AN INVESTIGATION OF TGF-β RECEPTORS AND THE ACTIONS OF TGF-β1 AND TGF-β2 IN PLACENTAL TROPHOBLAST CELLS

by

Kim Lee

A thesis submitted to the Faculty of Graduate studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Pharmacology & Therapeutics
McGill University
Montreal, Quebec

January 1993

TGF-β RECEPTORS AND ACTIONS OF TGF-β1 AND TGF-β2 IN TROPHOBLAST CELLS

by

Kim Lee

A thesis submitted to the Faculty of Graduate studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Pharmacology & Therapeutics
McGill University
Montreal, Quebec

January 1993

TABLE OF CONTENTS

ABSTRACT

Transforming Growth Factor Beta (TGF-β) is highly pleiotropic, involved in controlling cellular proliferation, differentiation extracellular matrix (ECM) remodelling. TGF-β is a potential mediator of placental trophoblast functions, including differentiation, hormone production, endometrial invasion and immunosuppression. Trophoblast cells are the epithelial-like covering of the chorionic villae at the fetal-maternal interface. In this study equilibrium binding assays and functional assays were used to investigate the binding characteristics and the actions of the TGF-β1 and TGF-β2 isoforms on an established human choriocarcinoma trophoblastic cell line, BeWo. Equilibrium saturation experiments indicated that the BeWo cells exhibited similar average affinities and total number of binding sites for TGF-β1 and TGF-β2. The K values obtained from Scatchard analyses were approximately 65 pM for I-TGF-β1 and 40 pM for I-TGF-β2, with 70,000 and 85,000 sites per cell respectively. Competitive equilibrium experiments indicated that TGF-β1 and TGF-β2 were equipotent (apparent half maximal inhibition (IC)~70 pM) and that all binding sites were capable of recognizing both isoforms. Two frequently used functional assays to determine dose responsiveness to TGF-β isoforms were investigated with BeWo cells. A H-Thymidine incorporation assay was used to measure TGF-β effects and this indicated that neither TGF-β1 nor TGF-β2 had any significant effect on BeWo cells. The synthesis of an ECM protein, fibronectin, was monitored using both gelatin-Sepharose affinity chromatography and immunoprecipitation with anti-human fibronectin polyclonal antibodies. However, neither TGF-β1 nor TGF-β2 had any effect on fibronectin synthesis in BeWo cells as measured by these two methods. The significance of the results obtained from the functional assays are discussed in terms of differences in TGF-β responsiveness between BeWo choriocarcinoma cells and other cell types, and in terms of the low levels of Type I and Type II TGF-β receptors expressed in BeWo cells.

RÉSUMÉ

Le facteur de croissance TGF-β (Transforming Growth Factor Beta) est un agent pléiotrope impliqué dans le contrôle de la prolifération cellulaire, de la différenciation cellulaire et du remodelage de la matrice extracellulaire (ECM). Le TGF-β est un médiateur potentiel des activités des trophoblastes placentaires incluant leur différenciation, leur production d'hormones, leur potentiel d'invasion de l'endomètre et d'immunosuppression. Les cellules trophoblastiques sont de type épidermal et recouvrent les villosités choriales, à l'interface embryo-utérine. Dans la présente étude, des analyses de liaison à l'équilibre et des études fonctionnelles sont utilisées pour comprendre les différences au niveau des caractéristiques de liaison et d'action des isoformes, TGF β1 et TGF β2, chez les cellules BeWo, lignée cellulaire choriocarcinômique trophoblastique humaine. Des études de saturation à l'équilibre et l'analyse de Scatchard indiquent que les cellules BeWo ont un K_d apparent semblable pour les isoformes du TGF-β soit 65 pM dans le cas de β1 et 40 pM pour β2. Le nombre de sites de liaison est du même ordre pour TGF-β1 et TGF β2 soient, respectivement 70,000 et 85,000. Des expériences de compétition à l'équilibre indiquent que TGF β1 et TGF-β2 sont équipotents (IC_{50} ~ 70 pM) et que tous les sites de liaison sont capables de reconnaître les deux isoformes. Deux essais fonctionnels ont été utilisés pour déterminer la dose-réponse des cellules BeWo aux isoformes de TGF β de la présente étude. L'incorporation de ^{3}H-Thymidine et l'effet des TGF β sur la prolifération cellulaire chez les cellules BeWo n'indiquent aucune différence entre les effets des deux isoformes sur la prolifération. Le métabolisme de synthèse de la fibronectine, protéine de type ECM, a été étudié dans les cellules BeWo par chromatographie d'affinité et par immunoprécipitation à l'aide d'anticorps polyclonaux anti-fibronectine humaine. Les résultats de ces deux méthodes de dosage n'ont démontré aucun effet significatif des facteurs de croissance TGF-β1 et TGF-β2 sur la synthèse de la fibronectine par les cellules BeWo. La pertinence des résultats obtenus suite à ces analyses fonctionnelles est discutée en terme de différences dans la réponse aux isoformes de TGF β, par les cellules BeWo comparée à celle de cellules d'autres types, de même qu'en terme de niveaux d'expression des récepteurs de TGF β de type I et II exprimés chez les cellules BeWo.

INTRODUCTION

Transforming Growth Factor Beta (TGF-β) was originally described for its ability, in the presence of epidermal growth factor (EGF) to enhance the growth of fibroblast in soft agar (Roberts et al., 1981). Today, TGF-β is recognized as a family of multifunctional growth factors. Five distinct TGF-β isoforms have been described, TGF-β 1 through 5. The human TGF-β family consists of three isoforms TGF-β1, -β2, and β3, which are highly homologous. TGF-β is highly pleiotropic, being able to control proliferation, differentiation and extracellular matrix deposition (for reviews see Roberts & Sporn, 1990; Massague, 1990). Generally, TGF β1, -β2, and -β3 are interchangeable in in vitro biological assays, however, in some in vitro assays they do demonstrate differential activity (Ohta et al., 1987; Ottman & Pelus, 1988; Tsunawaki et al., 1988; Jenning et al., 1988; Rosa et al., 1988; Graycar et al., 1989; Cheifetz et al., 1990; Qian et al.,1992). Furthermore, recent work indicates that the expression of TGF β1, -β2, and -β3 is differentially regulated, which suggests that these isoforms have different functions in vivo (Pelton et al., 1989; 1991; Schmid et al., 1991; Paria et al., 1992; Roberts & Sporn, 1992).

TGF-βs, as with other growth factors, act by binding to specific cell surface receptors that transduce the regulatory signal. It has been possible to identify putative TGF-β receptors by chemical cross linking of ^{125}I-TGF-β to specific, high affinity binding components on cell surfaces (Massague, 1985; Massague & Like, 1985; Fanger et al., 1986; Segarini, 1989). Initially affinity-labelling techniques demonstrated three distinct types of TGF-β cell surface binding components, known as the Type I, Type II, and Betaglycan (also known as Type III), TGF-β receptors (for reviews see Segarini, 1991; Massague, 1992). The Type I and Type II receptors are glycoproteins and are thought to be true signalling receptors, (Boyd & Massague, 1989; Laiho et al., 1990;

1991). The Betaglycan component, a proteoglycan, exists in both membrane-anchored and soluble forms and thought to serve non signalling roles (Andres et al., 1989). Generally, the Type I and Type II receptors display much higher affinities for TGF β1 and TGF-β3 than for TGF-β2, however, subtypes that have high affinity for TGF-β2 have been demonstrated (Cheifetz, 1990; Cheifetz & Massague, 1991; Mitchell et al., 1992 a,b). The predominant population of Betaglycan components show equal affinities for TGF β1 and TGF-β2 although Betaglycan subtypes having a higher affinity for TGF-β2 than for TGF-β1 also exist (Segarini et al., 1987; Mitchell & O'Connor-McCourt, 1991; Mitchell et al., 1992 a,b). The Betaglycan and Type II TGF-β receptor genes have been recently cloned and sequenced (Lopez-Casillas et al., 1991; Wang et al., 1991; Lin et al., 1992). Other TGF-β binding proteins, distinct from the Type I, Type II and Betaglycan receptors, have also been described (MacKay et al , 1990; 1992; MacKay & Danielpour, 1991; O'Grady et al., 1991 a,b; Segarini et al., 1992; Hannah et al., 1992; Ichijo, 1992; Cheifetz & Massague, 1991).

