectin Matrix Assembly

Human fibronectin can be detected *in vivo* in human blood as a soluble dimer or in the ECM of many tissues as an insoluble polymer (Christopher et al., 1997). It is the prevailing view that the latter is the biologically active form of fibronectin (Morla and Ruoslahti, 1992; Wu et al., 1998). Polymerization of fibronectin is strictly dependent upon ligation of two cellular receptors, the matrix assembly site that recognizes the N-terminal domain of fibronectin and the $\alpha_5\beta_1$ integrin that binds to its RGD-containing domain (Morla et al., 1994; Sakai et al., 1996). Inhibition of $\alpha_5\beta_1$ by the addition of anti- $\alpha_5\beta_1$ monoclonal antibodies abrogates fibronectin polymerization (Fogerty et al., 1990). Since CEA/CEACAM6 expression in L6 myoblasts seems to induce an activation of the $\alpha_5\beta_1$ integrin receptor, we determined using immunofluorescence methods (Wu et al., 1998) whether CEA- and CEACAM6-transfected L6 myoblast cells have a greater ability to polymerize fibronectin than L6 parental cells. CEA/CEACAM6 expressing L6 myoblasts were in fact found to polymerize fibronectin remarkably more than control cells (L6 parental and CEACAM1, NCAM-125, Δ N-CEA, and Bcl-2 transfectants) as shown by staining with a specific anti-fibronectin mAb (Fig. 7A). Identical results were obtained when L6 transfected myoblasts were incubated in the presence of exogenous labeled fibronectin (Fig. 7B). The number of fibers that incorporated the labeled fibronectin was scored under the fluorescence microscope: the amount of fibrilogenesis was dramatically higher in CEA expressing L6 cells (Fig 7B) and the size and width of the fibronectin fibers was significantly larger than in L6 parental and L6-NCAM control cells (Fig 7A). We therefore propose that CEA and CEACAM6 expression in L6 myoblasts increases fibronectin matrix assembly through activation of the $\alpha_5\beta_1$ integrin receptor. This assembly envelops the cell and can be thought as a “cocoon” of polymerized fibronectin.

Figure 7

CEA and CEACAM6 expression in L6 myoblasts increases the polymerization of endogenous and exogenous soluble fibronectin into an insoluble matrix. (A) Polymerization of fibronectin, seen as intensely stained fibrillar material over the cells, was detected in L6-CEA, L6-CEA-2 (independent pooled population of CEA transfectant clones), and L6-CEACAM6, but not in any of L6 parental, L6-ΔN-CEA, L6-CEACAM1, L6-NCAM-125 nor L6-Bcl-2 transfected myoblast controls, although longer exposure times than 6 seconds (see M&M) will show fibrillogenesis also in L6 control myoblasts, the level was much less than L6-CEA transfected cells. The size and width of the fibronectin fibers was significantly larger in L6-CEA transfected myoblasts. This figure represents a typical experiment of three independent experiments with identical results. (B) Quantification of matrix assembly using biotinylated fibronectin (see M&M). The amount of fibrils in 10 random microscopic fields was scored; averages and standard deviation are indicated.

A**L6****L6-CEA****L6-CEA-2****L6-NCAM****L6-ΔNCEA****L6-CEACAM6****L6-CEACAM1****L6-bcl-2****B**

CEA/CEACAM6 Expression Inhibits Anoikis by Activating the $\alpha_5\beta_1$ Integrin

The $\alpha_5\beta_1$ integrin has been implicated in the regulation of apoptosis in various cell lines (Frisch and Ruoslahti, 1997). The forced expression of the $\alpha_5\beta_1$ integrin in HT29 human colonic epithelial cells by cDNA transfection, for example, inhibited apoptosis of these cells when cultured under serum free conditions (O'Brien et al., 1996). We have recently shown that CEA and CEACAM6 expression on the surface of L6 myoblasts and Caco-2 colonocytes inhibits anoikis (Ordoñez C., R. Screaton, C. Ilantzis and C.P. Stanners, submitted for publication). To test for a possible involvement of the $\alpha_5\beta_1$ integrin in this effect, we used mAbs that recognize the rat (HM α_5 -1) and human (IIA1) homologues of the α_5 integrin subunit, block cell adhesion to fibronectin, and could potentially revert the CEA/CEACAM6-mediated activation of this integrin receptor.

L6 and Caco-2 transfected cells were cultured in suspension (anchorage-independent) on polyHEMA-coated plastic surfaces in the presence and absence of the anti- α_5 mAbs for a period of 16 to 72 hours, and the percentage of apoptotic cells measured by DAPI DNA staining. In the absence of these mAbs, CEACAM6 transfected L6 myoblasts (Fig. 8A) and CEA/CEACAM6 doubly transfected Caco-2 colonocytes (Fig. 8B) showed a markedly lower apoptotic index than L6 parental and Caco-Hygro control cells. Similar results were obtained with CEA transfected L6 myoblasts. These results were confirmed using the TUNEL assay (Ordoñez C, R. Screaton, C. Ilantzis, C.P. Stanners, submitted for publication). The addition of anti-rat and anti-human α_5 mAbs rescued anoikis of L6-CEACAM6 and Caco-CEA/CEACAM6 transfected cells, respectively (Fig. 8A&B), whereas control Ha1/29 and 36.3 mAbs had no significant effect (data not shown). Interestingly, the percentage of apoptotic cells did not change when L6 myoblasts transfected with bcl-2 cDNA were treated with the anti-rat α_5 mAb, indicating that the effects of this mAb on L6-CEACAM6 cells were specific (Fig. 8A).

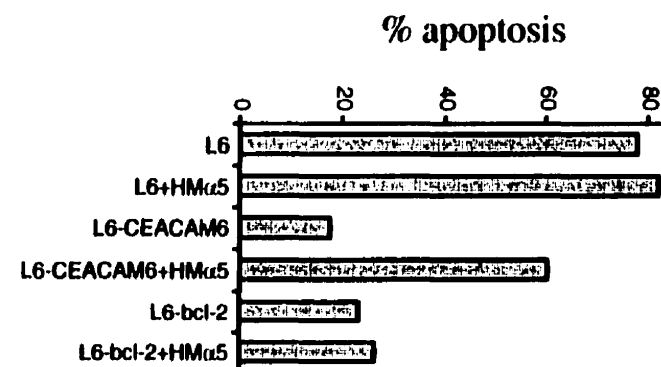
These results support the hypothesis that CEA/CEACAM6-mediated inhibition of anoikis is due to activation of the $\alpha_5\beta_1$ integrin.

Binding to fibronectin by the $\alpha_5\beta_1$ integrin is known to promote survival of many cell types (Frisch and Ruoslahti, 1997). To investigate whether CEA effects on fibronectin matrix assembly were contributing to the inhibition of anoikis, soluble rat fibronectin (10 $\mu\text{g/ml}$) was added to suspended transfected L6 cells. The apoptotic index of L6 parental and L6-bcl-2 transfected cells was not affected by the addition of soluble fibronectin, whereas CEA-transfected L6 cells showed a lower apoptotic index (Fig. 8C). Likewise, the addition of a mAb that specifically recognizes rat fibronectin, completely rescued anoikis of L6-CEA transfected myoblasts without affecting the apoptotic index of L6 parental (not shown) and L6-bcl-2 transfected myoblasts (Fig 8D), presumably by interfering with fibronectin matrix assembly on the surface of L6-CEA cells. Based on these results we propose that CEA-mediated increase in fibronectin polymerization due to the activation of the $\alpha_5\beta_1$ integrin receptor causes the formation of a fibronectin cocoon responsible for the observed resistance to anoikis (Fig 9).

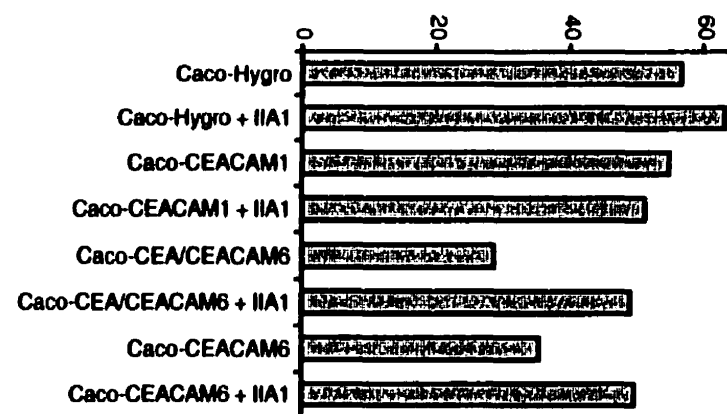
Figure 8

The addition of HM α 5-1 (recognizes rat α_5 integrin subunit) and IIA1 (recognizes human α_5 integrin subunit) mAbs rescued anoikis of L6-CEACAM6 (A) and Caco-CEA/CEACAM6 (B) to almost the same level as their respective control cells. L6-bcl-2 cells (A), on the other hand, were resistant to anoikis but were unaffected by the presence of the HM α 5-1 mAb. Anoikis was measured at 24 hours in serum free DMEM (A) and at 72 hours in GM (B) following the procedure described in M&M. These results were reproduced in three independent experiments. (C) CEA-mediated inhibition of anoikis increased in the presence of soluble fibronectin. L6 transfectants were suspended in GM as described (see M&M) in the presence of 10 μ g/ml of soluble rat fibronectin for 24 hours. The apoptotic index of L6-CEA transfected cells but not L6-parental and L6-bcl-2 control cells decreased further in the presence of soluble fibronectin. (D) The addition of mAb clone-10 that recognizes rat fibronectin rescued anoikis (measured in GM for 16 hours as described in M&M) of L6-CEA transfected myoblasts but did not affect the apoptotic index of L6-bcl-2 transfected cells.

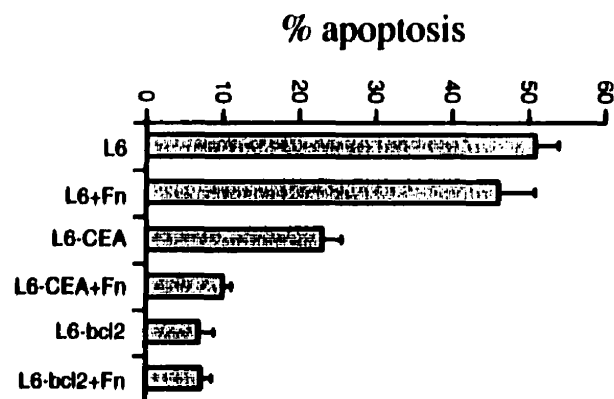
A



B



C



D

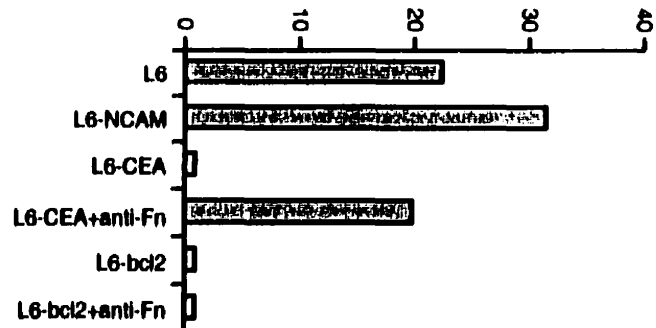
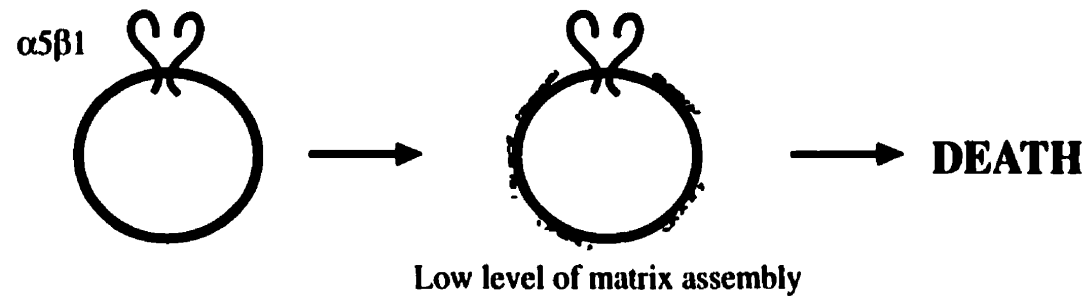


Figure 9

Model of CEA effect on anoikis of L6 cells. CEA-expressing L6 myoblasts produce an activated form of the $\alpha_5\beta_1$ integrin receptor, resulting in an increase of fibronectin matrix assembly that envelops the cell as a cocoon. This polymerized fibronectin provides a survival signal in suspended CEA-expressing L6 cells (**B**) that is absent from L6 parental cells (**A**).

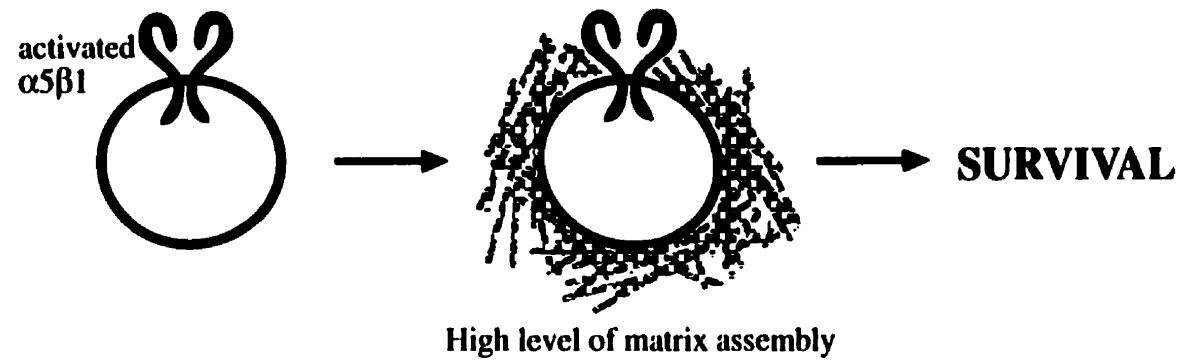
A)

L6



B)

L6-CEA



DISCUSSION

The over-expression of CEA/CEACAM6 by cDNA transfection of a number of quite different cell types profoundly inhibits their differentiation programs, blocks cell polarization, distorts tissue architecture, inhibits anoikis and increases tumorigenicity (see Introduction and Table I). In order to understand the mechanism of these rather diverse effects, a molecular process common and required for all was sought. One such process is the cellular recognition of the stimulatory and inhibitory signals that lie on their ECM (Adams and Watt, 1993; Lukashev and Werb, 1998). We propose that perturbation of cell-ECM adhesion due to activation of the $\alpha_5\beta_1$ integrin is responsible.

This hypothesis is supported by the following: (1) CEA and CEACAM6 expression on the surface of L6 rat myoblasts and Caco-2 human colorectal cancer cells, which inhibited their differentiation and anoikis programs, induces aberrant adhesion to whole extracts of their respective ECM. (2) Insulin rescued both myogenic differentiation and cell-ECM adhesion of L6-CEACAM6 transfected L6 myoblasts specifically. (3) CEA/CEACAM6 expression in L6 myoblasts early in their growth cycle caused an increase in cellular binding to fibronectin, and a progressive increase in fibronectin matrix assembly on their surfaces, providing the cells with an aberrant "cocoon" of fibronectin later in the growth cycle when differentiation normally occurs; this would presumably interfere with the recognition of positive differentiation signals from the ECM (Lukashev et al., 1994). Furthermore, the fibronectin cocoon could render the cells resistant to anoikis (Fig. 9); in that the cells might sense that they are still properly anchored when suspended. (4) The addition of soluble fibronectin to CEA expressing L6 myoblasts accentuated the anoikis resistance phenotype, presumably due to an observed increased incorporation of the added fibronectin to the insoluble fibronectin matrix surrounding the cell. (5) The addition of a mAb that recognizes

fibronectin to CEA/CEACAM6 expressing L6 myoblasts rescued both myogenic differentiation and anoikis. (6) The addition of a mAb that recognizes the α_5 integrin subunit (mAb HM α 5-1) and prevents cell adhesion to fibronectin, rescued anoikis of CEA/CEACAM6 over-expressing L6 myoblasts and Caco-2 colonocytes. (7) The expression of CEA/CEACAM6 in L6 myoblasts increased cellular binding to immobilized HM α 5-1 mAb without affecting cell surface expression levels of the $\alpha_5\beta_1$ integrin receptor. We propose, therefore, that the effects on cell differentiation and anoikis of the anti- α_5 and anti-fibronectin mAbs are due to interference with the integrin $\alpha_5\beta_1$ -fibronectin interaction on the surface of CEA/CEACAM6 expressing cells, thus inhibiting fibronectin matrix assembly and thereby preventing the formation of the fibronectin cocoon that surrounds these cells.