Trophoblast cells are the epithelial-like covering of the chorionic villae at the fetal-maternal interface. Trophoblasts play an important role in the physical attachment of the placenta to the uterus, in the exchange of nutrients and wastes, the production of hormones, and the regulation of the maternal immune response. The placenta is a rich source of growth factors and growth factor receptors and has been utilized as a source for the purification of growth factors (Goldstein et al., 1978; Wu & Fischer, 1980; Gospodarowicz et al., 1985) including TGF β (Frolik et al., 1983), and growth factor receptors (Hock et al., 1979; 1980; Downward et al., 1984; LeBon et al., 1986; Maly & Luthi, 1986). What role growth factors play in placental function are unclear, however, it is assumed that they act via autocrine and paracrine circuits (for reviews see Blay & Hollenberg, 1989; Pollard, 1990). Growth factor receptors have been studied on human trophoblast cells in primary cultures (Deal & Guyda, 1983; Lai &

Guyda, 1984), trophoblast cell lines derived from primary cultures (Goustin et al., 1985) and on trophoblastic cell lines such as BeWo (O'Connor-McCourt & Hollenberg, 1983; Deal & Guyda, 1983).

The activities of TGF-β, notably in extracellular matrix deposition, cellular proliferation and differentiation, and immunosuppression point to its potential roles as a mediator of trophoblastic invasiveness, differentiation and for the maintenance of pregnancy (Tamada et al., 1990; Lala & Graham, 1990; Morrish et al., 1991; Altman et al., 1990; Kelly et al., 1990). Moreover, recent murine studies suggest that TGF-β2 in particular, is important both in placental function and embryonic development (Miller et al., 1989; Pelton et al., 1989; Clark et al., 1990; Altman et al., 1990; Kelly et al.,1990). In order to understand the possible roles of different TGF-β isoforms in placental trophoblast function, the characterization of the placental TGF-β receptors is necessary.

Affinity labelling studies carried out in this laboratory have shown that human placental membrane preparations (Mitchell and O'Connor McCourt, 1991), human primary trophoblast cells (Mitchell et al., 1992a), and the human choriocarcinoma trophoblast-like cell line, BeWo, (Mitchell et al., 1992b) express a unique class of Betaglycan receptor which exhibits a higher affinity for TGF-β2 than for TGF β1, yet a higher capacity for TGF-β1 than for TGF-β2. Minor subtypes of both the Type I and Type II receptors with equal or higher affinity for TGF-β2 than -β1 were also detected on these trophoblastic cells, similar to subtypes detected on a few other cell types (Cheifetz et al., 1990; Cheifetz & Massague, 1991).

BeWo cells provide a more consistent model than primary trophoblast cells for studying the effects of TGF-β, its isoforms, its receptors, and its signalling pathways. In this study, TGF-β receptors on BeWo cells were further characterized by equilibrium binding assays. Equilibrium binding assays are able to give a more

quantitative measurement of overall binding affinity and binding capacity for the different TGF-β isoforms than affinity labelling studies. The functional role(s) of TGF-β1 and TGF β2 in BeWo cells were also studied. Two functional assays, a proliferation assay and an assay for measuring fibronectin synthesis, both of which are frequently used to determine dose responsiveness to the TGF β isoforms, were investigated. The aim of this study was to try to correlate the binding affinities for the TGF-β1 and β2 isoforms to the trophoblast TGF-β receptors with the biological potencies of these isoforms.

SELECTED REVIEW OF THE LITERATURE

TRANSFORMING GROWTH FACTOR BETA

The first indication of Transforming Growth Factor Beta's (TGF-β) true importance as a mediator of normal cellular physiology was described by De Larco and Todaro (1978) just a little over a decade ago. It was found that murine sarcoma virus-transformed rat kidney fibroblasts secreted growth stimulating polypeptides into their extracellular medium that had the ability to induce changes in normal rat fibroblasts giving them a neoplastic phenotype, including the ability to grow and form colonies in soft agar. These peptides were termed sarcoma growth factors (SGFs)(De Larco & Todaro, 1978). However, similar peptides with similar activities were then isolated from various sources, other than sarcomas, neoplastic and non-neoplastic, (Roberts et al., 1981) and the peptides' name changed to transforming growth factors.

TGFs were later found to be a mixture of two different peptides which were consequently named TGF-α and TGF-β (Roberts et al, 1982; Anzano et al., 1982). The two TGFs were distinguished by their marked differences in biological properties, particularly with respect to their relationship to epidermal growth factor (EGF)(Anzano et al., 1982). TGF-α is an immunologically distinct analog of EGF with high affinity for the EGF receptor. EGF and TGF-α by themselves induce only a small number of colonies that are able to grow in soft agar. TGF-β does not compete for the EGF receptor, and alone does not induce the formation of colonies in soft agar. However, in the presence of either EGF or TGF-α, TGF-β induces a dose-dependent formation of colonies that surmounts that response elicited by EGF or TGF-α up to 10-fold (Anzano et al.,1982).

When it was shown that TGF-β is synthesized and secreted by a wide variety of normal and neoplastic cells and tissues, it became

clear that this factor is not tumor specific, but rather it plays a fundamental role in the growth and physiology of normal cells. Subsequently TGF-β was isolated and purified to homogeneity from several sources, human platelets (Assosian et al., 1983), human placenta (Frolik et al., 1983), and bovine kidney (Roberts et al., 1983), by monitoring TGF-β activity in an assay which measured its ability, in the presence of EGF, to induce normal rat kidney fibroblasts to grow and form colonies in soft agar. The purification of TGF-β from different sources revealed that it is a protein with an apparent molecular mass of 25,000 daltons (Assosian et al., 1983; Roberts et al., 1983; Frolik et al., 1983). Reduction of the disulfide bridges converts the 25 kDa protein into a 12.5 kDa species, as assessed by SDS-polyacrylamide gel electrophoresis. It is the dimeric form which is biologically active. Now, TGF-β is known to exist in at least five different forms, TGF-β1 through TGF-β5 (for reviews see Roberts & Sporn, 1990; Massague, 1990). The second form of the peptide TGF β2 was isolated and purified from bovine bone (Seyedin, 1985; 1987), human glioblastoma cells (Wrann et al., 1987), porcine platelets (Cheifetz et al., 1987) and monkey BSC-1 cells (Hanks et al., 1988). TGF-β1 and TGF-β2 have been cloned and their amino acid sequence deduced. They are both homodimers with each monomer unit consisting of 112 amino acids. The mature 112 amino acid monomers are derived from the carboxyl-terminal portion of a 390 amino acid precursor for TGF-β1 (Derynk, 1985), and a 412 amino acid precursor for TGF-β2 (DeMartin, 1987), and are 72% identical (for review see Roberts & Sporn, 1990). Recently, by crystallography, the tertiary structure of TGF-β2 has been deduced and reveals that the nine conserved cysteine residues are involved in disulfide bonds, one of which links the two monomers together (Daopin et al., 1992; Schlunegger & Grutter, 1992) . The other three forms TGF β3, TGF-β4, and TGF-β5 have been identified by screening cDNA libraries but have yet to be isolated from natural sources. However, northern blots demonstrate that the corresponding mRNA's are expressed. All five TGF-β's are 64 - 82 % homologous to one

another, and conserve nine cysteine residues in the active peptide. Moreover, each of the different TGF-βs is more than 98% conserved between species; for example TGF-β1 is identical in man, monkey, pig and chicken. This strict conservation of sequence between species emphasizes the importance of TGF-β in mediating normal cellular actions (for reviews see Roberts & Sporn, 1990; Massague, 1990; Derynk, 1987).

All of the TGF-β isoforms are processed from a long precursor, in which a remarkably high degree of sequence homology also occurs. (for reviews see Roberts & Sporn, 1990; Massague, 1990). The precursor structure of all TGF-βs consist of a N-terminal signal sequence, a pro-region, and a C-terminal bioactive domain, except for TGF-β4 which lacks a discernable signal sequence (Jakowlew et al., 1988). TGF-β is secreted from cells in a biologically latent form which consist of the cleaved pro-region non-covalently associated with the TGF-β dimer. The pro-region cleavage site is 4 or 5 amino acids immediately preceding the bioactive domain. The latent complex from platelets and certain cell lines is covalently associated with a 125 - 160 kDa TGF-β modulator protein via a disulfide bond (Figure 1). The role of the modulator protein is unclear, however, the absence of this protein in the latent form of TGF-β expressed in chinese hamster ovary (CHO) cells (recombinant) and human renal carcinoma cells (naturally occurring) indicates that it is not necessary to confer latency on TGF-β (Gentry et al., 1987; Wakefield et al., 1988). The latent complex can not bind TGF-β receptors, is immunologically different from TGF-β and must be activated before it can exert an effect (Miyazona et al., 1988; Wakefield et al., 1988). The precise mechanism to activate TGF-β in vivo is still unknown. In vitro studies show that TGF-β can be activated by extreme pH (1.5 or 12) and by plasmin treatment (Lyons et al., 1989). This suggest that acid microenviroments and proteases secreted by activated macrophages in sites of wound healing could contribute to the activation of latent TGF-β (Roberts & Sporn, 1990; Massague, 1990).

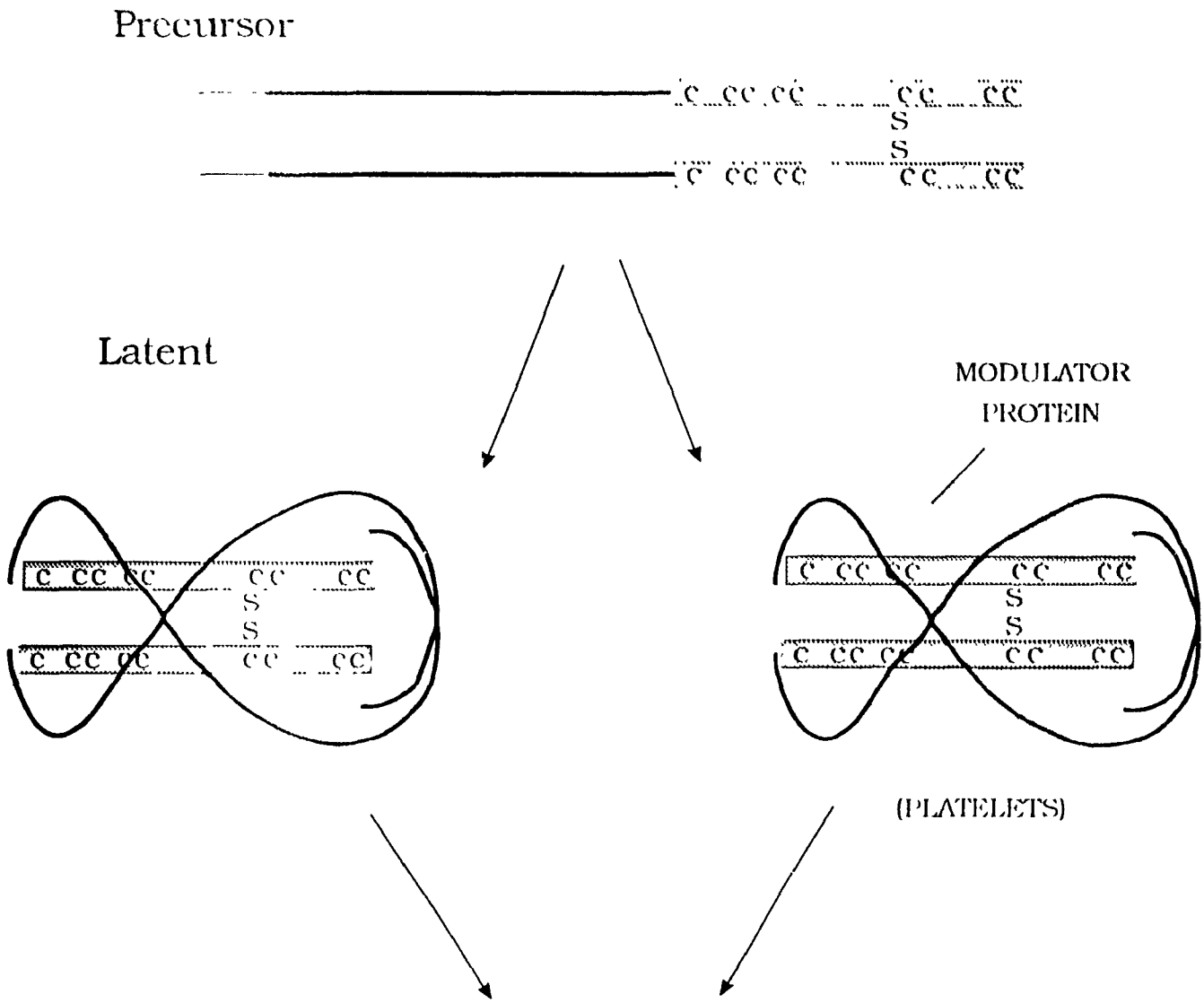

Precursor
S
S
Latent
MODULATOR
PROTEIN
S
S
S
S
(PLATELETS)
Bioactive
S
S

FIGURE 1. Processing of TGF-β from precursor to bioactive dimer. The TGF-β precursor consists of an N-terminal signal sequence (thin line), a pro region (thick line) and the C-terminal bioactive domain (box). (C) indicates the approximate location of the 9 conserved cysteines in the different forms of TGF-β. After secretion, the cleaved pro-region remains associated with the TGF-β dimer forming a latent complex. The TGF-β monomers are covalently linked via a disulfide bond. In platelets and certain cell lines, the complex is covalently associated with a 125 - 160 kDa modulator protein of unknown function. Bioactive TGF-β is released by disassembly of the latent complex including the modulator protein in platelets. (Adapted from Massague, 1990)

Of the five TGF-β isoforms TGF-β1, TGF-β2, and TGF-β3 are found in mammals. The two most obvious reasons why there are so many forms of TGF-β may be that: 1) The different forms may be processed and activated differently. Although in vitro all latent TGF-β types can be activated by acid treatment (Roberts & Sporn, 1990), the in vivo physiological mechanisms of activation may be isoform specific. 2) The different forms of TGF β may have unique biological activities in vivo. In most in vitro biological assays TGF-β1, TGF-β2, and TGF-β3 are interchangeable (Cheifetz et al., 1987; Seyedin et al., 1987; Graycar et al., 1889). However, in some in vitro assays the TGF-β isoforms do demonstrate differential activity (Ohta et al., 1987; Ottman & Pellus, 1988; Tsunawaki et al., 1988; Jenning et al., 1988; Rosa et al., 1988; Graycar et al., 1989; Cheifetz et al 1990; Qian et al., 1992) For example TGF β1 is about 100 times more potent than TGF-β2 in inhibiting the growth of hematopoietic progenitor cells (Ohta et al., 1987) and aortic endothelial cells (Jennings et al., 1988) whereas, TGF β2 is more potent than TGF-β1 at inducing mesodermal differentiation markers in Xenopus embryo cells (Rosa et al., 1988). It has now been shown that the TGF-βs 1, 2, and 3 each have separate genes on separate chromosomes (Fujii et al., 1986; Barton et al., 1988; ten Dijke et al., 1988) and the promoter structures and regulatory elements for these three TGF-β forms are different from each other (Kim et al., 1989; Lafyatis et al., 1990; Mona et al., 1991). Moreover, there is a growing body of evidence that these three types of TGF βs are expressed in different spatial and temporal patterns as demonstrated by in situ and Northern hybridization studies (for review see Roberts & Sporn, 1992), supporting the belief that these isoforms may have specific roles in vivo. The elucidation of the differential biological activities of the various types of TGF-βs may give insight to how these forms are differentially regulated, and represents an exciting area of research.

In addition to the TGF-βs, many other proteins have now been found to belong to the TGF-β supergene family by virtue of amino

acid homologies, particularly with respect to conservation of seven out of nine cysteine residues of TGF-β. These proteins have only 30 - 40% homology to the TGF-βS and are functionally distinct. Members of the TGF-β supergene family include: activins, inhibins, Mullerian inhibiting substance, bone morphogenetic proteins, and the decapentaplegic gene complex in Drosophila. These proteins appear to have their own receptors, thus do not bind to TGF-β receptors (for reviews see Roberts & Sporn 1990; Massague, 1990).