Previous reports support the view that the $\alpha_5\beta_1$ integrin-fibronectin interaction plays a negative regulatory role in cell differentiation and anoikis. (1) The ectopic expression of the α_5 integrin subunit in primary cultures of quail skeletal muscle cells by cDNA transfection accelerates cell proliferation and inhibits myogenic differentiation (Sastry et al., 1996). (2) The level of expression of the $\alpha_5\beta_1$ integrin receptor in human skeletal muscle cells decreases upon myogenic differentiation (Gullberg et al., 1995). Similarly, the $\alpha_5\beta_1$ integrin receptor becomes inactive upon myogenic differentiation of chicken myoblasts (Boettiger et al., 1995). (3) The forced expression of the $\alpha_5\beta_1$ integrin in HT29 human colonic epithelial cells by cDNA transfection inhibited apoptosis of these cells when cultured under serum free conditions (Frisch and Ruoslahti, 1997; O'Brien et al., 1996). (4) Proteolytic degradation of fibronectin is required for myogenic differentiation of rat myoblasts (Dourdin et al., 1997). (5) Fibronectin triggers a negative signal for myogenic differentiation (Podleski et al., 1979; von der Mark and Ocalan, 1989), adipogenic differentiation (Castro-Munozledo et al., 1987), colonic differentiation (Vachon et al., 1995), and for anoikis of many cell types (Frisch and Ruoslahti, 1997).

Further support for the view that the effects on cell-ECM binding and integrin function are necessary and sufficient for the inhibition of cell differentiation by CEA/CEACAM6 comes from further studies on the inhibition of retinoic acid-induced neurogenic differentiation of P19 embryonal carcinoma cells by CEA and CEACAM6 (Malette B. & C.P. Stanners, submitted for publication). In this case, CEA and CEACAM6 interfere with the regulation of cell surface levels during differentiation of both $\alpha_5\beta_1$ and a related integrin, $\alpha_4\beta_3$ (Yang et al., 1996). The binding of CEA and CEACAM6 transfectants of P19 cells to vitronectin, a component of the ECM that serves as a ligand for $\alpha_4\beta_3$, is inhibited (Malette B. & C.P. Stanners, submitted for publication). Vitronectin binding, regulation of endogenous $\alpha_4\beta_3$ levels and, importantly, neurogenic differentiation could all be restored by transfection with human α_4 cDNA (but, as a control, not α_2 cDNA). Interestingly, neurogenic differentiation, unlike myogenic, adipogenic and colonic differentiation, appears to be stimulated by fibronectin (Lewandowska et al., 1990). Thus, although the particular integrins affected may vary with the system, taken together the results show a consistent interference of CEA and CEACAM6 expression with the functions of specific integrins.

The mechanistic nature of the CEA/CEACAM6-mediated activation of the $\alpha_5\beta_1$ integrin receptor is presently unknown. One potential mechanism is that CEA-CEA external domain interactions, known to be required for the myogenic differentiation block (Eidelman et al., 1993), may promote the clustering of the $\alpha_5\beta_1$ integrin on the cell surface. It has been previously reported that integrins of the α_5 integrin receptor subfamily are regulated by membrane clustering rather than by a conformational change (Bazzoni and Hemler, 1998). The lateral mobility of integrins has been observed to be influenced by other cell surface glycoproteins such as the Four Transmembrane (TM4) proteins (Hemler et al., 1996). Other mechanisms are, of course, possible.

A similar phenotype of anoikis resistance has been observed in transfected rat intestinal epithelial cells that over-express the integrin-linked kinase (ILK) (Wu et al., 1998). ILK-over-expressing transfected cells, like CEA transfectants, show: (1) a functional activation of the $\alpha_5\beta_1$ integrin, (2) an increase and decrease in cell adhesion to fibronectin early and late in the growth cycle respectively, (3) an increase in fibronectin matrix assembly and (4) resistance to anoikis (Wu et al., 1998). These observations raise the possibility that CEA might inhibit anoikis through regulation of ILK. This hypothesis is currently under investigation.

In conclusion, we propose that CEA and/or CEACAM6 over-expression on the cell surface interferes with the functions of certain integrin receptors resulting in aberrant cell-ECM adhesion, leading to inhibition of cell differentiation and anoikis and a distortion of tissue architecture, thus promoting malignant progression. Considering the large proportion of human tumors showing aberrant expression of CEA/CEACAM6, this finding could have significant implications for the understanding of malignant progression in human cancer.

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In the two previous chapters, as well as in Appendix-A, we have presented data indicating that CEACAM-1, unlike CEA/CEACAM6, do not inhibit either cell differentiation and/or anoikis. These results agree with the hypothesis that CEACAM1, in contrast to CEA/CEACAM6, may inhibit tumor formation.

The next chapter contains data supporting this notion. Using a murine model, the authors demonstrated that the expression of the long tail isoform of the murine homologue of CEACAM1, Bgp1, on the surface of the CT51 mouse colonic carcinoma cell line inhibits the tumorigenic properties of these cells.

Chapter 4

Inhibition of colonic tumor cell growth by biliary glycoprotein

Abstract

Biliary glycoproteins (BGPs) are members of the carcinoembryonic antigen (CEA) family. These glycoproteins function *in vitro* as intercellular adhesion molecules and, in the mouse, serve as receptors for the mouse hepatitis viruses. In previous studies, BGP expression has been reported to be generally down-regulated in colon and liver carcinomas of human, rat and mouse origins. We now demonstrate that introduction of murine *Bgp1* cDNA isoforms into a mouse colonic carcinoma cell line, negative for endogenous *Bgp1* expression, significantly alters the growth properties of these cells. Cells bearing the *Bgp1* isoforms were growth-retarded and exhibited a reduced ability to form colonies in an *in vitro* transformation assay, when compared to parental or control neo^r cells. Furthermore, tumor formation was inhibited by 80% when cells bearing the full-length *Bgp1* were injected into BALB/c syngeneic mice, while cells expressing a *Bgp1* isoform lacking most of the intracytoplasmic domain produced tumors as readily as the parental cells. These results indicate that a biliary glycoprotein isoform is involved in negative regulation of colonic tumor cell growth, by a process which requires its intracytoplasmic domain. The precise mechanisms causing *Bgp*-dependent tumor growth inhibition remain, however, to be defined.

Introduction

The biliary glycoproteins (BGPs) are members of the carcinoembryonic antigen (CEA) family, which is part of the immunoglobulin (Ig) superfamily (Thompson et al., 1991). CEA is widely used to detect recurrences and progression of gastro-intestinal tumors in patients (Gold and Freedman, 1965). The human *BGP* gene, located in the same chromosomal cluster as the *CEA* gene (19q13.1-3) (Thompson et al., 1991), is subjected to alternative splicing mechanisms generating twelve mRNAs (Barnett et al., 1993; Barnett et al., 1989). The encoded proteins exhibit heavily glycosylated extracytoplasmic Ig-like domains with a N-terminal domain resembling the Ig variable region and one or three Ig C2-set constant regions (Barnett et al., 1989). Amongst CEA family members, BGPs are unique in that they possess cytoplasmic domains that are either short (10 amino acids) or long (71-73 amino acids) (Barnett et al., 1989; McCuaig et al., 1993). Inclusion of a 53 bp exon between exons 6 and 8 of the mouse *Bgp1* gene shifts the open reading frame of the encoded cytoplasmic domain and an additional 63 amino acids is inserted into the protein. Investigations on the expression patterns of the mouse Bgps have revealed that these glycoproteins are expressed in epithelial cells of many different tissues, in endothelial cells of large blood vessel walls and in B cells, macrophages, monocytes, platelets and granulocytes (Coutelier et al., 1994; McCuaig et al., 1992; Obrink, 1991). They are however, absent in T lymphocytes (Coutelier et al., 1994). Although they are abundant in normal colon and liver (McCuaig et al., 1993; McCuaig et al., 1992), these proteins are generally down-regulated in colonic and hepatic tumors (Neumaier et al., 1993; Rosenberg et al., 1993). Rat Bgp homologs, called C-CAMs, are also decreased in expression in primary and transplantable hepatocarcinomas (Hixson et al., 1985). The *Bgp1* transcriptional block is most likely an early event in the progression

to malignancy, since in the mouse, this glycoprotein is absent from colonic stage A tumors (Rosenberg et al., 1993).

These glycoproteins behave as intercellular adhesion molecules in *in vitro* aggregation assays; at low levels of expression in transfected cells, Bgps require calcium and physiological temperature for aggregation (Oikawa et al., 1992; Rojas et al., 1990; Turbide et al., 1991), whereas high levels of Bgp expression abrogate the calcium-dependency (McCuaig et al., 1992; Obrink, 1991). The adhesion function is postulated to be instrumental in hepatocyte aggregation during embryonic development and for adoption of colonic tissue architecture (Benchimol et al., 1989; Ocklind and Obrink, 1982). Mouse Bgps have also been recognized as the receptors for colono-, hepato- and meningo-tropic strains of mouse hepatitis viruses (Dveksler et al., 1991; Yokomori and Lai, 1992). Rat Bgps have also been shown to function as ecto-ATPases (Lin and Guidotti, 1989) and the 71 amino acid Bgp cytoplasmic tail is known to be responsible for bile acid efflux from hepatocytes (Sippel et al., 1993). Bgps may also be involved in signal transduction events since they are phosphorylated on either serine/threonine and tyrosine residues subsequent to activation of either protein kinase C (PKC) or the insulin receptor (Afar et al., 1992; Lin and Guidotti, 1989; Rees-Jones and Taylor, 1985).

To understand the role played by these proteins in tumorigenesis, we have inserted two cDNA isoforms of the mouse *Bgp1* gene bearing either a 10 or 73 amino acid intracytoplasmic domain into mouse colon carcinoma cells, negative for endogenous Bgp expression, and evaluated the properties of the resulting transfectant cells. Expression of either the short or the long-tailed Bgp protein in these cells reduced their rate of proliferation and their ability to form colonies in clonogenic assays, as compared to parental or control infected cells. Furthermore, expression of the long-tailed Bgp variant inhibited tumor formation in syngeneic mice by 80%. Notably, the short-tailed

Bgp had no inhibitory effect *in vivo*. This data indicates that Bgps may play a role in growth and/or differentiation of colonic epithelial cells.

Materials and Methods

Cell Culture

Mouse colonic carcinoma CT51 cells were generously provided by Dr. Michael G. Brattain, Baylor College of Medicine, Texas (Brattain et al., 1980). These cells were established from chemically-induced BALB/c transplantable tumors and they readily form tumors when injected subcutaneously into syngeneic mice. The CT51 and CT51-derived cells were grown in α -modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 units/ml of penicillin and 50 μ g/ml of streptomycin at 37°C in a 5% CO₂-air humidified incubator (Rosenberg et al., 1993). Transfectant cells were selected and grown in the presence of 750 μ g/ml of active geneticin (G418). Tumor cells were excised from the mice, minced and disaggregated in α -modified Eagle's medium (without FBS) containing 200 U/ml of collagenase 1, 270 U/ml of DNase 1 and 35 U/ml of hyaluronidase type IV. These cells were subsequently passed through 70 μ m cell strainers (Kimball et al., 1978), suspended in medium containing 10% FBS and submitted to cytofluorometric analysis.

Antibodies

The polyclonal rabbit anti-mouse Bgp antibody (serum 231) used to detect mouse Bgp1 proteins in immunoblot analyses or immunoselection procedures has previously been described (McCuaig et al., 1992). For cytofluorometric analyses, a monoclonal rat anti-mouse Bgp1-specific antibody called B10 was generously provided by Drs. Kuprina and Rudinskaya, Moscow, Russia (Kuprina et al., 1990).

Infection of CT51 Cells and Selection of Bgp-Positive Cell Clones

Insertion of the *BgpA* [natural variant with a short tail (McCuaig et al., 1992)] or the full-length *BgpD* [natural variant with a long tail (McCuaig et al., 1993)] into CT51 cells was performed *via* retroviral-mediated infections. The cDNAs were cloned into the *EcoRI* site of the pLXSN retroviral vector under the transcriptional control of the Moloney murine leukemia viral LTR (Miller and Rosman, 1989). 5 µg of this construct was transfected by calcium phosphate coprecipitation into ψ 2 packaging cells. Viral stocks from supernatants of Bgp-positive ψ 2 cells resistant to G418 selection (500 µg/ml) were used to infect 2×10^5 CT51 cells in 5 ml of α -modified Eagle's medium supplemented with 10% FBS and 40 µg of sterile polybrene for 4 hr at 37°C. Bgp-positive populations were immunoselected ten days after viral infections using the 231 polyclonal anti-Bgp antibody (231) and Dynabeads. The resulting populations were grown for three days and single cells were then manually cloned. One hundred and seventy five BgpA- and BgpD-expressing clones were analysed by immunoblotting to determine the relative amount of expressed Bgp. The clones selected for further experimentation remained resistant to G-418 selection and were continuously maintained in selection medium throughout all experimental procedures. The clones have maintained approximately the same Bgp cell surface expression (as determined by cytofluorometric analyses) as when originally cloned. Control neo-resistant clones were generated using the same procedure, but with an empty pLXSN vector. Ten neo^r clones were manually cloned from the G-418-resistant population and three of these were used in further experiments. The CT51 parental cells were used as a cell population.

Cytofluorometric Analyses

Bgp-expressing cells were analyzed by cytofluorometry with the B10 MAb and fluorescein-conjugated affinity-purified goat anti-rat IgG Fab₂ fragments using a FACScan program. Fold expression of cell surface-expressed Bgp populations or clones was calculated by deriving the ratio of the median fluorescence values of positive cells versus the median of the same cells treated with the secondary fluorescent-labelled antibody only.

Immunoblot Analyses

Western analyses were performed essentially as described (Rosenberg et al., 1993). Cells detached from the dishes using a solution of PBS-citrate were divided in two portions and each half was processed simultaneously for cytofluorometric analyses and preparation of protein lysates. 150 µg of cell lysate proteins were separated on 7.5% SDS-PAGE, the proteins were transferred to Immobilon membranes and Bgp glycoproteins were revealed by incubation with the 231 polyclonal antibody and [¹²⁵I]-labeled protein A. Quantification of the radioactive bands was performed on a Fuji BioAnalyzing system 2000 and are reported as fold over background.

Growth Curves

Cells were plated in duplicate at 10⁴ cells per well (9.64 cm²) in α-modified Eagle's medium supplemented with 10% FBS (and 750 µg/ml G418 for infected cells). Cells were detached with trypsin-EDTA and counted on various days of culture using a Coulter counter. Experiments were repeated four times.

Anchorage-Independent Growth Assays

Soft agar assays were essentially performed as described with some modifications (Kimball et al., 1978). Briefly, cells were passaged through 70 μm cell strainers and 15 μm filters (Brattain et al., 1980) and seeded in duplicate wells or plates at densities of 10^2 , 10^3 cells or 5×10^3 per well or plate in a 0.32% top agarose layer dissolved in α -modified Eagle's medium containing either 0, 1, 2.5, 5, 10 or 20% FBS (and 750 $\mu\text{g/ml}$ G418 for infected cells) over a bottom 0.5% agarose layer dissolved in medium containing 10% FBS. Fresh medium (containing G418 where indicated) was replenished over the agarose layer every week. Colonies were counted 21 days after seeding of cultures. Experiments were repeated five times.

Syngeneic Mice Tumor Formation Assays

All animals were maintained in accordance to the Canadian Council on Animal Care Committee recommendations and experimental protocols were reviewed by the McGill University Animal Care Committee. Six to eight week old female BALB/c mice (Charles River Inc.) were injected s.c. in the posterior flank with viable cells (either 1×10^6 or 4×10^6) resuspended in 200 μl of α -modified Eagle's medium on day 0 (Brattain et al., 1980). Animals were checked every second day for appearance of tumors and sacrificed either after 28 days or when tumors reached approximately 1.0-1.5 cm in diameter. Experiments were repeated three times.

Statistical Analysis

Since the growth rates of all clones were linear over the first six days, regression analysis was used to determine the initial slope of each growth curve. Colonies in *in vitro* transformation assays were counted and standard error deviations were computed. The statistical significance of tumor incidence was determined by comparing incidence of all

clones to that of the CT51 parental cell line. Two-by-two contingency tables were constructed and the chi-square statistic was computed with a continuity correction. *P* values were determined at one degree of freedom. Differences in tumor latency were evaluated using the Student's *t* test and *P* values were determined at the appropriate degree of freedom.

Results

Expression of Bgp Proteins in Colon Carcinoma Cells Alters their Growth Properties

We have previously described that several mouse *Bgp1* splice variants (Nedellec et al., 1995) are expressed in normal colon and liver, but are absent from colon tumors or colonic and hepatic tumor cell lines (Rosenberg et al., 1993). A survey of expression patterns in mouse tissues has also shown that, as in colon, the long intracytoplasmic tail (73 amino acids)-bearing variant (named BgpD) is always expressed in a lower ratio than the short-tailed variant (named BgpA) produced by the same *Bgp1* gene (McCuaig et al., 1993; Nedellec et al., 1995). To understand the role played by each of these glycoproteins in tumorigenesis, we introduced their respective cDNAs into BALB/c-derived CT51 mouse colon carcinoma cells (Brattain et al., 1980) using retroviral-mediated infections. As shown in Figure 1A, the parental (CT51) or the control infected cell population (neo pop.) or clones (neo1, neo2, neo3) expressing the empty vector exhibited undetectable expression of the Bgp proteins at the cell surface. The small peak or shoulder observed in these cytofluorometric profiles is a consequence of the two types of epithelial cells which are present in this clonal cell line. As reported by Brattain *et al.*, attempts to resolve the heterogeneity of this cell line by cloning have not been successful and it is thought that the heterogeneity may be due to the state of differentiation of the

cells or the phase of the cell cycle of particular cells (Brattain et al., 1980). This cell line (Figure 1B, CT51) or control infected population or clones (Figure 1B, neo pop., neo1, neo2, neo3) did not express detectable amounts of Bgp by immunoblot analyses. We have, however, detected faint transcription of the *Bgp1* mRNAs in CT51 cells using nested reverse transcription-polymerase chain reaction (RT-PCR) methods with *Bgp1*-specific primers (data not shown).