TGF-β FUNCTIONS

Today, TGF-β is considered the prototypic multifunctional growth factor and is also recognized as the most potent polypeptide growth inhibitor isolated from natural sources. TGF β has far reaching actions on epithelium, muscle, bone, and cartilage, the immune system, angiogenesis, wound healing and embryogenesis (for review see Sporn & Roberts, 1989). The nature of TGF β's action on a particular target cell is context-dependent, meaning that it is critically dependent on many parameters including cell type, its state of differentiation, growth conditions, and on the presence of other growth factors. For example, NRK rat fibroblast require both EGF and TGF-β for anchorage-independent growth. However, when NRK cells are grown in anchorage-dependent monolayer culture, EGF stimulates their growth, but TGF-β significantly blocks this EGF growth stimulatory effect in a dose-dependent manner (Roberts et al., 1985). In addition to its growth regulatory activities, TGF-β has been shown to modulate cellular differentiation and a variety of other biological activities including extracellular matrix synthesis, and degradation. Most of the effects of TGF β have been studied with <u>in vitro</u> systems. However, in a few cases, TGF β responses have been measured <u>in vivo</u> (Roberts et al., 1986; Silberstein & Daniel, 1987; Russell et al., 1988). Since most of the cells studied to date have the ability to respond to TGF β, it suggests that TGF-β plays a fundamental role in normal and pathological cellular physiology. TGF-β is very potent in eliciting its <u>in vitro</u> cellular actions, with half maximal effects observed at picomolar concentrations. Some of the major effects of TGF-β are discussed in greater detail below. It should be noted, however, that the majority of studies on TGF β functions so far, have used TGF-β1 due to the low availability of TGF β2 and TGF-β3 protein from natural sources.

CELL PROLIFERATION

As noted above, TGF-β may have stimulatory, inhibitory, or no effects on cell proliferation depending on the cell type, its state of differentiation and its environmental conditions. Inhibition of cell growth is the eminent action of TGF-β, it inhibits the growth of some normal and transformed epithelial, erdothelial, neuronal, lymphoid, fibroblast and hematopoietic cells in culture (for reviews see Roberts & Sporn, 1990; Massague, 1990). The antiproliferative action of TGF-β on B-lymphocytes, T-lymphocytes (Kehrl et al., 1986a) and thymocytes (Ristow et al., 1986) *in vitro* is consistent with TGF-βs immunosuppressive activity *in vivo* (Wrann et al., 1987; DeMartin et al., 1987). Evidence for TGF-β's antiproliferative effects have been shown *in vivo* whereby TGF-β implants inhibited the growth of the developing mammary gland in mice (Silberstein & Daniel, 1987) and intravenous injections of TGF-β1 or TGF-β2 abrogated the proliferative response of regenerating rat liver (Russel et al., 1988).

TGF-β antiproliferative effects *in vitro* occur by lengthening or arresting the G1 phase of the cell cycle. Studies with flow microfluorimetry with regenerating endothelial cells (Heimark et al., 1986) and flow cytometry and autoradiography with primary cultures of human prokeratinocytes (Shipley et al., 1986) and rat hepatocytes (Lin et al., 1987) show that TGF-β1 delays the progression of cells from G1 to S phase. Moreover, TGF-β1 represses several cell cycle markers characteristic of the late G1 phase and early S phase (Smeland et al., 1987; Chambard & Pouyssegur, 1988; Laiho et al., 1990). TGF-β represses the induction of: thymidine kinase, a marker of entry into S phase, in chinese hamster lung fibroblast; the transferrin receptor, a marker of late G1 phase, in activated B-lymphocytes (Smeland et al., 1987); and phosphorylation of the retinoblastoma gene product, an event that occurs late in G1 (Laiho et al.,1990). In contrast, TGF-β exerts a stimulatory effect on the growth of some mesenchymal

cells in vitro, such as rat fibroblasts (Roberts et al., 1981) and human embryo (Hill et al.,1986) and rat osteoblasts (Centrella et al., 1987). As mentionned above, the mechanism that leads to the dual effect of TGF-β on cell proliferation in some instances is apparently due to the interaction between TGF β and other growth regulatory proteins, such as EGF (Roberts et al., 1985) and PDGF (Leof et al., 1986). However, most of the diverse effects of TGF β on cell proliferation cannot be so simply explained and important cell-specific determinants, such as a cell's differentiative state, must be considered in trying to predict a particular cell's response to TGF-β.

CELL DIFFERENTIATION

Depending on the cell type, TGF-β also has stimulatory or inhibitory effects on the differentiation of cells. In vitro, TGF β has been shown to induce the differentiation of prechondrocytes (Seyedin et al., 1985), intestinal epithelial cells (Kurokowa et al., 1987; Barnard et al., 1989), and bronchial epithelial cells (Masui et al., 1986; Jetten et al., 1986) but it can also inhibit the differentiation of other cells including skeletal muscle myoblast (Massague et al., 1986; Olson et al 1986; Florini et al., 1986), preadipocytes (Ignotz & Massague, 1985), and hematopoietic progenitor cells (Ohta et al., 1987; Ottman & Pelus, 1988). In cartilage, TGF-β1 and TGF-β2 can act both as an inducer and an inhibitor of differentiation in vitro (Rosen et al., 1988). In some instances, the effects of TGF-β on cell differentiation are coupled with an effect on proliferation. For example, TGF-β stabilizes the differentiated state of kidney epithelial cells induced by insulin and hydrocortisone by blocking further cell proliferation stimulated by these hormones (Fine et al., 1985). In contrast, the antiproliferative effects of TGF-β may in some instances be coupled to an inhibition of further cell differentiation, as in the case of B-lymphocytes; the differentiation of these cells to a state where they secrete

immunoglobulins is blocked by TGF-β (Kehrl et al., 1986b). However, TGF-β effects on differentiation are not always accompanied by its effects on proliferation. In a manner similar to its actions on cell proliferation, TGF-β coordinates the nature of the cellular response with various other differentiation signals.

Nevertheless, TGF-β is present at relatively high levels in centers of active tissue differentiation, including cartilage canals, osteocytes, bone marrow, and hematopoietic stem cells in fetal liver. The highest levels of TGF-β1 and TGF-β2 are found in blood platelets thus, TGF-β may be physiologically delivered to sites of wound healing. Furthermore, it has been shown that TGF-βs 1, 2, and 3 are localized in characteristic spatial and temporal patterns indicating their role in controlling morphological and histological events through embryonic development (Roberts & sporn, 1992; Paria et al., 1992; Pelton et al., 1991). Together, these observations suggest an active involvement of TGF-βs in the genesis of many types of tissues in normal development as well as in wound healing.

In addition, many mesenchymal and epithelial cells whose differentiation and proliferation are affected by TGF-βs respond to these factors with elevated expression of various cell adhesion proteins, such as fibronectin, and the various types of collagen (Ignotz & Massague, 1986) . Since it is known that the addition of fibronectin and collagen to several types of cells can inhibit their differentiation in vitro, TGF-β may affect differentiation in these cells by controlling the abundance and architecture of the extracellular matrix as well as the ability of cells to interact with it (Pfeilschifter, 1990).

EXTRACELLULAR MATRIX SYNTHESIS

The extracellular matrix (ECM) is a dynamic entity,

continually cycling between synthesis and degradation, where many factors play a role in dictating whether there is to be net synthesis or net degradation. Since the composition and organization of the ECM plays an important role in controlling cellular adhesion, migration, proliferation and differentiation, ECM remodelling is implicated in morphogenesis, embryogenesis, tissue repair and normal and pathological physiology. TGF β is an important regulator of ECM remodelling, in general, inducing a net deposition of ECM proteins. TGF-β can regulate ECM deposition through different mechanisms. TGF-β has been shown to activate gene transcription, to increase the synthesis and processing, and to increase the secretion of matrix proteins (for reviews see Roberts & Sporn, 1990; Massague, 1990). In vitro, TGF β increases the synthesis of various types of collagen (Ignotz & Massague, 1986; Wrana et al., 1986; Varga et al., 1987; Penttinen et al., 1988; Madri et al., 1988), fibronectin (Ignotz & Massague, 1986; Wrana et al., 1986), thrombospondin (Penttinen et al., 1988), osteopontin (Noda et al., 1988), tenascin (Pearson et al., 1988), elastin (Liu & Davidson, 1988) and glycosaminoglycans (Chen et al., 1987; Morales & Roberts, 1988; Bassols & Massague, 1988). In vivo, TGF-β, when injected subcutaneously in new born mice, causes the formation of granulation tissue, which is the induction of angiogenesis and activation of fibroblasts to produce collagen (Roberts et al., 1986). Furthermore, in vitro, TGF-β increases the expression of cell adhesion receptors on the surface of target cells, such as integrin. Thus, TGF-β may increase the interaction between a cell and its ECM and modify cell adhesion and morphological events which in turn are likely to affect cell migration, proliferation and differentiation.

TGF-β also increases net ECM deposition by decreasing the synthesis of proteolytic enzymes that degrade matrix proteins and by increasing the synthesis of protease inhibitors that block the activity of these enzymes. TGF-β's activity in controlling ECM proteases and their inhibitors has been shown to be a direct role

of TGF-β in altering mRNA levels of the affected genes (for review see Roberts & Sporn, 1990; Massague, 1990). TGF-β treatment has been shown to decrease the synthesis of a thiol protease (Chiang & Hamilton, 1986), and a plasminogen activator, both of which are serine proteases (Laiho et al., 1986; Lund et al., 1987), as well as the metalloproteinases including, collagenase (Edwards et al. 1987), elastase (Redini et al.,1988), and transin/stromelysin (Matrisan et al., 1986; Kerr et al., 1988). As mentionned above, TGF β may further decrease protease activity by increasing the expression of protease inhibitors such as plasminogen activator inhibitors-1 (PAI-1; Laiho et al., 1987; Lund et al., 1987; Allan et al., 1991), and tissue inhibitor of metalloproteinases (TIMP; Edwards et al, 1987; Kubota et al., 1991) In contrast, a recent report has demonstrated that TGF-β1 can enhance plasminogen activator activity and the expression of its mRNA (Hamilton et al., 1991), again demonstrating TGF-β'S ability to elicit either positive or negative responses. TGF-β's capacity to modulate the expression of these ECM modulating enzymes thus implicates its therapeutic potential for wound repair and cancer treatment.

TGF-β RECEPTORS

Polypeptide growth factors act through specific cell surface receptors that are stimulated by the ligand to initiate a cellular response. Like other growth factors, TGF-β binds specifically to cell surface receptors that do not recognize any other growth factors. The binding of TGF-β to almost 150 different cell lines and cell types have been examined thus far, and with only a very few exceptions almost all cells bind TGF-β (Wakefield et al. 1987; Massague et al., 1990). Several distinct cell-surface binding components, operationally defined as receptors, have been demonstrated by the electrophoretic analyses of affinity labelled complexes of iodinated TGF-β covalently linked, with cross linking agents such as bis (sulfosuccinimidyl) suberate (BS), to cell surface molecules (for reviews see Massague et al., 1990; Massague, 1992; Segarini, 1991). TGF-β has been iodinated by various methods including those based on using reagents such as chloramine T. Under carefully controlled conditions, these methods have been shown to have no affect on the biological activity of the ligand (Frolik et al., 1984; Wakefield et al., 1987; Wakefield, 1987).

The most widely studied and the most widespread forms of TGF β receptors are the: Type I, Type II,and Type III (or Betaglycan) receptors, which usually coexist on cells. The Type I and Type II receptors are glycoproteins, with N-linked sugars, that are not required for TGF-β binding (Cheifetz et al., 1988a). The Type I and Type II receptors appear as affinity-labelled complexes of 65 kDa and 85 kDa respectively, on SDS-PAGE under reducing conditions (Cheifetz et al., 1988a). In general, these two TGF β receptors bind TGF-β1 with higher affinity than TGF β2, but subtypes of Type I and II receptors with similar (Segarini et al., 1987) or higher affinity for TGF-β2 than TGF-β1 (Cheifetz et al., 1990; Cheifetz & Massague, 1991; Mitchell & O'Connor-McCourt, 1991; Mitchell et al., 1992a,b) have been recognized. Betaglycan (Type III) is a proteoglycan, containing glycosaminoglycan (GAG) sugars of heparin

sulfate and chondroitin sulfate, and it migrates as a broad smear ranging from 250 - 350 kDa on SDS-PAGE (Segarini & Seyedin, 1988; Cheifetz et al., 1988a). As assessed by enzymatic removal of the GAG chains, Betaglycan consists of a core protein ranging from 120 - 140 kDa containing the binding site for TGF-β (Segarini & Seyedin, 1988). The GAG chains appear to be dispensable for both ligand binding to the core, and for Betaglycan cell surface expression (Cheifetz & Massague, 1989). Betaglycan, unlike the Type I and Type II, receptors generally, binds TGF-β1 and TGF-β2 with similar affinity, albeit at a somewhat lower affinity (~300 pM) than Type I and Type II (~30 pM)(Cheifetz et al., 1988b). A soluble form of Betaglycan has also been detected in extracellular matrices, the culture media of several cell types,and in serum. Soluble Betaglycan binds to TGF-β1 and TGF-β2 with similar characteristics as the membrane bound form (Andres et al., 1989). Subtypes of Betaglycan having a higher affinity for TGF-β2 than for TGF-β1 have also been detected (Segarini et al., 1987; Mitchell & O'Connor-McCourt, 1991; Mitchell et al., 1992 a,b).

Several other TGF-β binding proteins have been demonstrated by affinity-labelling although they are not as widely studied. The Type IV receptor has only been detected on a pituitary tumor cell line. It is different from other TGF-β receptors in that it can bind activin and inhibin as well as TGF-β and it does not contain N-linked sugars. It migrates as a 60 kDa protein on SDS-PAGE (Cheifetz et al., 1988c). The Type V receptor is approximately 400 kDa, as assessed by affinity-labelling (O'Grady et al., 1991a). The Type V receptor has been found to be present in membrane preparations of several tissues including human placenta and it is also present on the surface of various cell in culture, transformed and nontransformed, (O'Grady et al., 1991a,b). The Type VI receptor is a glycoprotein of 180 kDa and its distribution appears universal among cell types (Segarini et al.,1992). Various novel receptors have been recently demonstrated from diverse sources. Novel TGF-β receptors include the 180 kDa, 60 kDa, and 140 kDa,

proteins identified on fetal bovine heart endothelial cells and on human osteosarcoma cells, which are attached to the cell membrane through phospholipid anchors (Cheifetz & Massague, 1991). In addition, a 53 kDa protein from rat lung membranes (Hannah et al., 1992) and a 38 kDa protein from a human choriocarcinoma trophoblastic-like cell line (Mitchell et al., 1992b) have been reported as well.

The role of each individual TGF-β receptor is still unresolved. Correlation of the differential binding of TGF-β1 and TGF-β2 to the receptors and the differential potency of the ligand in biological assays originally led to the hypothesis that Betaglycan mediates TGF-β effects where TGF β1 and β2 are equipotent. Such effects include the inhibition of epithelial cell proliferation (Cheifetz et al.,1987), regulation of the expression of certain phenotypes, and elevated expression of cell adhesion proteins (Ignotz & Massague, 1987; Cheifetz et al., 1988a). At that time, it was proposed that, the Type I and II receptors were responsible for mediating biological activities specific to TGF-β1 such as, TGF-β1's selective inhibition of mouse hematopoietic progenitor cells (Ohta et al.,1987), B6SUt-A multipotential hematopoietic progenitor cells (Cheifetz et al., 1988a), and endothelial cells (Jenning et al., 1988). However, the observation that cells, such as L-6 myoblasts (Segarini et al., 1989) and primary lymphocytes (Kehrl et al., 1986b) which respond equally well to TGF-β1 and β2, and which do not express Betaglycan and only have Type I and II receptor suggest that Betaglycan may not be necessary for TGF-β activity. Furthermore, more recent studies with chemically induced mink lung epithelial cell (MvILu) mutants resistant to TGF-β actions implicate Type I and Type II receptors as the true signalling receptors (Boyd & Massague, 1989, Laiho et al., 1990). In these studies several types of mutants were described: R (Resistant) mutants, lacking Type I receptors; LR (Low levels of type I, Resistant) mutants, having low levels of Type I; DR (Double, Resistant) mutants, either lacking or having low levels