Bgp-positive cell clones were isolated by immunoselection with an anti-Bgp polyclonal antibody (Ab 231) and Dynabeads. The Bgp-expressing populations as well as derived clones were evaluated for Bgp expression by both cytofluorometric analyses and immunoblotting (Figures 1A and 1B). The BgpA protein exhibiting a short intracytoplasmic domain migrates as a broad 120 kDa band while the BgpD protein migrates slightly slower due to an additional 8 kDa provided by the 73 amino acid intracytoplasmic tail. As shown in Figure 1A, Bgp-infected populations or clones selected in this study overexpressed Bgp at the cell surface by a factor of 1.7-3.3 fold over background, while quantification of the radioactive bands on the immunoblots (Figure 1B) indicate that Bgp was overexpressed by factors of 4.5-9.3 fold over background. The discrepancy found between the amount of Bgp expressed at the cell surface and the total amount found in cell lysates could indicate that not all of the Bgp proteins synthesized in these cells reached the cellular membrane. However, the amount of Bgp expressed in these infected cells corresponds to approximately the concentration of Bgp found in normal mouse colon ((McCuaig et al., 1993) and data not shown).

Figure 1

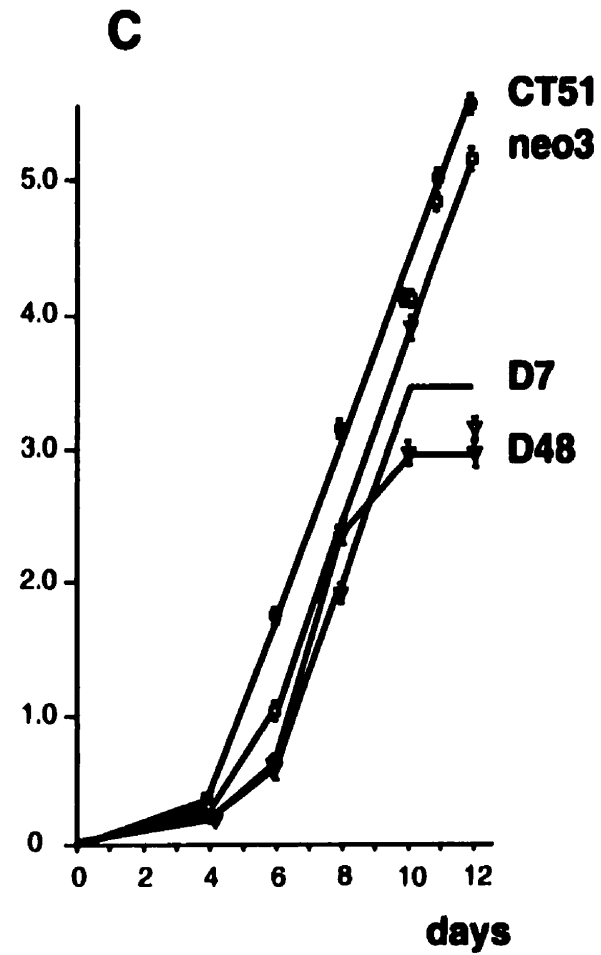
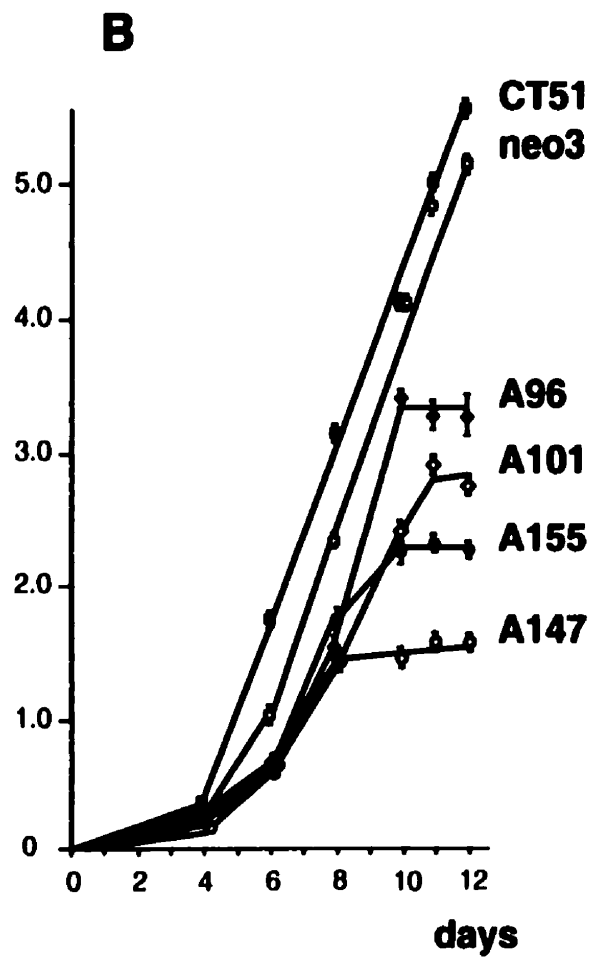
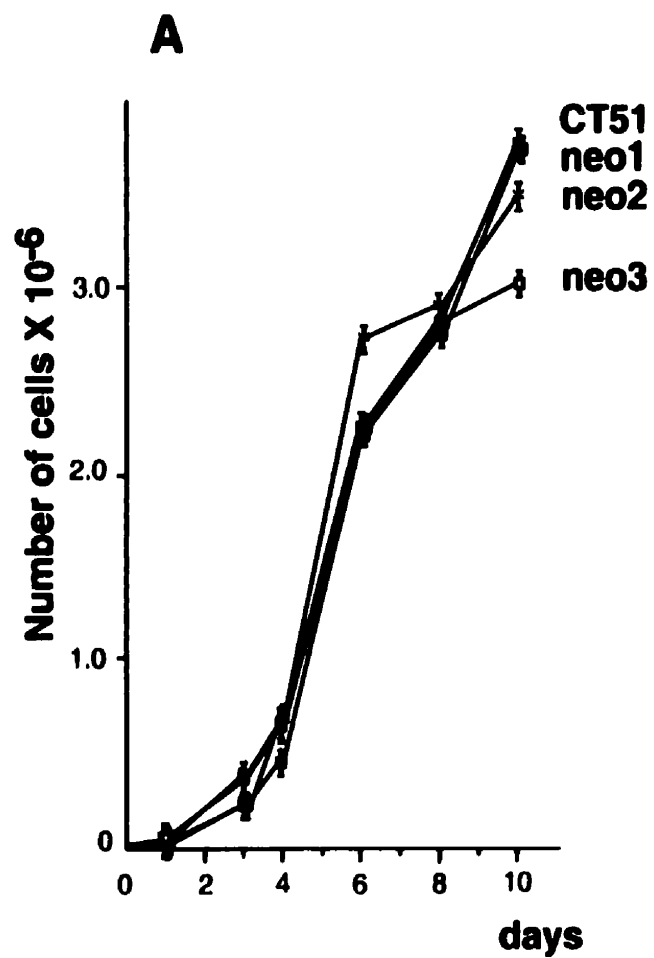
Expression of Bgp in CT51 parental and infected cells. CT51 parental, neo^r control or Bgp-expressing infected cell populations or clones described in Materials and methods were analyzed for Bgp expression by cytofluorometric analysis using a Bgp-specific B10 MAbs. (A). Fluorescence was evaluated on a log scale. CT51 parental (CT WT). neo pop. or neo1, neo2 and neo3 represent CT51 populations and clones infected with an empty vector while BgpA pop., A96, A101, A147 and A155 are populations or clones expressing the short-tailed BgpA protein. BgpD population (pop.) or D7, D48 and D66 clones express the long-tailed BgpD protein. Dashed lines depict cells incubated with the secondary fluorescein-labeled antibody only and solid lines represent cells incubated with both primary (B10) and secondary antibodies. Fold expression of the clones over background is indicated in the top right corner of each profile and represents the ratio of the positive over negative medians. (B) Cells were lysed and 150 µg of total cellular proteins were separated on 7.5% SDS-PAGE gels. After transfer of the proteins, the membranes were incubated with a rabbit anti-mouse Bgp polyclonal antibody (serum 231). The radioactive bands were quantified on a Bas 2000 BioAnalyzing system and are reported as fold expression over background. Molecular weight markers are indicated on the left of the autoradiograms.

As cell clones were being established, we observed that most Bgp-expressing cells grew more slowly than the parental cells or the neo^r control cells. To quantify this observation, equal numbers of CT51 parental or infected cells were plated and growth curves were derived. Typical experiments are shown in Figure 2. No significant differences in growth were noticed between three neo control clones (Figure 2A) indicating that these differences were not due to clonal variability. However, significant growth differences of the BgpA- or BgpD-expressing cells were observed by day 6 when compared to the CT51 parental cells or neo control clones (Figure 2A, 2B and 2C). Moreover, the difference in growth between the parental and control cells and the Bgp-expressing cells became more apparent over the course of the next four to six days due to a lower saturation density of the BgpA- and BgpD-expressing cells. The parental cells and neo transfectant control cells exhibited three dimensional growth and tended to stack up whereas the Bgp transfectant cells exhibited a reduced ability to do so; they either reduced their growth rate or shedded off and died. Furthermore, growth retardation of Bgp clones was somewhat correlated with the amount of Bgp protein expressed at the cell surface; the A147 and A155 clones, expressing 2.5-2.8 fold of BgpA over background, grew significantly slower than the the A96 or A101 clones expressing 1.7-1.9 fold, respectively (Figure 2B). The D7 and D48 clones were similarly growth-retarded relative to the control cells (Figure 2C). The D66 clone was not used in this assay. This suggested a lower saturation density for cells expressing a higher Bgp concentration at the cell surface. Similarly, the growth rate of the Bgp-positive cells, calculated by deriving the slope of the growth curve drawn on a semi-logarithmic scale (data not shown), was reduced when compared to that of the parental cells in the first 6-8 days (1.05×10^5 cells/day for BgpA155-expressing cells, 1.53×10^5 cells/day for BgpD7-expressing cells *versus* 3.13×10^5 cells/day for parental CT51 cells) ($P < 0.05$). These data indicate that

the presence of Bgp at the surface of colonic carcinoma cells affects the growth rate and the saturation density of these cells.

Figure 2

Growth Curves of CT51 Parental, Neo^r control and Bgp Transfectant Cells. 10^4 cells of CT51 parental (CT51), control (neo), Bgp short-tailed (A) or long-tailed (D) derived cells were plated in duplicate 9.64 cm² wells and grown for 10-12 days. Cells were collected at various time points with trypsin-EDTA and counted. Expression of Bgp was verified by cytofluorometric analysis at the beginning and the end of every growth curve. Experiments were repeated four times and representative experiments are plotted. Experiments in **B** and **C** were performed tandemly but were plotted separately for clarity. Variability indicated here is the standard error, which is smaller than the symbols in many cases.

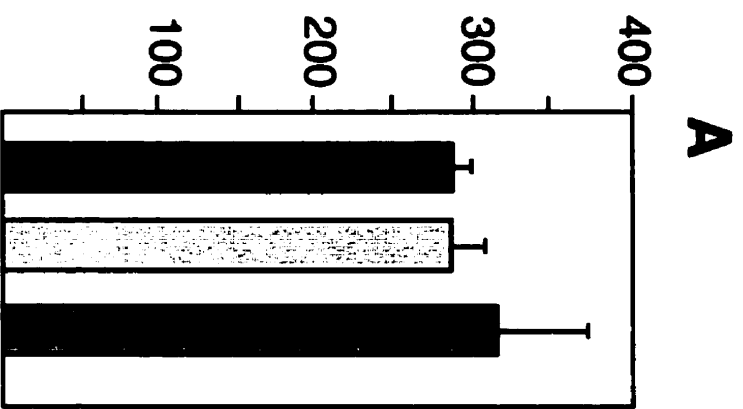


Bgp Expression Inhibits Anchorage-Independent Growth of CT51 Cells

Since the growth of Bgp-bearing CT51 cells was affected, we tested if Bgp expression had effects on anchorage-independent growth of CT51 cells. As shown in Figure 3A, no significant differences in the ability to form colonies in soft agar were noticed when either neo control populations or BgpA- and BgpD-expressing populations were compared. When three neo control clones were analysed and compared to the parental cells (Figure 3B), the neo1 clone exhibited the same properties as the parental cells, whereas, in this experiment, the neo2 clone showed a 27% increase and the neo3 clone a 41% decrease in the ability to form colonies relative to the parental cell line. Bgp-positive cells, as shown in Figure 3C, irrespective of whether the protein included a short or a long intracytoplasmic tail, formed a reduced number of colonies in soft agar (35-83% inhibition) when compared to the CT51 parental or neo3 control cells. In addition, the colonies that did form with the Bgp-positive clones contained fewer cells (data not shown). Furthermore, cells expressing higher amounts of BgpA at the cell surface produced a greater inhibition (Figure 3C, A147 and A155 *versus* A96 and A101), suggesting that this phenotype was in fact due to the presence of Bgp. Decreasing the serum concentration from 10% to 2.5% had no effect on anchorage-independence (data not shown). These results suggest that the relatively abundant expression of Bgp in normal colon epithelial cells could be instrumental in normal cellular growth and/or differentiation, since Bgp expression in colon carcinoma cells considerably reduced their transformed properties in *in vitro* assays.

Figure 3

Bgp-dependent growth inhibition of colon carcinoma cells in soft agar. 10^2 , 5×10^2 or 10^3 CT51 parental, control neo^r or Bgp-expressing cells were seeded in duplicate 9.64 cm² wells in soft agar with α -minimal essential medium containing 10% as described in Materials and methods. Colonies were counted after 21 days of growth and counts were plotted accordingly. Variability indicated here is the standard error. Experiments were repeated five times and typical results are plotted.



CT51
 neo 1
 neo 2
 neo 3
 A96
 A101
 A147
 A155
 D7
 D48

Expression of the Bgp Isoform Bearing a Long Intracytoplasmic Domain Inhibits Tumor Formation in Syngeneic Mice

To corroborate these *in vitro* results, either the parental CT51, neo control or Bgp-bearing cell populations and clones were injected into BALB/c syngeneic mice. When CT51 parental, neo control or BgpA-expressing cell populations were used (Table 1), a statistically significant number of mice (8/10, 9/10 or 10/10) developed tumors within a two week period and these tumors increased in size until the end of the experimental period ($P > 0.1$). However, only 4/10 mice developed tumors when BgpD-expressing cell populations were injected into mice, which was a statistically significant result ($P < 0.02$) when compared to that of the control neo' population. This result indicated that the Bgp cytoplasmic domain influenced the *in vivo* growth properties of these colonic carcinoma cells.

To corroborate the results obtained with the cell populations, cell clones were used in a similar experiment (Table 2). Two cell concentrations were used for this assay, as done previously (Brattain et al., 1980) and the length of the experimental period was prolonged until the tumors had reached approximately 1.0-1.5 cm in diameter. When 4×10^6 CT51 parental cells or neo' control clones were injected s.c., 92% of the mice developed tumors that reached 1.0-1.5 cm in diameter in 31.4 ± 7.2 or 34.0 ± 5.3 days (neo3, Table 2). When a lower cell concentration was used (1×10^6 cells/injection), 44% of the mice developed tumors within 45.4 ± 2.3 (CT51) or 43.6 ± 3.0 days (neo3 clone). Similarly, when CT51 cells bearing the short-tailed Bgp variant (BgpA) were injected at 4×10^6 cells/injection, 100% of the mice developed tumors if the cells expressed high amounts of BgpA at the cell surface (Table 2: A147 and A155 clones). BgpA clones expressing lower amounts of this protein at the cell surface (Table 2: A96 and A101 clones) were capable of inducing tumors at an incidence of 60%, which was not statistically different than that of the parental CT51 cells ($P = 0.35$). Interestingly, the

latency of tumor appearance was decreased (23.6 ± 4.2 days) relative to parental cells (45.4 ± 2.3 days) with 1×10^6 BgpA155-bearing cells ($P < 0.00001$), but not with other BgpA clones. The metastatic status of BgpA-induced tumors remains to be investigated.

Transfectant cells expressing the Bgp isoform exhibiting a 73 amino acid cytoplasmic domain (BgpD) significantly suppressed tumor formation. No tumors formed when 1×10^6 cells were injected and 13 mice out of 44 produced tumors with 4×10^6 cells/injection (Table 2). This was a statistically significant decrease in tumor formation when compared to the parental CT51 or neo^r control cells ($P < 0.01$). In addition, we verified if tumors appeared in some of these mice as a result of loss of Bgp expression from the injected cells. BgpA155- or BgpD7-borne tumors were disaggregated and tested for Bgp expression by cytofluorometric analysis. The cells had retained Bgp expression (data not shown). The few tumors that did form after injection of the BgpD-expressing cells were delayed in appearance (48.8 ± 2.6 days) as compared to tumors derived from the parental cells (31.4 ± 7.2 days) ($P < 0.0001$). The mice that did not form tumors were monitored for over four months to detect late-onset tumor formation; none was observed. These results indicate that the Bgp cytoplasmic domain was capable of significantly inhibiting tumor formation.