of Type I and II receptors; and S mutants, which have both Type I and II as determined by affinity-labelling. All mutants failed to respond to TGF-β1 and TGF-β2, and Betaglycan is apparently normal in all of them. No mutants were found that lacked only the Type II receptor. Moreover, the high relative frequency of DR mutants suggest that Type I and II interact with each other, i.e. DR mutants may have Type I receptors that can not bind TGF-β in the absence of Type II receptors. Genetic complementation experiments support this hypothesis, since somatic cell hybrids between R and DR mutants have essentially normal expression of Type I and II receptors (Laiho et al., 1991). However, no physical evidence of Type I and Type II receptor interaction has yet been reported. Furthermore, a recent report of human carcinoma cell lines which express Type I receptors and low levels of Type II receptors, yet can still respond to TGF-β action on gene expression (Geiser et al. 1992), refutes this hypothesis. The present hypothesis is that the Type I and Type II receptors are the true signalling receptors. Whereas, Betaglycan is thought to serve non-signalling roles, possibly concentrating and transferring ligand to the signalling receptors.

The intracellular pathway through which TGF-β exerts its effects has been studied extensively, although not intensively. The classical enzymatic activities and second messengers signal transduction mechanisms used by other hormones and growth factors have been investigated and have met without clear results. TGF-β binding proteins do not appear to be associated with phosphotyrosine activity (Fanger et al., 1986), nor is TGF-β activity associated with changes in a kinase activity of the 40S ribosomal protein S6 (Like & Massague, 1986). However, guanine nucleotide-binding proteins (G proteins) seem to be involved in mediating TGF-β effects in AKR-2B fibroblasts (Mulder et al.,1988; Speigel et al.,1987). Demonstration of the activation of glycolysis, amino acid uptake, intracellular calcium levels and phosphatidyl inositol turnover have been observed in rat fibroblasts in response to TGF-β (Boerner et al., 1985; Inman &

Colowick, 1985; Muldoon et al., 1988). Furthermore, TGF-β1 has been shown to stimulate prostaglandin E_2 production in lung fibroblasts (Diaz et al., 1989), and cultured mouse calvaria (Tashjian et al., 1985). Whether these responses are a direct effect of TGF-β, directly coupled to TGF-β receptors, or are downstream effects remains unclear. The strongest indication that TGF-β binding proteins mediate signal transduction by TGF β was presented upon the expression cloning of the human TGF β Type II receptor (Lin et al., 1992). The TGF-β Type II receptor has been found to be a member of a novel class of transmembrane signalling molecules, the protein serine/threonine kinases. Other members of this family include Caenorhabditis elegans Daf-1 gene product (Georgi et al., 1990) and two mouse Type II activin receptors, ActR-II (Mathew & Vale, 1991) and ActR-IIB (Attisano et al., 1992).

Cloning of the TGF-β Type II receptor gene reveals its product to be a membrane anchored protein with a cysteine rich extracellular domain and a predicted cytoplasmic serine/threonine kinase domain. Its extracellular domain shares some sequence homology with the extracellular domain of the recently cloned Activin receptors ActR-II (Mathews and Vale, 1991), ActRIIB (Attisano at al., 1992) and the Daf-1 gene product (Georgi et al., 1990). The intracellular region contains the serine/threonine sequence. Fusing the TGF-β Type II receptor kinase domain to glutathione S-transferase gene has shown that bacterially expressed Type II can autophosphorylate (Lin et al., 1992), although ligand induced activation of the kinase has yet to be demonstrated. Nevertheless, it has been recently shown that serine/threonine kinase inhibitors can block TGF-β responses (Ohtsuki & Massague, 1992).

The rat Betaglycan gene has also recently been cloned and sequenced (Lopez-Casillas et al., 1991; Wang et al., 1991). It has been found to encode an 853 amino acid protein. The protein has a large extracellular domain, a transmembrane domain and a relatively

short cytoplasmic tail of 43 amino acids. There is lack of a discernable signalling sequence in the intracellular domain of Betaglycan. This suggest that Betaglycan does not play a direct role in TGF-β signalling, consistent with the mutant studies mentionned above. Since its ectodomain contains putative sites for proteolytic cleavage and release into the pericellular environment it is plausible that Betaglycan may serve a as reservoir transferring TGF-β to signalling receptors. The availability of cDNA clones of the Type II and Betaglycan TGF-β receptors will definitely permit the further analysis of their structures, binding properties, individual functions and mechanisms of action.

THE HUMAN PLACENTA

The placenta is probably the most important organ in maintaining the welfare and the survival of the developing fetus. It is the organ of exchange between the mother and the fetus, providing nutrients and oxygen for the fetus and removing wastes from the developing organism for excretion by the mother. In addition, the placenta serves as an endocrine organ, providing hormones that maintain pregnancy and support the growth and maturation of the fetus. In fact, from the time the embryo first begins to implant itself, placental trophoblast cells release human chorionic gonadotropin (hCG) which signals the corpus luteum that pregnancy has begun, without hCG the corpus luteum would degenerate and the embryo would be aborted and flushed out with the menstrual flow (Spence and Mason, 1987).

The human placenta is classified as hemochorial type placenta which is invasive, in comparison to the non-invasive epitheliochorial type placenta of ungulates (Arey, 1965). On approximately the seventh day of development the embryo begins to implant in the endometrium of the uterus (Spence & Mason, 1987; Wynn, 1975). The trophoblast cells, of the outer blastocyst, in contact with the uterine lining secrete proteolytic enzymes thus degrading local ECM. Subsequently, the blastocyst will penetrate the basement membrane of the uterine epithelium implanting itself into the endometrium. With continued placental development, placental trophoblast cells invade uterine glands and blood vessels (Figure 2; Boyd & Hamilton, 1970).

The placenta may be considered analogous to an invasive tumor where, immunologically recognized as fetal tissue, it grafts itself upon maternal tissue (Redman 1986). Successful implantation and development of the blastocyst depends on a series of complex, coordinated interactions between maternal and fetal tissues that are mediated by trophoblast cells. Thus, in turn the placental