Table 1. Tumor growth in syngeneic mice using cell populations.

<i>Cell populations</i>	<i>Incidence</i>	<i>Average size of tumors (cm³)</i>
CT51	9/10	0.6-0.2
neo ^r	10/10	0.7-0.2
BgpA	8/10	0.5-0.2
BgpD	4/10	0.5-0.2

CT51 parental, control neo^r or BgpA and BgpD-expressing cell populations were injected s.c. in the right posterior flank with 4×10^6 cells resuspended in 200 μ l of α -minimum essential medium without serum on day 0. Mice were checked every two to four days for appearance of tumors and sacrificed on day 28. Tumors were excised, measured and statistical analyses were performed.

Table 2. Inhibition of tumor growth in syngeneic mice produced by BgpD-expressing cell clones.

<i>Cell line</i>	<i>No. of cells X 10⁶ per injection</i>	<i>Incidence</i>	<i>Latency (1.0-1.5 cm tumor produced)</i>
CT51	1	5/10	45.4±2.3
	4	12/13	31.4±7.2
Neo1	4	5/10	33 ^a
Neo2	4	5/5	43.0 ^a
Neo3	1	6/15	43.6±3.0
	4	11/12	34.0±5.3
A96	1	1/5	46.0
	4	3/5	46.0 ^b
A101	1	3/5	29.0 ^b
	4	3/5	35.0 ^b
A147	1	1/5	35.0
	4	4/4	38.0±4.2
A155	1	5/5	23.6±4.2
	4	13/13	27.2±5.9
D7	1	0/10	-
	4	9/27	48.8±2.6
D48	1	0/5	-
	4	2/5	*
D66	1	0/4	-
	4	2/12	*

CT51 parental, neo or Bgp-expressing derived cells were injected s.c. in the right posterior flank with the indicated amount of cells in 200 μ l of α -minimum essential medium without serum on day 0. Mice were checked every two days for appearance of tumors and sacrificed when the tumors reached approximately 1.0-1.5 cm. ^a Indicates that mice were sacrificed arbitrarily on these days. ^b Only one tumor reached the minimum 1.0 cm threshold at the time of sacrifice of these animals. Dashes indicate that no tumors formed after 4 months. * Indicates that tumors did not reach 1.0 cm within 45-55 days.

Discussion

In this paper, we have evaluated the effect of two Bgp protein isoforms in tumor formation using *in vitro* and *in vivo* transformation assays. Although these two protein variants are usually expressed coordinately in many cell types, we first questioned whether Bgp protein isoforms independently affected tumor formation. Using colony formation in soft agar and subcutaneous injection of Bgp-positive transfected CT51 cells into syngeneic mice, we demonstrate that the short-tailed BgpA-bearing clones inhibited colony formation in soft agar, but surprisingly had no effect on inhibition of tumor development in syngeneic mice. The discrepancy between results obtained with BgpA-bearing CT51 cells in *in vitro* soft agar and *in vivo* tumor formation is noteworthy and may reflect the fact that the *in vitro* model does not accurately mimic the *in vivo* situation. Cellular interactions, such as putative ligand binding or cell-cell interactions, may be required for *in vivo* tumor inhibition to occur in this case. The syngeneic BALB/c model is a very reliable animal model to study the biology of tumor development because of an intact immune system. The BgpD variant, possessing a long intracytoplasmic tail, had a dramatic effect on the parental cells' tumorigenic properties which leads to an overall 80% inhibition of tumor development in syngeneic mice. These data strongly suggest that the long intracytoplasmic domain of Bgp is involved in inhibition of cell growth and tumor formation. Similar findings have recently been reported in a prostatic carcinoma model where the human PC-3 cells transfected with the rat BGP isoform bearing a 71 amino acid cytoplasmic domain (called C-CAM1) exhibited reduced tumorigenic properties *in vitro* and *in vivo* (Hsieh et al., 1995). The phenotype elicited by the biliary glycoprotein isoform, shown to function as an adhesion molecule *in vitro*, is therefore another example of cell adhesion molecules actively involved in cellular proliferation or migration; a purified preparation of N-CAM

consisting of mainly the polysialylated embryonic form of N-CAM has been shown to inhibit proliferation of astrocytes (Sporns et al., 1995) and E-cadherin as well as the α 4 β 1 integrin act to suppress metastatic invasion (Chen and Obrink, 1991; Navarro et al., 1991; Qian et al., 1994; Vleminckx et al., 1991).

The Bgp proteins studied here are identical except for their intracytoplasmic domains (McCuaig et al., 1993; McCuaig et al., 1992). The long intracytoplasmic tail is inserted into the Bgp structure through alternative splicing of the *Bgp1* gene. The exon structure in this region of the *BGP* genes is well conserved throughout evolution as the mouse, rat and human *BGP* long tail mRNAs are synthesized using the same alternative splicing mechanism (Barnett et al., 1989; Najjar et al., 1993; Nedellec et al., 1995). This conservation implies functional necessity. The results presented here emphasize the importance of this cytoplasmic tail on cell growth and/or differentiation of normal colonic cells. Interactions of Bgp with cytoplasmic proteins may lead to activation of signal transduction pathways, establishing a connection between the cell surface and the nucleus. Transcriptional activation or silencing due to signals produced through Bgp could result in the regulation of cell growth through modulation of the cell cycle. So far, only one protein, calmodulin, has been shown to associate *in vitro* with the long intracytoplasmic domain of the rat Bgp homolog (C-CAM) (Edlund and Obrink, 1993). The biological relevance of calmodulin binding to Bgp remains however, to be clarified.

Elements within the cytoplasmic domain may also be important for other cellular properties such as polarity of hepatocytes or colonic cells. In fact, Bartles *et al.* have shown that the rat Bgp homolog (also identified as HA4) is first destined to the basolateral membrane and then transported to the apical pole of the hepatocyte (Bartles et al., 1987). In other receptors, such as the low density lipoprotein (Davis et al., 1986), poly-immunoglobulin (Vega and Strominger, 1989), mannose-6-phosphate (Lobel et al., 1989)

or the β -adrenergic receptors (Valiquette et al., 1990), tyrosine residues in their intracytoplasmic domains are crucial for agonist-induced internalization.

In summary, our data indicates a clear correlation between the presence at the cell surface of the Bgp isoform bearing a long tail, the lower saturation density observed in the growth curve and the inhibition of tumor formation in syngeneic mice. The cytoplasmic domain of this Bgp isoform may therefore be a negative regulator of normal colonic growth and absence of Bgp from tumors may result in the uncontrolled growth of the cancerous cells and may possibly abrogate contact inhibition. However, it should be noted that the two Bgp isoforms studied here are usually expressed coordinately in normal colon and liver cells in a defined ratio (~1-3 BgpD:~7-10 BgpA) (McCuaig et al., 1993) and that this tandem expression of the short- and long-tail Bgp may influence normal cellular growth. Several consensus phosphorylation sites (Ser/Thr and Tyr) have been identified within the long intracytoplasmic domain and these may be important for cell growth and differentiation processes (Afar et al., 1992; Lin and Guidotti, 1989; Rees-Jones and Taylor, 1985; Sippel et al., 1993). We are presently generating transfectants expressing mutations and deletions of the cytoplasmic domain as well as expressing both constructs together to better define the mechanisms responsible for such a phenotype.

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General Discussion

We have previously proposed that the CEA family plays an instrumental role in human cancer (Benchimol et al., 1989; Eidelman et al., 1993; Screaton et al., 1997; Stanners, 1998). CEA and CEACAM6 are over-expressed in more than 50% of all human cancers including major sites such as breast, colon and lung cancer (Hammarström et al., 1998). In contrast, CEACAM1 expression is down-regulated in early stages of prostate and colon cancer (Kleinerman et al., 1995a; Kleinerman et al., 1995b; Rosenberg et al., 1993). This data led to the hypothesis that CEA/CEACAM6 and CEACAM1 have opposite roles in human cancer (Obrink, 1997). CEA/CEACAM6 may promote tumor formation and progression, whereas CEACAM1 may function as a tumor suppressor protein, as demonstrated in Chapter 4 of this thesis. One potential mechanism contributing to the tumorigenic effects of CEA and CEACAM6 is the capacity of these cell surface glycoproteins to block cell differentiation and disturb normal tissue architecture (Stanners, 1998). This Ph.D. thesis presents a second mechanism that could well promote CEA/CEACAM6 tumorigenic activity. CEA and CEACAM6 over-expression on the surface of cancer cell lines inhibited anoikis *in vitro* (Chapter 2), a tissue architecture quality control mechanism that triggers apoptosis of cells losing contact with their extracellular matrix (ECM) (Frisch and Ruoslahti, 1997). As we will discuss here, CEA/CEACAM6-mediated inhibition of cell differentiation and anoikis might instrumentally contribute to tumor formation and progression.

CEA and CEACAM6 over-expression on the surface of a variety of cell lines inhibited anoikis (Chapter 2). L6 rat myoblasts and Caco-2 human colorectal cancer cells over-expressing CEA and/or CEACAM6 on their surfaces, and both MDCK epithelial cells and SW1222 human colorectal cancer cells over-expressing CEA or CEACAM6, respectively, were less prone to undergo anoikis than their respective control parental cell lines (Chapter 2, Fig. 1-3). Likewise, anoikis was inhibited in L6 myoblasts expressing

bcl-2, a known inhibitor of apoptosis (Adams and Cory, 1998; Frisch and Ruoslahti, 1997). In contrast, the expression of CEACAM1 and GPI-bound NCAM on the surface of L6 myoblasts did not inhibit anoikis. A mutated version of CEA lacking a portion of the N-terminal domain of CEA, unlike wild-type CEA, failed to inhibit anoikis suggesting a mechanistic role for the N-domain of CEA. As we will discuss below, CEA/CEACAM6-mediated inhibition of anoikis may allow the persistence of the aberrant tissue architecture pattern caused by CEA/CEACAM6 over-expression on the surface of cancer cells.

Anoikis is an essential mechanism in the maintenance of normal tissue architecture restricting cells to their normal spatial constraints (see Introduction in Chapter 1). Cells abandoning these constraints are forced to commit suicide (Frisch and Ruoslahti, 1997). For instance, most epithelial cells exist as monolayers of cells in intimate contact with their basement membranes or ECM (Lelievre et al., 1996; Nguyen et al., 1992; Ronnov-Jessen et al., 1996). This epithelial-like tissue architecture is actively preserved by anoikis (Frisch and Ruoslahti, 1997). Epithelial cells that lose contact with their ECM are forced to die of anoikis thus preventing the invasion and colonization of surrounding tissues by these cells. Cancer cells seem to be resistant to anoikis, acquiring the capacity to grow as a disorganized multilayered group of cells.

Stanners previously proposed that CEA/CEACAM6 over-expressing cancer cells have an increased capacity for intercellular adhesion leading to a disruption of normal tissue architecture and inhibition of cell differentiation (see Introduction in Chapter 1). According to this model, CEA/CEACAM6 over-expressing cells have the capacity to grow as a multilayered group of cells, instead of the monolayered architecture adopted by normal epithelial cells. However, this model did not explain why CEA/CEACAM6 over-expressing cells arranged in such multilayered architecture, and presumably lacking contact with the basement membrane, did not die of anoikis. In this thesis, we have

modified this tissue architecture model to include the effect of CEA/CEACAM6 on anoikis (Chapter 2, Fig. 4).

The inhibition of anoikis caused by CEA/CEACAM6 cell surface over-expression may promote the survival of cancer cells that have lost contact with their ECM. This molecular mechanism may both promote and preserve the arrangement of cancer cells into an aberrant tissue architectural pattern (Chapter 2, Fig. 4). We propose that CEA/CEACAM6-mediated inhibition of anoikis, together with inhibitory effects of these molecules on cell differentiation, could contribute to tumor formation and progression. In support of this hypothesis, our group has demonstrated that both CEA/CEACAM6 over-expressing Caco-2 human colorectal cancer cells (Ilantzis C., L. DeMarte, R. Screaton, C.P. Stanners, submitted for publication) and CEA over-expressing L6 rat myoblasts (Screaton et al., 1997) form tumors in nude mice with a shorter latent period and grow faster than their respective control untransfected cell lines.

CEACAM1, unlike CEA and CEACAM6, does not inhibit cell differentiation (Rojas et al., 1996) and anoikis (Chapter 2), and is thought to function as a tumor suppressor (Hsieh et al., 1995; Kleinerman et al., 1995b). Both isoforms of CEACAM1 containing either long or short cytoplasmic tails are simultaneously expressed in human colonocytes. The cell surface expression of the murine homologue of CEACAM1-L (long tail) alone in CT51 murine colorectal cancer cells, a CEACAM1 negative cell line, inhibited both their growth in soft agar and the formation of tumors when these cells were injected into syngeneic mice (Chapter 4). In contrast, CEACAM1-S (short tail) alone had no effect in this system (Chapter 4). However, it has been proposed that in human cancer the ratio of CEACAM1-L versus CEACAM-S needs to be optimal for such tumor suppressor effect to occur (Turbide et al., 1997).

As indicated above, CEACAM1, unlike CEA and CEACAM6 that are bound to the cell surface by a glycosylphosphatidyl inositol (GPI) anchor, contains a

transmembrane domain followed by either long or short cytoplasmic tails. This structural difference might explain the distinct functions of CEA/CEACAM6 and CEACAM1. We hypothesize that the mode of anchorage to the plasma membrane, GPI-bound versus transmembrane, determines the role of the CEA family members in human cancer (Screaton et al., 2000). We propose that GPI-bound CEA and CEACAM6 are oncogenic whereas transmembrane CEACAM1-L plays a tumor suppressor role. Data supporting this hypothesis will be discussed below.

CEACAM1, unlike CEA, inhibits tumor growth in animal models (Chapter 4). The ratio of CEACAM1-L cell surface expression versus CEACAM1-S in cancer cells seems to determine its tumor suppressor function (Turbide et al., 1997). This suggests that interactions of both isoforms of CEACAM1 are required to inhibit tumor growth.

Recently, it has been demonstrated that CEACAM1 has a stimulatory effect on angiogenesis (Ergun et al., 2000). This result suggests that CEACAM1 may be oncogenic by increasing tumor angiogenesis. This hypothesis seems contradictory to our model and others claiming a tumor suppressor role for CEACAM1. The tumor growth inhibitory properties of CEACAM1 reside on its long cytoplasmic tail, whereas its pro-angiogenic domain is in its extracellular domain. This possible dual role of CEACAM1 in human cancer needs further revision. Dr. Sue Hwa Lin from the Anderson Cancer Centre in Houston, Texas, is collaborating with Introgen, a US Biotechnology company, to develop anti-cancer gene therapies based on CEACAM1. This approach will consist of introducing into cancer cells only the domain corresponding to the long cytoplasmic tail of CEACAM1. The rationale behind this design is to exclude the CEACAM1 extracellular domain responsible for its angiogenic properties.

Using chimeric proteins as a model, our group has previously demonstrated that the GPI anchor of CEA plays a crucial role in the inhibition of L6 myogenic differentiation (Screaton et al., 2000). The expression of both chimeric proteins BC and

NC, consisting of the CEA-GPI anchor bound to the extracellular domains of CEACAM1 and NCAM, respectively, on the surface of L6 myoblasts inhibited myogenic differentiation (Screaton et al., 2000). These results indicate that the GPI anchor of CEA carries specific information necessary to inhibit myogenic differentiation since cell surface expression of the wild type versions of both CEACAM1 and GPI-bound NCAM molecules has no effect. The fact that NCAM is GPI-bound but has no effect indicates that the effects of the CEA-GPI anchor are specific. It has been suggested that these GPI anchors are structurally and functionally different (Screaton et al., 2000). Furthermore, cell surface expression of the chimeric protein CB, consisting of CEACAM1 cytoplasmic tail and transmembrane domains fused to the extracellular domains of CEA, had no effect on myogenic differentiation. Based on these results, we hypothesized that the CEA inhibitory effect on anoikis, like its effect on cell differentiation, might also depend on the mode of membrane anchorage.

In Appendix-A of this thesis, we demonstrated that the expression of the BC chimeric protein on the surface of L6 myoblasts not only inhibited myogenic differentiation (Screaton et al., 2000) but also anoikis of these cells. Conversely, the expression of the CB chimeric protein consisting of the substitution of the CEA-GPI anchor for the transmembrane domain of CEACAM1 had no effect. These results support the hypothesis that the GPI anchor of CEA is required for the inhibition of anoikis. Such an effect of the CEA-GPI anchor is specific since the GPI-anchor of NCAM had no effect.

However, in contrast to the inhibitory effect of NC on myogenic differentiation, the surface expression of NC did not inhibit anoikis despite of the presence of the CEA-GPI anchor. One interpretation of this result is that the inhibition of anoikis by CEA requires at least two molecular events. The first event could depend on the functions of the CEA-GPI anchor, whereas the second event could require specific molecular

interactions of the extracellular domains of CEA. This hypothesis differs slightly from a model previously proposed by our group to explain the role of the CEA-GPI anchor on the inhibition of L6 myogenic differentiation (Screaton et al., 2000).

In this model, two structural features of CEA are required for the inhibition of cell differentiation: 1) the CEA-GPI anchor and 2) extracellular domains with homotypic adhesive functions (e.g. CEA, CEACAM1 or NCAM extracellular domains). In contrast, CEA-inhibition of anoikis specifically requires CEA-like extracellular domains. Note that CEACAM1, but not NCAM, extracellular domains inhibit anoikis when bound to the cell surface through the CEA-GPI anchor. The possibility of a specific heterotypic interaction between CEA and another membrane protein immediately comes to mind. This hypothesis is currently under investigation. Based on this model, BC is effective in inhibiting anoikis because its CEACAM1 extracellular domain exhibits close homology with the corresponding domain of CEA. This homology may be sufficient to allow the GPI-bound extracellular domain of CEACAM1 and wild-type CEA to recognize the same heterotypic partner.

According to our model, NC does not inhibit anoikis because its NCAM-extracellular domain lacks sufficient homology with the corresponding domain of CEA. Therefore, it is unlikely that the extracellular domains of NCAM and CEA will interact with the same heterotypic partner. The mechanistic differences between CEA effects on cell differentiation and anoikis of L6 rat myoblasts might be due to the distinctive nature of both cellular phenomena. Nevertheless, the discovery of the role of the GPI anchor of CEA in the inhibition of anoikis and cell differentiation could provide a specific structural target for the development of anti-cancer drugs.

The discovery of the structural features of the CEA family that are responsible for the inhibition of cell differentiation and anoikis is of crucial importance for the understanding of the role of this family in human cancer. An alternative approach to

speed up this discovery process was based on functional assays to directly test CEA/CEACAM6 effects on cell differentiation and anoikis.

The CEA and CEACAM6-induced pan-inhibitory effects on cell differentiation (see Introduction to Chapter 3), anoikis and cell polarization extend to a number of cell lines including: L6 rat myoblasts, MDCK epithelial cells, 3T3-L1 and C3H10T1/2 fibroblasts, P19 teratocarcinoma cells, SW1222 human colorectal cancer cells, and Caco-2 human colorectal cancer cells (Chapter 3). Based on the variety of cell lines affected, we sought a molecular mechanism common to cellular functions, such as cell differentiation, anoikis and cell polarization, operative in many cell types. One such mechanism is the regulation of cell adhesion to the extracellular matrix (ECM). Many cell types require adhesion to their ECM to survive, to attain cell polarization and to differentiate (see Introduction in Chapter 1). In Chapter 3 of this thesis, we demonstrated that the over-expression of CEA or CEACAM6, but not CEACAM1 and GPI-bound NCAM, on the surface of L6 myoblasts increases cellular binding to fibronectin, a major ECM component. The regulation of cell-ECM adhesion by CEA/CEACAM6 might be responsible for the inhibitory effects of these molecules on cell differentiation and anoikis (see below).

Fibronectin functions, at least *in vitro*, as a negative regulator of myogenic, adipogenic, neurogenic and colonic differentiation (Chapter 1). These are the same differentiation processes found to be inhibited by CEA over-expression. The inhibition of these types of cell differentiation by both fibronectin and CEA supports the hypothesis that CEA-mediated increase in fibronectin binding could be responsible for such effects. To test this hypothesis, an anti-fibronectin monoclonal antibody was added to CEA and CEACAM6 expressing myoblasts. This antibody released the CEA/CEACAM6-imposed inhibition of myogenic differentiation (Chapter 3, Fig. 4). This result suggests that

CEA/CEACAM6-mediated inhibition of myogenic differentiation could be due to increased binding to fibronectin of CEA/CEACAM6 over-expressing myoblasts.

The main cellular receptor for fibronectin is the $\alpha_5\beta_1$ integrin receptor (Argraves et al., 1986; Argraves et al., 1987; Hautanen et al., 1989; Hocking et al., 1998; Hynes, 1992). CEA/CEACAM6 expressing L6 myoblasts bound to an immobilized anti- α_5 antibody to a greater extent than control L6 parental myoblasts or transfected L6 myoblasts expressing GPI-bound NCAM (Chapter 3, Fig.6). In addition, CEA and CEACAM6-expressing myoblasts showed an increase in their avidity for fibronectin. These results suggest that CEA/CEACAM6 over-expression could cause either an increase in the cell surface amount of $\alpha_5\beta_1$ and/or an activation of this integrin receptor. Since no increase in the cell surface levels of $\alpha_5\beta_1$ could be detected, we suggest that CEA/CEACAM6 over-expression activates the $\alpha_5\beta_1$ integrin receptor.

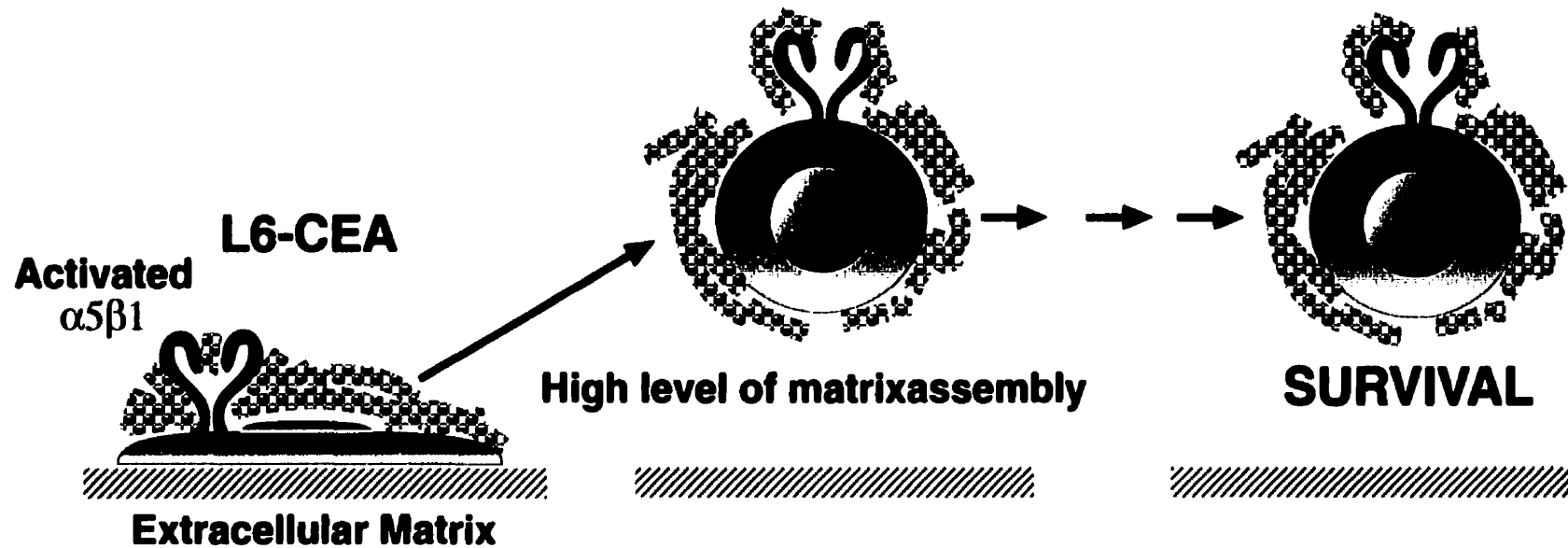
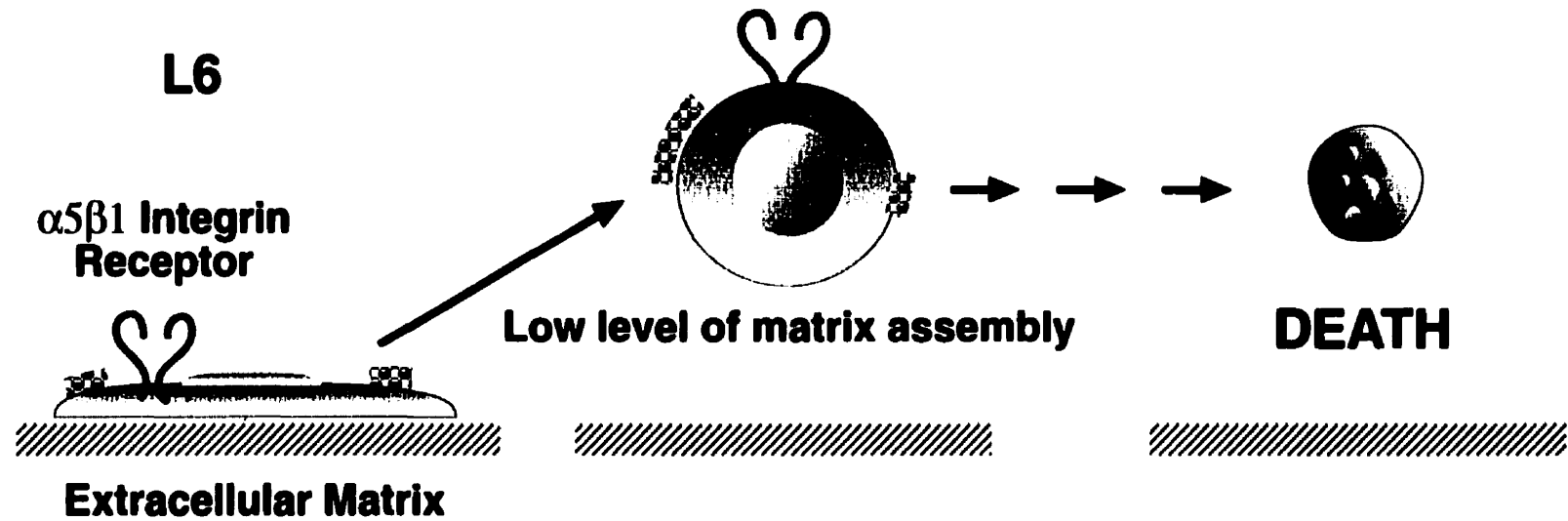
The $\alpha_5\beta_1$ integrin receptor plays an instrumental role in the polymerization of fibronectin, a process known as fibronectin matrix assembly (Schwarzbauer and Sechler, 1999; Wu, 1997). Polymerized fibronectin is the active isoform of this ECM component (Morla et al., 1994). The expression of CEA/CEACAM6 on the surface of L6 myoblasts causes a significant increase on fibronectin matrix assembly (Chapter 3, Fig. 7). We hypothesize that this dramatic increase in matrix assembly causes the formation of a *cocoon* of fibronectin coating the surface of CEA/CEACAM6 expressing myoblasts. This fibronectin cocoon impedes the fusion of L6 myoblasts thus completely abrogating myogenic differentiation. It has been shown that C2C12 myoblasts degrade their fibronectin matrix with the aid of proteases prior to the fusion event (Dourdin et al., 1997). According to our model, the elimination of the fibronectin cocoon should reestablish the myogenic differentiation of CEA/CEACAM6 expressing myoblasts. We found that the degradation of the fibronectin cocoon with mild trypsinization rescued the myogenic differentiation of these cells.

A more sophisticated way to measure the inhibitory role of the fibronectin cocoon is to interfere with the $\alpha_5\beta_1$ integrin receptor binding to fibronectin. The inhibition of the $\alpha_5\beta_1$ integrin-fibronectin interaction with specific monoclonal antibodies should significantly block matrix assembly (Fogerty et al., 1990). As mentioned above, the addition of an anti-fibronectin monoclonal antibody rescued the myogenic differentiation of CEA/CEACAM6 expressing myoblasts (Chapter 3, Fig. 4), presumably by inhibiting fibronectin matrix assembly.

Theoretically, the fibronectin cocoon should not only block myogenic differentiation but also protect these cells against anoikis (Fig. 1). Suspended cells lacking contact with their ECM undergo cell death. However, the coating of the plasma membrane with a fibronectin cocoon could provide a survival signal allowing the suspended cells to overcome the lack of anchorage.

Figure 1

Model of the inhibitory effect of CEA on the anoikis of L6 myoblasts. As described earlier, L6 myoblasts undergo anoikis in the absence of cell-ECM adhesion. We propose that the over-expression of CEA on the surface of L6 myoblasts causes the activation of the $\alpha_5\beta_1$ integrin receptor. As a result, the level of fibronectin polymerization on the surface of these cells is dramatically increased. This effect of CEA leads to the formation of a *cocoon* of fibronectin coating the cell surface and presumably replacing the survival signal normally provided by the ECM. As a consequence, CEA over-expressing L6 myoblasts become resistant to anoikis.



According to our model, the inhibition of fibronectin matrix assembly should rescue anoikis of CEA/CEACAM6 expressing cells. It has been previously shown that the addition of anti- α_5 monoclonal antibodies inhibit fibronectin matrix assembly *in vitro* (Fogerty et al., 1990; Pickering et al., 2000). Here, the addition of an anti- α_5 monoclonal antibody rescued anoikis of CEA/CEACAM6 expressing L6 myoblasts and Caco-2 human colorectal cancer cells (Chapter 3, Fig. 8). The same anti- α_5 monoclonal antibody did not rescue anoikis of transfected L6 myoblasts expressing bcl-2. The latter is a crucial control validating the specificity of CEA inhibitory mechanism. In the L6 system, the inhibition of anoikis by bcl-2 is independent of the $\alpha_5\beta_1$ integrin. Moreover, the simultaneous expression of CEA and bcl-2 in cotransfected L6 myoblasts had an additive but not a synergistic effect on the resistance of these cells to anoikis (data not shown).

In light of the fact that CEA and CEACAM6 are over-expressed in human colorectal cancer, the transfected Caco-2 cell line is a more relevant medical model to study CEA/CEACAM6 functions than transfected L6 myoblasts. However, the Caco-2 cell line is more difficult to study and some of the assays performed with L6 myoblasts are impossible to repeat with Caco-2 transfected cells. The fact that the inhibition of anoikis by CEA/CEACAM6 expression on the surface of Caco-2 cells is rescued by an anti- α_5 monoclonal antibody augments the significance of the results obtained with transfected L6 myoblasts.

The importance of the interaction of the $\alpha_5\beta_1$ integrin receptor with fibronectin for the survival of skeletal muscle cells has been previously demonstrated in knockout experiments (Taverna et al., 1998; Yang and Hynes, 1996). α_5 -null myoblasts showed defects in adherence and survival on fibronectin. The increased sensitivity of α_5 -null myoblasts to anoikis supports the notion that the $\alpha_5\beta_1$ integrin receptor provides a survival signal. We showed here that CEA/CEACAM6-mediated activation of the $\alpha_5\beta_1$ integrin receptor resulted in increased survival of L6 myoblasts.

The role of the $\alpha_5\beta_1$ integrin receptor in the regulation of apoptosis has been demonstrated in other cell systems than L6 myoblasts (Frisch and Ruoslahti, 1997). For instance, the $\alpha_5\beta_1$ integrin inhibits apoptosis of neurons in a model system for Alzheimer disease (Matter et al., 1998) and in colorectal cancer cells deprived of serum (O'Brien et al., 1996). Based on these results, we hypothesize that the regulation of the $\alpha_5\beta_1$ integrin receptor by CEA and CEACAM6 might affect the behavior of many cell types. This hypothesis is currently under investigation.

The demonstration of the inhibitory effect of CEA and CEACAM6 on anoikis raises the question of whether other types of apoptosis are affected by the over-expression of these cell surface molecules. In a previous report, CEA did not inhibit v-myc-induced apoptosis in doubly transfected L6 myoblasts (Screaton et al., 1997). This result suggests that v-myc triggers an apoptotic pathway that is independent of CEA regulation. However, the forced expression of CEA by cDNA transfection on the surface of Ha-ras-transformed NIH 3T3 fibroblasts caused resistance to adriamycin (Kawaharata et al., 1997), an anti-cancer drug known to induce apoptosis of the treated cells. The authors found an increase in the efflux of the drug in this model system.

In Appendix-B of this thesis, we showed that both CEA and bcl-2 expression in L6 myoblasts inhibited sodium orthovanadate and taxol-induced apoptosis. Sodium orthovanadate is an inhibitor of protein tyrosine phosphatases (Figiel and Kaczmarek, 1997), whereas taxol is a chemotherapeutic agent used in the treatment of cancers such as breast, ovarian, lung and other malignancies (Belani, 2000; Crown and O'Leary, 2000; Khayat et al., 2000). These observations support the notion that CEA functions as an inhibitor of certain apoptotic pathways. However, the precise nature of the mechanism of such resistance is presently unknown. The apoptotic mechanism triggered by sodium orthovanadate is not very well understood. Therefore, it is difficult to explain how CEA

inhibits vanadate-induced apoptosis. Whether there is a relationship between the inhibition of anoikis and vanadate-induced apoptosis remains to be seen.

The administration of taxol has been relatively successful in the treatment of cancer patients (Belani, 2000; Khayat et al., 2000). Therefore, the hypothesis that CEA over-expression might cause resistance to taxol is of significant clinical interest. Taxol targets the cytoskeleton of cancer cells inhibiting cell migration and mitotic division, and in addition, induces apoptosis (Perkins et al., 2000; Schmidt et al., 2000; Weigel et al., 2000). We do not know whether there is a link between CEA inhibition of anoikis and its effect on taxol sensitivity. However, it is possible that both phenomena relate to each other.

There are two common denominators between CEA-induced resistance to taxol and inhibition of anoikis:

- *Anoikis, like taxol, targets the cytoskeleton of the cell.* Anoikis can be induced by disruption of the cellular cytoskeleton (Rosen et al., 2000) (Chen et al., 1997a). Cytoskeletal proteins are one of the main targets of caspases (Kothakota et al., 1997).
- *Both resistance to taxol and anoikis depend on integrin functions.* Integrin receptors are key regulators of anoikis and are physically associated to the cytoskeleton (Giancotti and Ruoslahti, 1999).