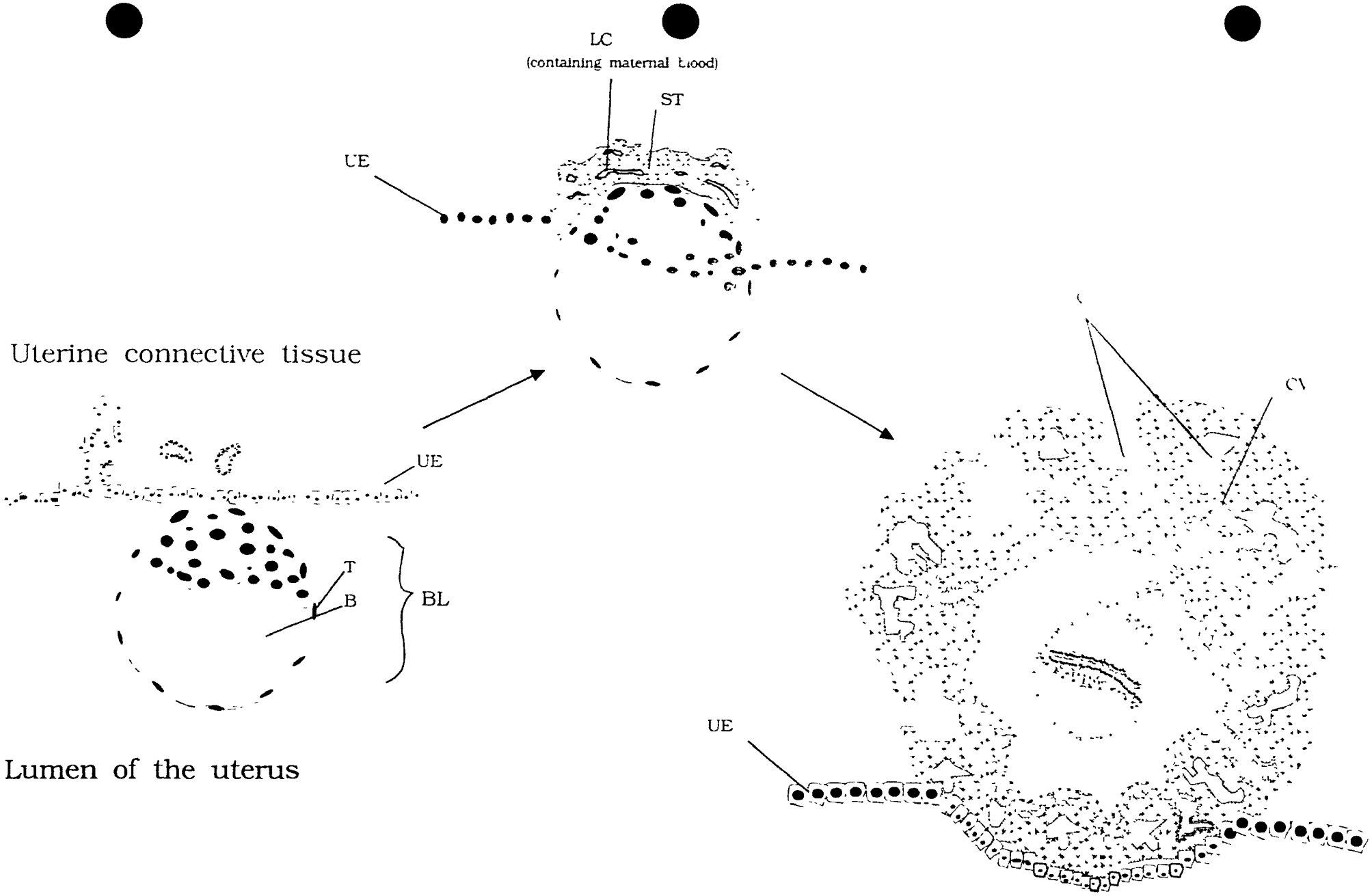

LC
(containing maternal blood)
ST
UE
Uterine connective tissue
UE
T
B
BL
UE
Lumen of the uterus

FIGURE 2. Diagram illustrating successive stages of placental implantation and development. B = Blastocoele, BL = Blastocyst, CV = Chorionic villus, LC = Lacuna, ST = Syncytiotrophoblast, T = Trophoblast, UE = Uterine epithelium, (Adapted from Spence & Mason, 1987)

trophoblast cells are similar to tumor cells in that they are invasive and secrete high amount of proteases. Trophoblast cells have been shown to degrade ECMs or penetrate basement membrane in vitro (Glass et al., 1983; Yagel et al., 1988; Fisher et al., 1989; Kliman & Feinberg, 1990; Librach et al., 1991), and secrete proteases such as serine proteases (Strickland et al., 1976; Mignatti et al., 1986; Queenan et al., 1987) and metalloproteinases (Fisher et al., 1989; Mignatti et al., 1986). Unlike tumor cells, trophoblast invasiveness is tightly controlled both spatially and temporally. A tight control and balanced degree of trophoblast invasion is absolutely essential during normal pregnancy. Poor invasiveness could result in inadequate fetal-maternal exchange and pathological conditions such as preeclampsia, whereas excessive invasiveness may result in the pathological destruction of the uterus that is associated with ectopic pregnancy, placenta accreta or choriocarcinoma.

Trophoblast cells are complex functionally and morphologically. There are three recognized types of trophoblast cells, i.e. cytotrophoblasts, intermediate trophoblasts and syncytiotrophoblasts. Intermediate trophoblasts consists of villous trophoblast and extravillous trophoblast. Cytotrophoblast which immediately overlie the chorionic villi, are undifferentiated, mononucleate stem cells which show no hormone production, and from which the other trophoblast cells are derived. Cytotrophoblast may follow two separate pathways of differentiation. In one pathway, the proliferative cytotrophoblast fuse together to form syncytiotrophoblast which are terminally differentiated multinucleated cells and are responsible for the synthesis of various steroid and protein hormones. This pathway of cytotrophoblast differentiation involves an abrupt transition through villous trophoblast. The other pathway of differentiation also begins on the surface of the chorionic villi. However, in this pathway there is no abrupt change from cytotrophoblast to syncytiotrophoblast, but a gradation of syncytiotrophoblast being

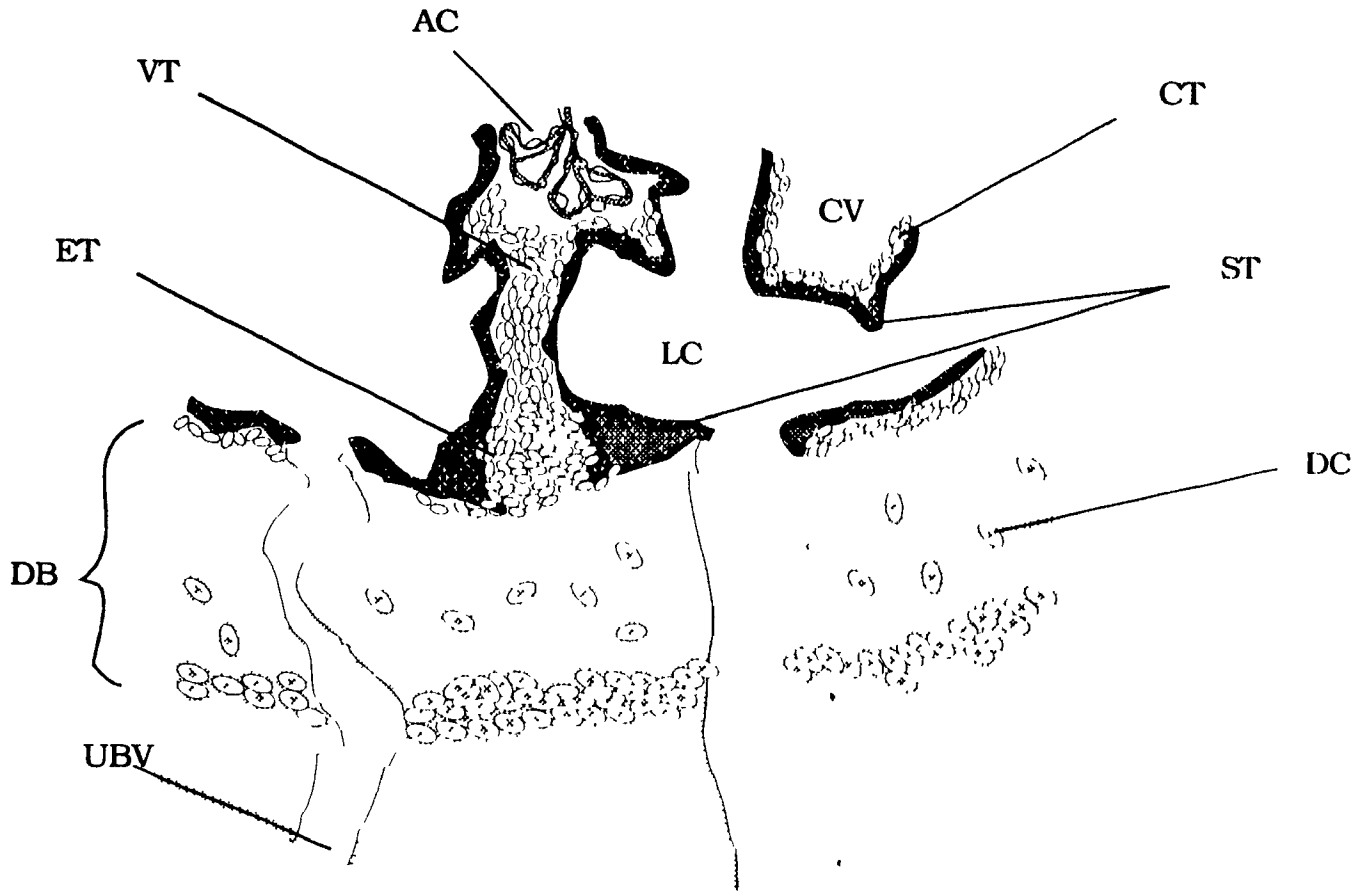

AC
VT
CT
CV
ET
ST
LC
DC
DB
UBV

FIGURE 3. Diagram illustrating the uteroplacental junction and the different kinds of trophoblast cells. Chorionic villi called anchoring villi maintain the attachment of the placenta to the uterine wall. Extravillous trophoblasts which migrate out from anchoring villi are highly invasive, they fuse and differentiate into syncytiotrophoblast, which are non-invasive cells. CV = chorionic villus, CT = cytotrophoblast, AV = anchoring villus, DB - decidual basalis, DC = decidual cell, VT = villous trophoblast, ST = syncytiotrophoblast, UBV = uterine blood vessel. (Adapted from Panigel, 1986).