As discussed above, integrin receptors are key regulators of anoikis. Recently, it has been suggested that integrin receptors could be a target of taxol. CD18, a component of the β_2 integrin family, is a taxol-binding protein (Bhat et al., 1999). In addition, Akt, a ser/thr kinase involved in integrin signaling and anoikis inhibition, is responsible for resistance to taxol in ovarian cancer cells (Page et al., 2000).

The relationship between integrin functions and drug resistance is not limited to taxol. Drug-sensitive 8226 human myeloma cells become resistant to the anti-cancer

drugs doxorubicin and melphalan when bound to fibronectin (Damiano et al., 1999). The level of expression of the $\alpha_5\beta_1$ integrin receptor, a member of the same integrin family as $\alpha_5\beta_1$, is higher in drug-resistant than in drug-sensitive 8226 human myeloma cells. In light of the fact that CEA regulates the function of the $\alpha_5\beta_1$ integrin receptor in L6 myoblasts (Chapter 3), we speculate that CEA-mediated resistance to taxol (Appendix-B) could be caused by CEA effects on integrin function.

We have identified the $\alpha_5\beta_1$ integrin receptor as a downstream target of CEA/CEACAM6. However, we do not know whether CEA or CEACAM6 directly interact with this integrin. CEA and CEACAM6 function *in vitro* as homotypic intercellular adhesion molecules (Chapter 1). It is possible that these molecules form *clusters* on the cell surface based on parallel and anti-parallel homotypic interactions. The entrapment of the $\alpha_5\beta_1$ integrin receptor in these clusters could cause activation of such a receptor. Integrin receptors of the β_1 subfamily are known to be activated by membrane clustering (Hemler et al., 1996).

A second candidate mechanism is the induction of a signal transduction pathway by CEA/CEACAM6 resulting in the activation of the $\alpha_5\beta_1$ integrin receptor. If this hypothesis is correct, the obvious step is to identify the molecular elements of such a pathway. One candidate is the integrin-linked kinase (ILK), a ser/thr kinase discovered through a two hybrid screening using the β_1 integrin as a bait (Hannigan et al., 1996). The over-expression of ILK in rat intestinal epithelial cells caused activation of the $\alpha_5\beta_1$ integrin receptor, increased fibronectin matrix assembly and inhibited anoikis (Wu et al., 1998), thus resembling the phenotype of CEA/CEA expressing L6 myoblasts (Chapter 3, this Thesis). Furthermore, ILK is endogenously expressed in C2C12 mouse skeletal myoblasts and its level of expression decreases upon the induction of myogenic differentiation (Huang et al., 2000). The forced over-expression of ILK, using cDNA transfection methods, inhibited myogenic differentiation of C2C12 myoblasts (Huang et

al., 2000). These results lead to the hypothesis that ILK might be a component of the CEA-induced signal transduction pathway. As it is the case for ILK, the forced expression of CEA in C2C12 myoblasts inhibits myogenic differentiation (Screaton et al., 1997).

The requirement of the CEA-GPI anchor, but not of the NCAM-GPI anchor, for the inhibition of anoikis suggests that the former may carry specific biological information regulating $\alpha_5\beta_1$ integrin functions. It is known that the $\alpha_5\beta_1$ integrin receptor binds Fyn, a member of the Src-family of protein tyr kinases, through the membrane adapter protein caveolin-1 (Giancotti and Ruoslahti, 1999; Pande, 2000; Wei et al., 1996). The latter is one of the main constituents of raft or DIG membrane microdomains rich in both GPI-bound glycoproteins and signal transduction molecules such as Lck and Fyn (Draberoova and Draber, 1993; Pande, 2000). Using specific detergents, Lck has been coimmunoprecipitated with CEA in a transfected basophilic leukemic cell line model system (Peter Draber and C.P. Stanners, unpublished observations). However, it is not yet known whether the $\alpha_5\beta_1$ integrin receptor colocalizes with Lck and CEA in this model system. The possible colocalization of GPI-bound CEA/CEACAM6 and the $\alpha_5\beta_1$ integrin receptor in the same membrane microdomains is a hypothesis currently under study in our laboratory.

The urokinase plasminogen activator receptor (uPAR) is an example of a GPI-bound cell surface protein that binds to and regulates the functions of the β_1 integrin (Ossowski and Aguirre-Ghiso, 2000). uPAR, like CEA, is over-expressed in human cancer (Ossowski and Aguirre-Ghiso, 2000; Wilhelm et al., 1999). Recently, the integrin binding site of uPAR has been identified (Simon et al., 2000). Other GPI-bound proteins that directly bind to and regulate integrins are: lipopolysaccharide (LPS) binding protein (LBP) receptor (CD14), and Fc γ receptor IIIB (CD16b) (Sendo et al., 1998).

The role of the CEA-GPI anchor in the inhibition of cell differentiation and anoikis raises the question of whether this mode of membrane anchorage represents a gain of function by the CEA family during evolution. GPI anchored protein members of the CEA family arose later in evolution just prior to the primate radiation (see Chapter 1). The inhibitory functions of GPI-bound CEA/CEACAM6 may play a role in tissue patterning during embryogenesis. There are other examples of GPI anchored proteins whose functions depend on the mode of membrane linkage. For instance, the transmembrane and non-pathogenic isoform of the prion protein becomes the infectious scrapie isoform when GPI-bound to the membrane (Kaneko et al., 1997). The lymphocyte surface antigen Qa-2 is only capable of T cell activation when GPI-bound whereas its transmembrane version is ineffective (Robinson et al., 1989).

As mentioned above, GPI anchored proteins are localized to the apical membrane whereas integrins are found in the basolateral membrane of polarized epithelial cells. In this context, it is unlikely that GPI-bound CEA/CEACAM6 affect integrin functions. However, cell polarization is disrupted in cancer cells. Therefore, we propose that CEA/CEACAM6 over-expression over the entire surface of a cancer cell interferes with integrin functions. The question arises as to whether CEA/CEACAM6 over-expression causes a disruption of cell polarization or it is a consequence of the lack of cell polarization of the cancer cell. Recently, our group has shed light into this problem by demonstrating that CEA/CEACAM6 over-expression on the surface of Caco-2 human colorectal cancer cells disrupted cell polarization (Ilantzis C., L. DeMarte, R. Screaton, C.P. Stanners, submitted for publication).

The specific nature of the structure and functions of the GPI anchors of CEA family members is complicated by the discovery of GPI-bound CEACAM7 (formerly CGM2). CEACAM7 is, like CEA and CEACAM6, normally expressed on the apical membranes of colonocytes at the upper third of the human colonic crypts (Thompson et

al., 1997a). However, the scenario is very different in cancer cells. The expression levels of CEACAM7, despite being a glycoprotein linked to the plasma membrane by a GPI anchor as CEA/CEACAM6, are down-regulated in human colorectal adenocarcinomas (Nollau et al., 1997) (Thompson et al., 1997a). Based on this, CEACAM7 is thought to function, like CEACAM1, as a tumor suppressor protein (Thompson et al., 1997a). Our current hypothesis is that CEACAM7 contains a specific GPI-anchor that is structurally and functionally different from the GPI anchors of CEA and CEACAM6.

The notion that CEA over-expression contributes to tumor formation has been challenged by data obtained in transgenic experiments (Eades-Perner et al., 1994; Hasegawa et al., 1991; Thompson et al., 1997b). Mice expressing CEA in their intestines appear to be normal and do not develop tumors. We believe this is because in these animals, CEA was expressed under the regulation of its natural promoter resembling the localization pattern observed in the normal human colon. CEA is normally expressed on the apical surface at the top portion of the human colonic crypts (Benchimol et al., 1989). In such a location, it is unlikely that CEA will regulate integrin functions. Integrins are expressed on the basolateral membrane of most epithelial cells including colonocytes (Eaton and Simons, 1995; Nigam et al., 1993; Ojakian and Schwimmer, 1994; Stallmach et al., 1992; Wang et al., 1990). However, in cancer cells CEA is over-expressed over the entire surface, including the basolateral membrane, of cancer cells (Benchimol et al., 1989). Our model contends that such over-expression of CEA over the entire surface of cancer cells perturbs integrin functions, thus inhibiting anoikis and cell differentiation (Chapter 2, Fig. 4). According to this model, the over-expression of CEA in colonic stem cells with mitotic potential should indeed contribute to tumorigenesis. The latter could be achieved by transgenically expressing CEA under the regulation of the fatty acid binding promoter (Simon et al., 1997). This experiment is currently ongoing in our laboratory.

The inhibition of cell differentiation, anoikis and cell polarization by CEA/CEACAM6 should contribute to the malignant phenotype of cancer cells. In addition to the experimental evidence provided in this thesis, the following reports support the oncogenic role of CEA/CEACAM6: 1) CEA/CEACAM6 over-expression on the surface of Caco-2 human colorectal cancer cells inhibited cell polarization and increased tumor formation when these cells were injected into nude mice (Ilantzis C., L. DeMarte, R. Screaton, C.P. Stanners, submitted for publication); 2) in freshly excised colonocytes from human tumors, the expression of CEA was inversely correlated with their degree of cell differentiation. In this study, higher levels of cell surface expression of CEA were found in poorly differentiated tumors (Ilantzis et al., 1997); 3) the disruption of the basolateral polarity in colon epithelial cells by the c-Ki-ras oncogen was accompanied by an increase in the amount of CEA over the entire cell surface, including the basolateral membrane, of these cells (Yan et al., 1997); 4) the amount of CEA on the surface of HD6 colon carcinoma cells was increased 3-fold when these cells were prevented from polarizing (Yan et al., 1993); 5) Our group reported previously that CEA cooperates with both v-myc and bcl-2 in the formation of tumors by cotransfected L6 myoblasts (Screaton et al., 1997). This report proposed that such a powerful combination of oncogenes, including inhibitors of cell proliferation (v-myc), apoptosis (bcl-2) and cell differentiation (CEA) was responsible for the dramatic increase in tumorigenicity. In this thesis, we demonstrated another potential mechanism contributing to such tumorigenic effects: the inhibition of anoikis by both CEA and bcl-2 (Chapter 2 &3).

In conclusion, we propose that CEA family members play an instrumental role in tumor formation and progression. CEACAM1 expression is down-regulated in early stages of human tumors of the prostate and colon (Chapter 1) and inhibits tumor formation in nude mice (Chapter 4 and references therein). Recently, CEACAM1 has been proposed as a potential gene therapy candidate (Kleinerman et al., 1995b). In

contrast, CEA and CEACAM6 over-expression on the surface of several cancer cell lines inhibits cell differentiation, cell polarization and anoikis (Chapters 2&3 and references therein), thus instrumentally contributing to tumor formation and progression. We contend that such inhibitory effects of CEA and CEACAM6 are due to the functional perturbation of the $\alpha_5\beta_1$ integrin receptor (Chapter 3). The precise molecular interactions responsible for such an oncogenic mechanism are currently the subject of intense scrutiny since CEA and CEACAM6 are potential valuable targets for the development of novel anti-cancer therapies.

Appendix-A

The Glycosylphosphatidyl Inositol (GPI) anchor of the Human Carcinoembryonic Antigen is Required to Inhibit Anoikis

Introduction

The extracellular domain of CEA is bound to the plasma membrane by a glycosylphosphatidyl inositol (GPI) anchor. This type of linkage to the plasma membrane distinguishes CEA from other members of the family such as CEACAM1 (formerly BGP_a or Biliary glycoprotein). Unlike CEA, CEACAM1 contains a transmembrane domain followed by a cytoplasmic tail. The extracellular domain of CEACAM1 has a relative high degree of homology to the corresponding domain of CEA.

Based on the structural features of both molecules, it has been proposed that the mode of binding to the plasma membrane is responsible for the distinctive and sometimes antagonistic functions of CEACAM1 and CEA. Although both CEA and CEACAM1 function *in vitro* as intercellular adhesion molecules, CEA but not CEACAM1 is over-expressed in more than 50% of all human cancers. In contrast, CEACAM1 expression is down-regulated in early stage tumors of the prostate and colon. CEA increases tumor formation when Caco-2 human colorectal cancer cells over-expressing CEA on their surfaces are injected into nude mice.

In contrast, the forced expression of murine CEACAM1 on the surface of murine colorectal and prostatic cancer cells decreases the formation of tumors when these cells are injected into syngeneic and nude mice respectively. In addition, CEA but not CEACAM1, functions as a pan-inhibitor of cell differentiation, cell polarization and anoikis *in vitro*. Furthermore, CEACAM6, another CEA family member that like CEA binds the plasma membrane through a GPI-anchor, shares the same pro-oncogenic properties of CEA.

The hypothesis that the mode of binding to the plasma membrane determines the antagonistic functions of CEA and CEACAM1 has been recently tested experimentally with chimeric proteins consisting of the CEA-specific GPI anchor fused to the

extracellular domains of the neural cell adhesion molecule (NCAM) and CEACAM1 (Screaton et al., 2000).

NCAM, another member of the Immunoglobulin Superfamily, is a key control in these experiments because unlike CEA do not inhibit but rather stimulates the myogenic differentiation of L6 myoblasts. The isoform of NCAM used in our studies is also bound to the plasma membrane by a GPI-anchor. However, the structural nature of the NCAM-specific GPI anchor seems to be functionally different than the CEA-specific GPI anchor (Screaton et al., 2000).

The expression of a chimeric protein consisting of NCAM extracellular domain fused to the CEA-specific GPI anchor on the surface of L6 myoblasts inhibited myogenic differentiation (Screaton et al., 2000). This result indicates that the CEA-specific GPI anchor carries biological information necessary to inhibit myogenic differentiation. This notion was supported by two additional chimeras, BC and CB, consisting of CEACAM1 (former BGP_a) extracellular domain fused to CEA-specific GPI anchor (BC) and the CEA extracellular domain fused to CEACAM1 transmembrane domain and cytoplasmic tail (CB). BC, bound through CEA-specific GPI anchor, but not CB containing a transmembrane domain, expression on the surface of L6 myoblasts inhibited myogenic differentiation of these cells.

We demonstrate here, utilizing the chimeric proteins described above, that the CEA-specific GPI anchor is not only required to inhibit myogenic differentiation but also anoikis of L6 myoblasts. However, unlike the effect of CEA on cell differentiation where the GPI anchor is sufficient to inhibit such process, an additional molecular event involving the extracellular domain of CEA is also necessary for the inhibition of anoikis.

Results and Discussion

CEA-specific GPI anchor is necessary for CEA-mediated inhibition of anoikis

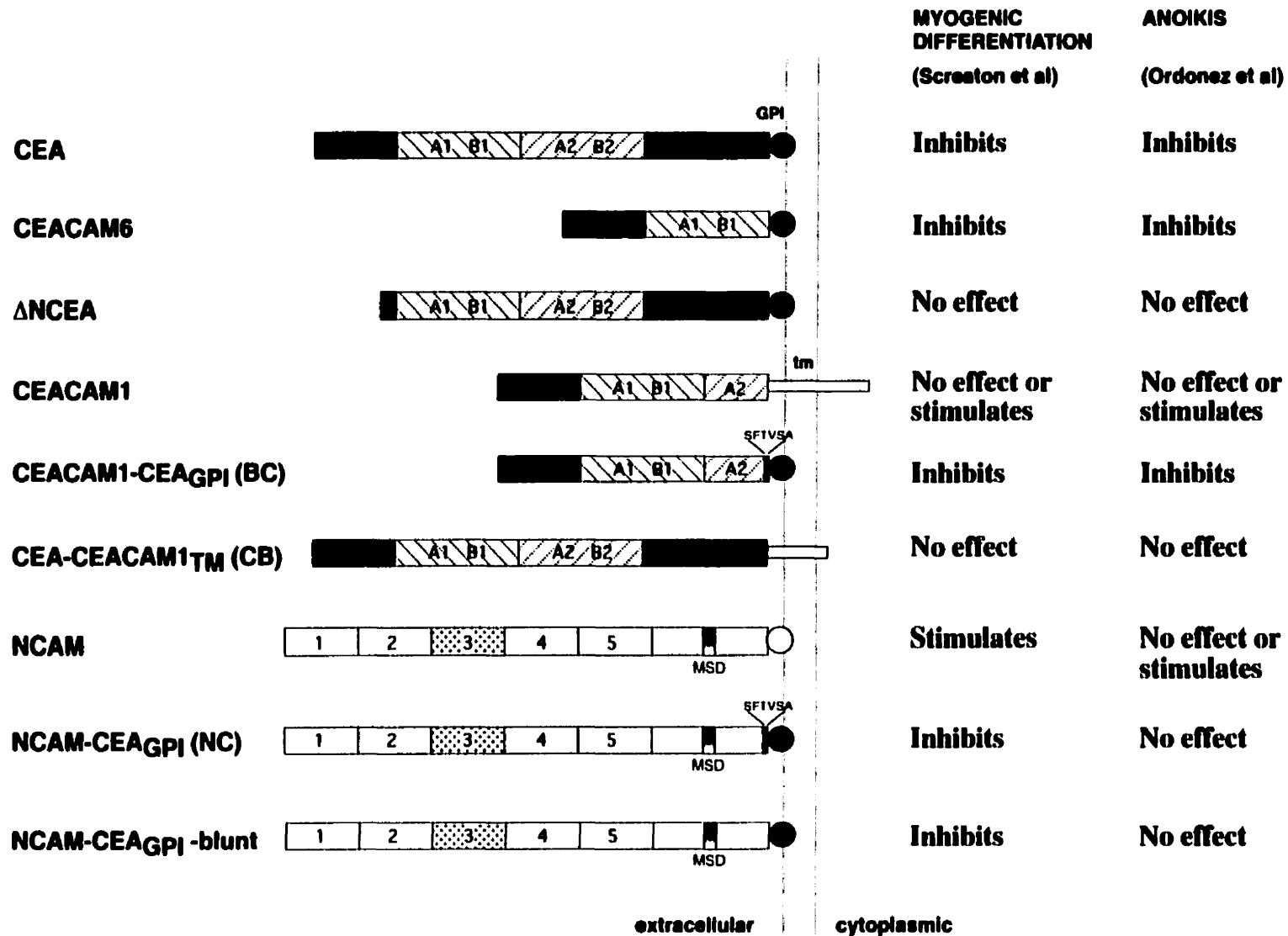
The GPI anchor of CEA seems to carry sufficient biological information by itself to modify cellular behavior (Screaton et al., 2000). This notion is supported by the inhibition of L6 myogenic differentiation by a chimeric protein consisting of the CEA-specific GPI anchor fused to the extracellular domain of NCAM (Screaton et al., 2000). NCAM is also a member of the Ig Superfamily and, like CEA, functions as an intercellular adhesion molecule *in vitro*. However, unlike CEA, NCAM do not inhibit but rather stimulates the myogenic differentiation of L6 myoblasts.

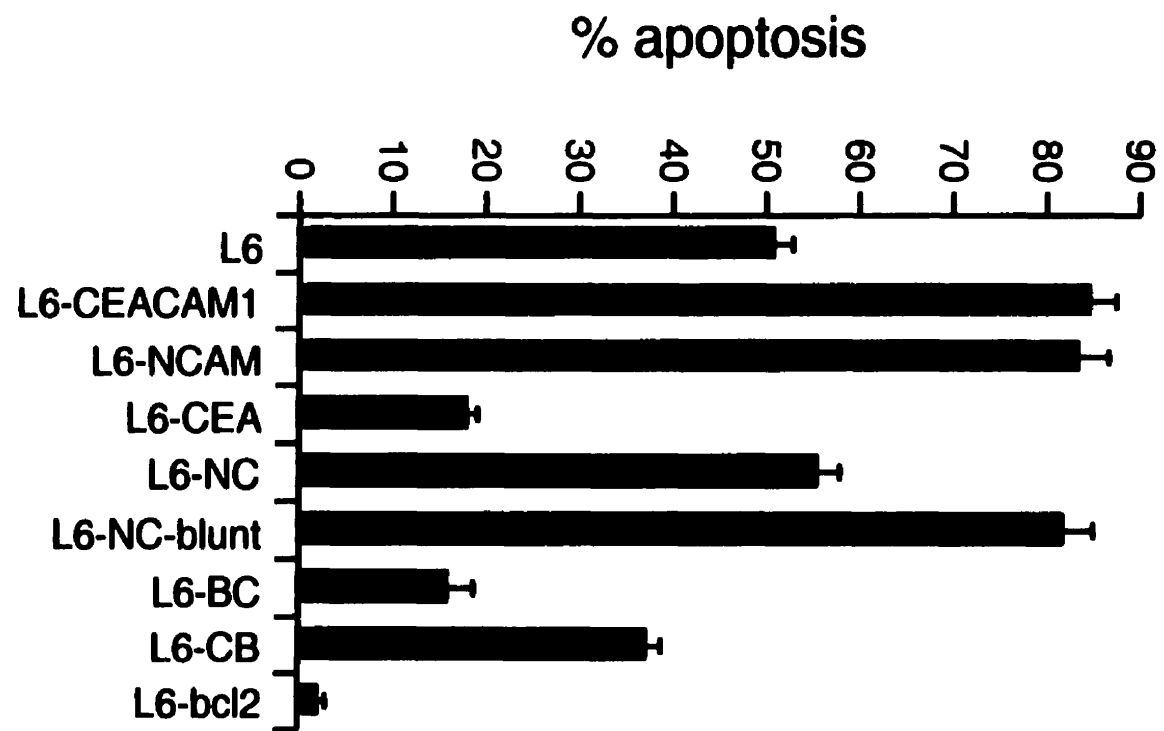
We have recently demonstrated that the ectopic expression of CEA on the surface of L6 myoblasts inhibits anoikis of these cells. To test whether the CEA-specific GPI anchor carries information required not only to inhibit myogenic differentiation but also anoikis, we utilized chimeric proteins consisting of the CEA-GPI anchor fused to either CEACAM1 (BC) or NCAM (NC) extracellular domains. These chimeric proteins were expressed on the surface of L6 myoblasts utilizing cDNA transfection procedures as previously described (Screaton et al., 2000). The composition and abbreviations of each chimera are presented in Figure 1A.

Figure 1

A) The diagram shows the structure of the glycoproteins encoded by the chimeric constructs utilized in this study. The extracellular domains N, A and B of each glycoprotein are shown. Only CEACAM1 and CB contain a transmembrane domain and a cytoplasmic tail. The rest of the glycoproteins are bound to the external surface of the plasma membrane through GPI-anchors. The effects of the transfection of each construct in L6 myogenic differentiation (Screaton et al) and anoikis is shown. B) DAPI staining of suspended L6 transfected myoblasts (see Experimental Procedures). The expression of the chimeric protein BC on the surface of L6 rat myoblasts inhibited anoikis of these cells when suspended on polyHEMA coated surfaces. Apoptotic cells showed characteristic fragmented nuclei while survivors showed intact nuclear morphology. The apoptotic index was calculated by scoring the percentage of apoptotic cells in three independent samples of 500-1000 cells each. The statistical average and standard deviation are indicated.

A)





B)

L6 myoblasts expressing the BC chimeric protein (CEACAM1 extracellular domain fused to CEA-specific GPI anchor) on their surfaces are less prone to undergo anoikis than control cell lines consisting of untransfected L6 parental myoblasts, and transfected L6 myoblasts expressing either NCAM (GPI- bound isoform of NCAM) or CEACAM1 on their surfaces (Fig. 1). This result indicates that the CEA-specific GPI anchor is necessary for the inhibitory effect of the CEA glycoprotein. A control cell line expressing CB, a chimeric protein consisting of CEA extracellular domain fused to CEACAM1 transmembrane domain and cytoplasmic tail, underwent anoikis as readily as the L6 parental cells (Fig. 1). L6 myoblasts expressing bcl-2, an inhibitor of anoikis and other types of apoptosis, and CEA on their surfaces were resistant to anoikis (Fig. 1).

The demonstration of the requirement of the CEA-specific GPI anchor for the inhibition of anoikis and cell differentiation raises the question of molecular mechanisms. It is possible that the GPI anchor has specific interactions with other cell surface molecules. The biochemical interactions of the GPI anchor of CEA are presently unknown. Another explanation for the effects of the GPI anchor is that it provides to the CEA glycoprotein an increased capacity for clustering relative to transmembrane proteins such as CEACAM1. A third but not excluding hypothesis is that the GPI-anchor provides CEA with a specific localization on unique microdomains in the plasma membrane allowing interactions with other cell surface proteins like the $\alpha_5\beta_1$ integrin receptor. We have recently demonstrated that CEA inhibits anoikis and cell differentiation through regulation of the $\alpha_5\beta_1$ integrin receptor (Chapter 3). The possible link between the function of CEA-specific GPI anchor and the $\alpha_5\beta_1$ integrin receptor is currently under study.

The CEA-specific GPI anchor is necessary but not sufficient to inhibit anoikis

The expression of NC, a chimeric protein consisting of the CEA-specific GPI anchor fused to the extracellular domain of NCAM, on the surface of L6 myoblasts inhibits the myogenic differentiation of these cells (Screaton et al., 2000). In contrast, the NC chimeric protein did not inhibit anoikis of L6 transfected myoblasts (Fig. 1). This result suggests that other molecular requirements besides the CEA-specific GPI anchor are necessary for the inhibitory effect.

Both NC and BC chimeric proteins contain the CEA-specific GPI anchor. However, unlike NC, the BC chimeric protein was capable of inhibiting anoikis of L6 transfected myoblasts. The extracellular domain of CEACAM1, but not the corresponding domain of NCAM, has striking homology with the extracellular domain of CEA. The latter suggests that CEACAM1 might be able to mimic a CEA-specific interaction that together with the functions of the CEA-GPI anchor leads to the inhibition of anoikis.

We hypothesize that the GPI- anchor of CEA is required for its inhibitory effect on anoikis, but a second molecular signal provided by the extracellular domain of CEA is required for the effect. This signal could be triggered by the homotypic interaction of two CEA extracellular domains or by the heterotypic interaction of CEA-extracellular domain with another cell surface receptor. Both possibilities are currently under study. It is intriguing that the mechanisms of the inhibitory effect of CEA on myogenic differentiation and anoikis differ. The biological significance of this difference is not known. Hypothetically, it could be beneficial to the organism to be able to regulate distinctively cell differentiation and anoikis.

We have previously proposed a model where the over-expression of CEA on the surface of cancer cells inhibits anoikis thus promoting the outgrowth of the malignant cells beyond their normal spatial constraints (Chapter 2). The discovery of the role of the

CEA-GPI anchor on the inhibition of anoikis not only shed light into the understanding of the molecular mechanism of such effect but also provides a structural target to develop new anti-cancer therapies.

Experimental Procedures

Cell Lines

L6 rat myoblasts were grown as monolayer cultures in DMEM containing 10% FBS (GIBCO BRL, Gaithersburg, MD) supplemented with 100 µg/ml streptomycin and 100 U/ml penicillin (GIBCO BRL) at 37°C in a humidified atmosphere with 5% CO₂. These cells were always subcultured in exponential phase of growth and seeded at a density of 1×10^4 cells/cm².

cDNA Transfections

The transfected cell lines used in this study have been previously described (Screaton et al., 2000). Briefly, stable transfectants of cell lines L6 were obtained by the calcium phosphate precipitation method using the p91023B expression vector containing full length cDNAs encoding cell adhesion proteins: CEA, CEACAM1-4L (formerly splice variant BGPa), CEA deletion mutant Δ NCEA (lacking the last 75 amino acids of the N domain), human NCAM-125 [GPI-linked NCAM splice variant with muscle specific domain, BC (cDNA encoding a chimeric protein consisting of CEACAM1 (former BGPa) extracellular domain fused to CEA-specific GPI anchor) (Fig. 1A), CB (cDNA encoding a chimeric protein consisting of CEA extracellular domain fused to CEACAM1 transmembrane domain and cytoplasmic tail), NC (cDNA encoding a chimeric protein consisting of NCAM extracellular domain fused to CEA-specific GPI anchor) (Fig. 1A), and pSV2neo plasmid as a dominant selectable marker.

Pooled populations of transfectant clones were used in this study to exclude clonal variation as a factor determining phenotypic properties of the transfected cells. Transfectants expressing the chimeric protein CB were the only exception. These cells were derived from a single high expressor clone. The failure to produce a total population could be due to the nature of the CB chimeric protein. The rest of the transfected cell lines used in this study consisted of pooled clones.

Pooled clones of stably transfected cells were selected with 400 µg/ml of Neomycin (G418). L6 myoblasts expressing high surface levels of the proteins encoded by the transfected cDNAs were selected by FACS using specific monoclonal antibodies. The transfectant populations (L6) were enriched for more stably expressing cells by culturing without G418 for ~20 doublings, followed by FACS re-selection for high expressors.

The preparation of L6 myoblasts ectopically expressing bcl-2 has been previously described. Briefly, L6 cells in the exponential phase of growth were infected with replication-defective recombinant retrovirus containing either pBabe(human bcl-2)puro or the vector alone as a control.

All cultures of stably transfected L6 used in the experiments described here were obtained from early passages of frozen stocks.

Anoikis Assays

Anoikis was measured as described in Chapter 2 of this thesis. Briefly, both L6 parental and transfected myoblasts were cultured in suspension in POLYHEMA coated 6-well tissue culture plates at a concentration of 0.2×10^6 cells/ml for a period of 48 hours. The percentage of apoptotic cells was estimated by staining the nuclei with DAPI. The slides were evaluated in a fluorescence microscope and cells with fragmented nuclei were

scored as apoptotic cells. The apoptotic index was calculated by scoring 1000 cells or more. All observations were reproduced in more than two independent experiments.

Appendix-B

Human Carcinoembryonic Antigen Inhibits Orthovanadate-Induced Apoptosis of L6 Rat Myoblasts

Introduction

Human Carcinoembryonic Antigen (CEA), a widely used tumor marker, and CEACAM6 (formerly NCA) are members of a family of intercellular adhesion molecules. Both CEA and CEACAM6 are over-expressed in many types of human cancers. The over-expression of CEA/CEACAM6 on the surface of many different cell types inhibit cell differentiation, anoikis, cell polarization and disrupt tissue architecture. Here, we show over-expression can also inhibit other types of apoptosis. The forced expression of CEA in L6 rat myoblasts markedly inhibit apoptosis induced by sodium orthovanadate or taxol. In addition, CEACAM6 also inhibited the formation of lumen-containing intercellular cysts by SW1222 human colorectal cancer cells when these cells were treated with sodium orthovanadate.

Results and Discussion

CEA inhibits orthovanadate-induced apoptosis of L6 myoblasts

The forced expression of CEA on the surface of many cell types inhibits anoikis (see Chapter 2). To test whether CEA could inhibit other types of apoptosis, L6 myoblasts were treated with 100 μ M of sodium orthovanadate, an inhibitor of phosphotyrosyl protein phosphatases (PTPPs). L6 parental myoblasts readily underwent apoptosis in the presence of orthovanadate (Fig. 1A, 2) and showed DNA laddering characteristic of apoptosis (Fig. 1B). In contrast, CEA expressing L6 myoblasts were resistant to orthovanadate treatment (Fig. 1A, 2) and did not show any signs of apoptosis including DNA laddering (Fig. 1B). L6 myoblasts expressing Bcl-2, a known inhibitor of apoptosis

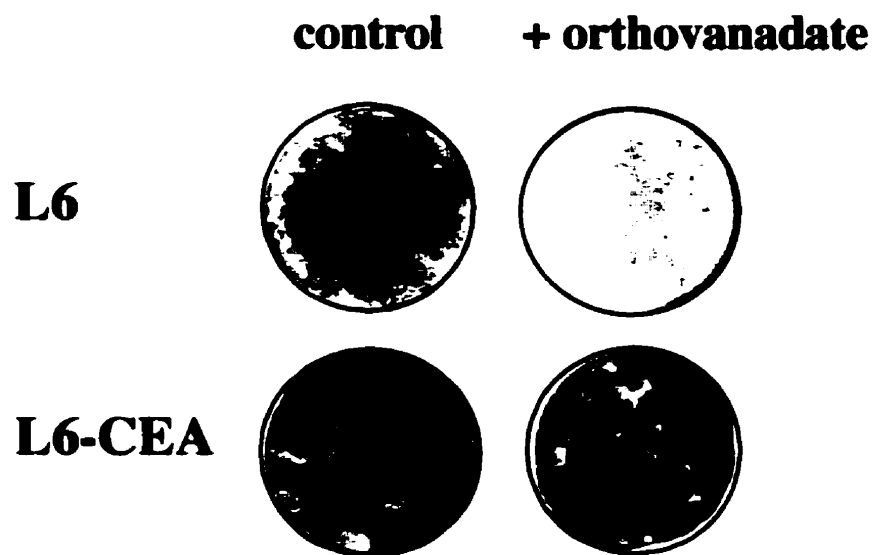
(Adams and Cory, 1998; Frisch and Ruoslahti, 1997), were also resistant to orthovanadate treatment (Fig. 1B, 2). Altogether, these results support the hypothesis that CEA over-expression in human tumors might not only inhibit anoikis, but also confers resistance to other types of apoptosis.

NCAM and CEACAM1, like CEA, are intercellular adhesion molecules, but in contrast to CEA, do not inhibit myogenic differentiation (see Introduction). These features make both NCAM and CEACAM1 suitable controls for the experiments described here. NCAM and CEACAM1 expressing L6 myoblasts underwent orthovanadate-induced apoptosis as readily as L6 parental myoblasts (Fig. 2). Since the isoform of NCAM used in this study is bound to the plasma membrane by a GPI-anchor, we propose that the CEA-mediated inhibition of apoptosis is specific and not due to an adventitious effect of its GPI-anchor.

Figure 1

Effect of CEA on orthovanadate-induced apoptosis of L6 transfected myoblasts. (a) Haematoxylin staining of L6 parental and transfected myoblasts treated with 20 and 100 μ M of sodium orthovanadate (see Experimental Procedures). CEA expressing L6 myoblasts were resistant to orthovanadate-induced apoptosis. This result was independently repeated five times. (b) Agarose gel electrophoresis showing DNA laddering characteristic of apoptotic cells in L6 parental myoblasts but not in CEA and Bcl-2 expressing myoblasts. This result was reproduced twice.

A



B

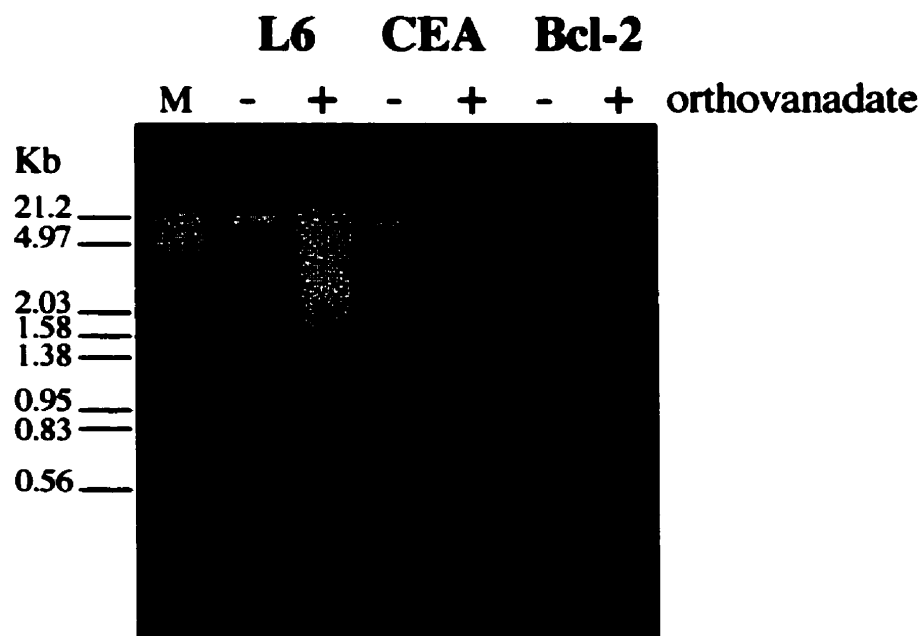


Figure 2

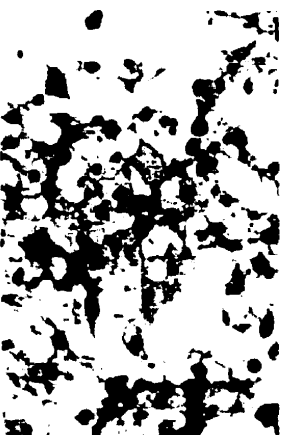
Effect of tyrphostin A30 on orthovanadate-induced apoptosis of L6 transfected myoblasts. Haematoxylin staining of L6 parental and transfected myoblasts treated with 100 μ M of sodium orthovanadate with and without tyrphostin A30. CEACAM1 expressing myoblasts are as prone to undergo orthovanadate-induced apoptosis as L6 parental cells. As demonstrated above, CEA and Bcl-2 expressing cells are resistant to orthovanadate treatment. However, when tyrphostin A30 was added simultaneously, CEA and Bcl-2 expressing L6 myoblasts became susceptible to orthovanadate-induced apoptosis. These results were reproduced three times.

control

+ orthovanadate

**+ orthovanadate
+ tyrphostin**

L6



L6-bcl-2



L6-CEA



L6-CEACAM1



The precise signaling pathway that leads to apoptosis of orthovanadate treated cells is unknown. The inhibition of PTPs by the drug must induce an increase in tyrosine phosphorylation that presumably triggers an apoptotic pathway. To demonstrate this hypothesis, the cells were treated simultaneously with orthovanadate and tyrphostin A30, an inhibitor of protein tyrosine kinases (see Experimental Procedures). Orthovanadate-induced apoptosis was prevented by the presence of tyrphostin A30, suggesting that apoptosis of L6 myoblasts was due to inhibition of PTPs and not to a secondary effect of the drug (Fig. 2).

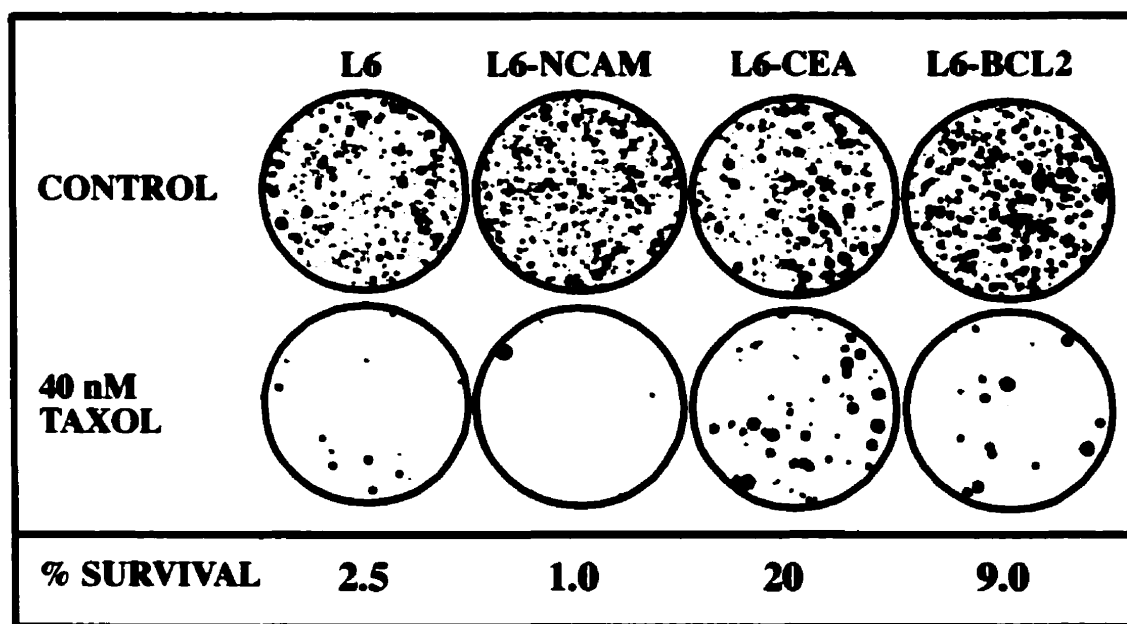
CEA inhibits taxol-induced apoptosis of L6 myoblasts

The precise nature of the anti-apoptotic event responsible for the CEA inhibitory effect is presently unknown. It seemed possible that, if CEA over-expression conferred resistance to apoptosis, it might also confer resistance to clinically important anti-cancer drugs. A previous report describing CEA-mediated resistance of 3T3 cells to adriamycin-induced apoptosis suggested that CEA might confer resistance to more than one apoptosis-inducing drug (Kawaharata et al., 1997). To test this hypothesis we treated L6 parental and transfected L6 myoblasts with paclitaxel (taxol).

Paclitaxel is one of the most effective chemotherapies used in the clinic today (Crown and O'Leary, 2000; Khayat et al., 2000). This drug is known to induce apoptosis of different cell lines in vitro (Fang et al., 2000; Gangemi et al., 2000; Page et al., 2000; Perkins et al., 2000; Schmidt et al., 2000; Weigel et al., 2000). The addition of 40 nM of taxol to the tissue culture medium induced apoptosis of L6 myoblasts (see Experimental Procedures). In contrast, CEA expressing L6 myoblasts were resistant to taxol (Fig. 3). L6 myoblasts expressing the human Bcl-2 protein survived the treatment with taxol significantly better than both L6 parental myoblasts and L6 myoblasts expressing GPI-bound human NCAM, but not better than CEA expressing myoblasts (Fig. 3).

Figure 3

Effect of CEA on taxol-induced apoptosis of L6 transfected myoblasts measured in a survival assay (see Experimental Procedures). Crystal violet staining of surviving colonies after 24 hours treatment with 40 nM of taxol. CEA and Bcl-2 expressing L6 myoblasts were less sensitive to taxol treatment than L6 parental myoblasts. (*% survival* was calculated using the initial number of cells seeded in the assay).



CEACAM6 inhibits orthovanadate-induced formation of lumen-type structures by SW1222 cells

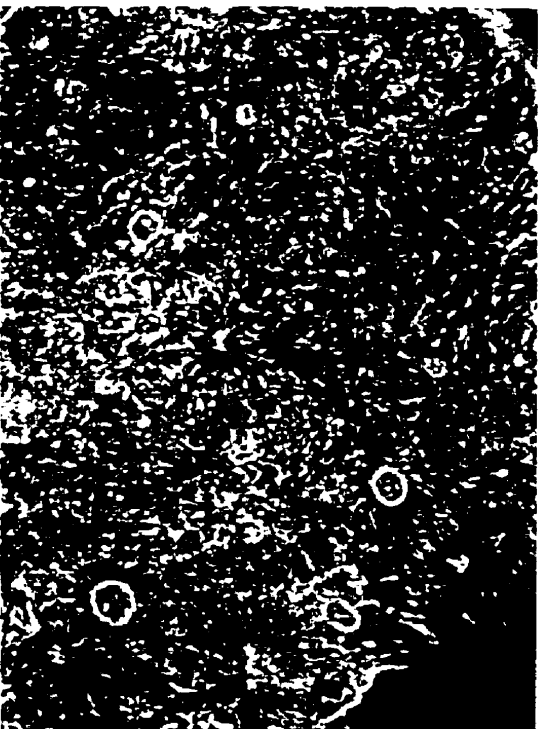
The formation of cysts of polarized epithelial cells facing a lumen is a complex morphogenetic process (Pignatelli and Bodmer, 1988; Richman and Bodmer, 1988). In the case of MDCK cells, the formation of the pseudolumen in the cyst includes an apoptotic event that is inhibited by Bcl-2 (Lin et al., 1999). By analogy, the formation of a pseudolumen by SW1222 may also include this apoptotic event. As demonstrated above, CEA and CEACAM6 inhibited orthovanadate-induced apoptosis of L6 myoblasts. Using CEACAM6 over-expressing SW1222 cells as a model, we tested the possibility that CEACAM6 might inhibit orthovanadate-induced apoptosis of SW1222 cells. CEA was not studied because we were unable to generate stable transfectants of SW1222 over-expressing CEA.

The treatment of SW1222 cells with orthovanadate markedly increased pseudolumen formation. CEACAM6 over-expression on the surface of SW1222 inhibited the formation of lumen containing cysts when these cells were treated with 100 μ M of sodium orthovanadate (Fig. 4). Based on these results, we hypothesize that the over-expression of CEA and CEACAM6 on the surface of cancer cells contributes instrumentally to tumor formation and progression by inhibiting not only anoikis but, in addition, other types of apoptosis as well.

Figure 4

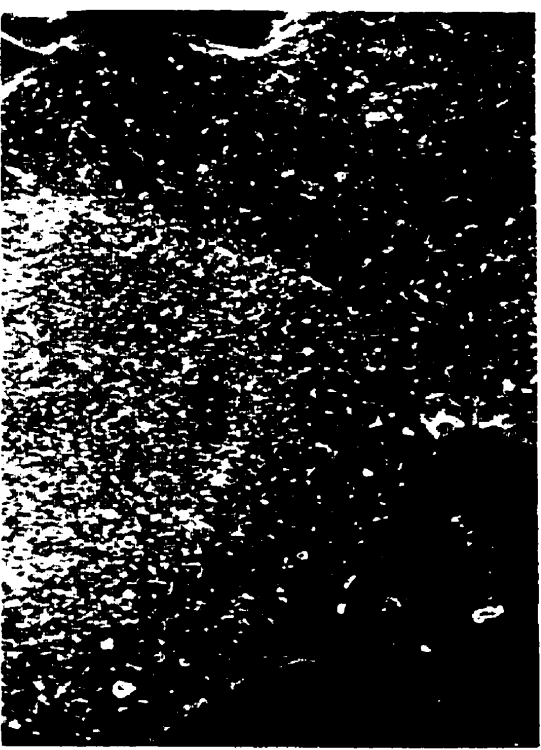
CEACAM6 over-expression on the surface of SW1222 inhibited the formation of lumen-containing intercellular cysts when these cells were treated with 100 μ M of sodium orthovanadate. This result was independently reproduced three times.

SW1222-Hygro

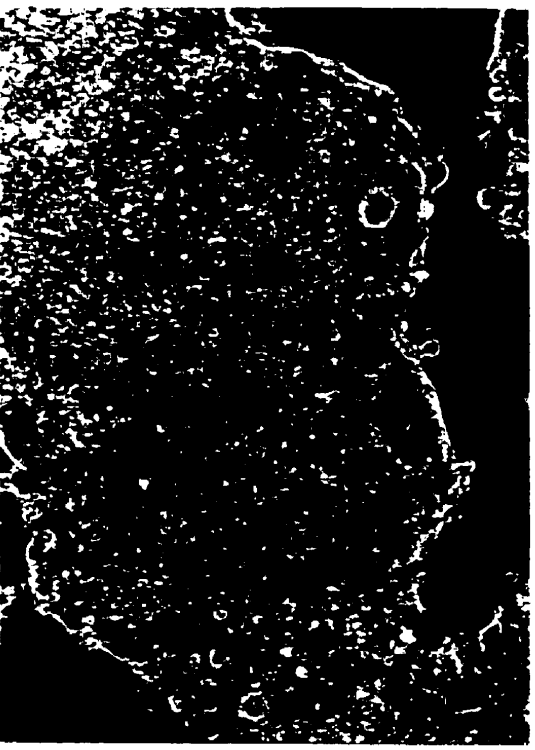
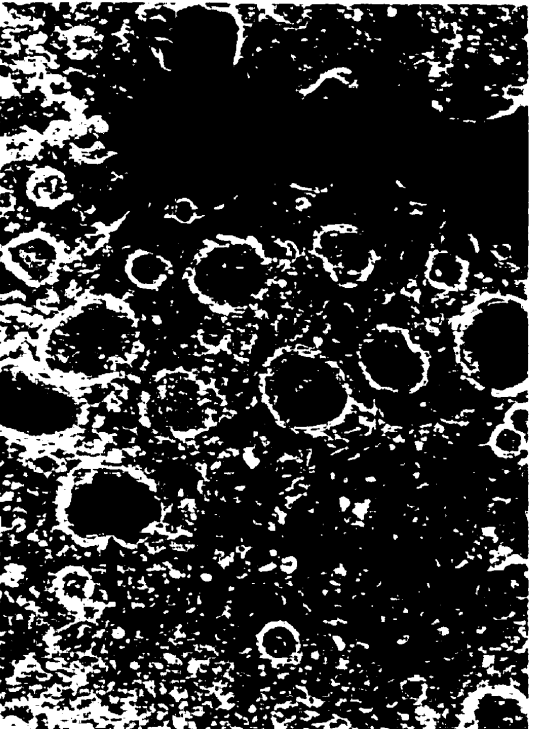


control

SW1222-CEACAM6



+ vanadate



Experimental Procedures

Cell Lines

L6 rat myoblasts and SW1222 human colorectal carcinoma cells were grown as monolayer cultures in DMEM (L6) or α -MEM (SW1222) containing 10% FBS (growth medium, GM; GIBCO BRL, Gaithersburg, MD) supplemented with 100 μ g/ml streptomycin and 100 U/ml penicillin (GIBCO BRL) at 37°C in a humidified atmosphere with 5% CO₂. All cell lines were subcultured before confluence and seeded at a density of 1×10^4 cells/cm².

cDNA Transfections and Infections

Our transfection procedures and transfected cell lines used in this study have been described above (See Introduction). Briefly, stable transfectants of cell lines L6 were obtained by the calcium phosphate precipitation method using the p91023B expression vector containing full length cDNAs encoding cell adhesion proteins: CEA and CEACAM1-4L (formerly splice variant BGPa), human NCAM-125 [GPI-linked NCAM splice variant with muscle specific domain] and pSV2neo plasmid as a dominant selectable marker. SW1222 single transfectants were obtained using the Zn²⁺-inducible episomal expression vector pML1 containing the mouse metallothionein promoter (mMT1) and the hygromycin-B resistance gene, alone (SW1222-Hygro population), and containing full length cDNAs a partial cDNA containing the coding region of CEACAM6 (pML1-CEACAM6). L6 cells in the exponential phase of growth were infected with replication-defective recombinant retrovirus containing either pBabe(human bcl-2)puro or the vector alone as a control.

Apoptotic Assays

L6 rat myoblasts were seeded at 0.7×10^3 cells/cm² in GM. After 3 days incubation, the culture medium was changed to DMEM supplemented with 5% horse serum and 100 μ M of sodium orthovanadate. This dose of the drug was repeated daily for the next 3 days. At this point the cells were fixed with 2.5 % glutaraldehyde and apoptosis was determined by hematoxylin staining.

Paclitaxel (Taxol) was added at 40 nM in the tissue culture medium to induce apoptosis of L6 myoblasts. The cell lines were treated with taxol for 24 hours. Afterward, the cells were collected by mild trypsinization, counted and seeded in tissue culture medium at 200; 1,000; and 10,000 cells per 60-mm petri dish. The cells were grown for 10 days, fixed in one- percent formaldehyde and stained with crystal violet. The number of surviving colonies was counted.

Central findings & major original contributions to knowledge in the field

- The discovery that CEA/CEACAM6 over-expression on the surface of cancer cells inhibits anoikis, and the demonstration of a specific requirement for the GPI-anchor of CEA for this effect.
- The finding of a molecular mechanism that can explain the pleiotropic effects of CEA/CEACAM6 on cell differentiation and apoptosis. CEA/CEACAM6 expressing cells are unable to differentiate and are resistant to apoptosis due to a functional perturbation of specific integrins.
- The direct demonstration that CEACAM1 has anti-tumorigenic functions *in vivo*, contrasting with the pro-tumorigenic functions of CEA and CEACAM6.

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