penetrated by sprouting solid masses of intermediate trophoblastic cells. It is presumed that it is via these sprouts that intermediate trophoblast invade the decidua, forming extravillous trophoblast. It appears to be the local environment that dictates the differentiation of intermediate trophoblastic cells in extravillous locations. After the intermediate trophoblasts have infiltrated the decidua, they then fuse into binucleate, trinucleate, and multinucleate cells corresponding to syncytiotrophoblastic giant cells which are not invasive (Figure. 3; for review see Yeh & Kurman, 1989; Aplin .1991). One of the proposed theories is that invasiveness is an intrinsic property of trophoblast cells and the lost in invasion at a certain point during pregnancy is not genetically preprogrammed (for review see Aplin, 1991; Billington, 1971). The control of trophoblast invasion is provided by decidua-derived and to some extent trophoblast-derived factors. This has been proven by experiments in which trophoblast cells transplanted to extrauterine sites and non-pregnant uteri showed a greater extent and duration of invasion (Kirby, 1960; 1963 a,b) Immunologically, trophoblast cells play an important role in placental implantation, contributing to the prevention of allograft-type rejection of the pregnancy by the mother. It is now well established that villous trophoblast cells fail to express Class I HLA antigens (Bulmer & Johnson, 1985). However, a novel Class I molecule HLA-G has been identified on cytotrophoblast, (Kovats et al., 1990) although its significance is not yet understood.

The factors that control trophoblast functions are pertinent for understanding uteroplacental homeostasis and controlled cellular invasion. Placental tissue expresses a variety of polypeptide and hormone receptors. Placenta has been a source for the purification of several growth factors including, nerve growth factor (Goldstein et al., 1978), granulocytic and macrophage colony-stimulating factors (Wu & Fisher, 1980), fibroblast growth factor (Gospodarowicz et al., 1985), and TGF-β (Frolik et al.,

1983). Placental tissue has also proven to be a rich source for growth factor receptors. The receptors for insulin-like growth factor I and II (IGF-I and II), epidermal growth factor (EGF) have been isolated using placental tissue as a source (for review see Blay & Hollenberg, 1989). Polypeptide growth factor receptors have been studied on human placental trophoblast cells as well. Insulin receptors and EGF receptors have been studied with primary trophoblast cells (Deal & Guyda, 1983; Lai & Guyda, 1984). PDGF receptors have been studied with trophoblast cell lines derived from primary trophoblast cells in culture (Goustin et al., 1985). Moreover, receptors have been studied with trophoblastic cell lines, such as BeWo. BeWo cells have been utilized to study EGF receptors (O'Connor-McCourt & Hollenberg, 1983), and to study transferrin receptors (van der Ende, 1989; 1990). The roles of TGF-β in cellular proliferation and differentiation, and extracellular matrix remodelling make it a very likely candidate for playing a fundamental part in regulating trophoblast functions. Human placenta is a rich source of TGF-β and is one of the initial sources from which TGF-β was purified to homogeneity (Frolik et al., 1983). TGF-β is expressed in human placenta (Dungy et al., 1991) in a spatial and temporal pattern further suggesting a role for TGF-β in placental functions during pregnancy. As demonstrated by immunocytochemistry, TGF-β appears to be localized largely within the cytoplasm of syncytiotrophoblasts at the human fetal-maternal interface (Dungy et al., 1991; Vuckovic et al., 1992; Graham et al., 1992).

Evidence that suggest that TGF-β is a factor involved in controlling trophoblast functions include recent results where conditioned medium from decidual cells or exogenous TGF-β abrogates the invasiveness of primary trophoblast cultures (Lala & Graham, 1990). These studies suggest that the anti-invasive effect of TGF-β may be due to an induction of TIMP expression. Both anti-TGF-β and anti-TIMP-1 antibodies are able to prevent the anti-invasiveness effects of exogenous TGF-β and decidual cell

conditioned medium. These studies also suggest that TGF-β may further control trophoblast invasiveness by stimulating their differentiation into syncytiotrophoblast (Lala & Graham, 1990; Graham et al., 1992). However, it has not been determined whether TGF-β1 or TGF-β2 is responsible for inhibiting trophoblast invasiveness.

Murine studies suggest that TGF-β2, in particular, via its immunosuppressive properties, is important for maintaining pregnancy (Clark et al., 1990; Altman et al., 1990). Clark et al., (1991) have demonstrated that anti-TGF-β2 antibodies are able to increase the incidence of spontaneous abortions in mice. In addition, this group has shown that certain decidual cells secrete a TGF-β2-like molecule, suggesting a paracrine mechanism of action. However, to date, studies on the expression of TGF β2 mRNA, in murine placenta have been controversial. As measured by in situ hybridization or Northern analysis, TGF-β2 mRNAs have been found at high levels in placenta (Miller et al., 1989; Pelton et al., 1989), whereas other groups detected very little or no TGF β2 mRNA in placenta (Altman et al., 1990; Schmid et al., 1991). The analysis of TGF-β2 protein expression in the placenta would be important to clarify its role in this tissue.

Affinity-labelling studies with radiolabelled TGF-β1 or TGF β2 have shown that placental membrane preparations (Mitchell & O'Connor-McCourt, 1991), primary trophoblast cells (Mitchell et al., 1992a), and the choriocarcinoma trophoblast-like cell line, BeWo, (Mitchell et al., 1992b) express TGF-β receptor types that demonstrate complex patterns of TGF β binding. For example, Betaglycan in BeWo cells have a higher capacity for TGF β1 than for TGF-β2 yet exhibits a higher affinity for TGF-β2, as shown by affinity-labelling saturation and competition experiments, respectively. The studies with BeWo cells are of particular interest because BeWo cells provide a more uniform model, as compared to primary trophoblast cells, with which to study TGF β,

its receptors, its isoforms and its signalling pathways. Furthermore, the TGF-β binding characteristics demonstrated by primary trophoblast cells (Mitchell et al., 1992a) are retained in BeWo cells (Mitchell et al., 1992b). Moreover, BeWo cells display many morphological and biochemical properties common to placental trophoblasts (van der Ende et al., 1987; 1990; Wice et al., 1990). BeWo cells also provide an interesting differentiative model because in response to methotrexate, these cells undergo a complex response that resembles the differentiation of cytotrophoblast into syncytiotrophoblast (Friedman & Skehan, 1979; Burres & Cass, 1987). In addition, as recently shown, BeWo cells may be cultured on permeable filter supports forming a polarized monolayer in which receptors can be studied from either the apical or basolateral domain (Corneus & van der Ende, 1991)

Taken all together these studies imply that TGF-β, and TGF-β2 in particular, are important in placental function and embryonic development during pregnancy. Further studies of placental TGF-β receptors and the actions of the different TGF-β isoforms will lead to a greater understanding of the crucial roles of trophoblast cells in placental implantation, fetal-maternal nutrient/waste exchange, hormone production and the maternal-immune response. The treatment of certain conditions of pregnancy, such as the overinvasiveness of choriocarcinoma, the underinvasiveness of preeclampsia, and recurrent otherwise unexplained spontaneous abortions, requires a greater understanding of the factors, such as TGF-β, that may be involved in controlling trophoblast functions. Furthermore, the increased understanding of TGF-β functions and its receptors will precede the application of TGF-β's therapeutic potential in cancer treatment, wound repair, and immunosuppression.

MATERIALS AND METHODS

Cell culture:

The human trophoblastic cell line BeWo, as well as the mink lung epithelial cell line, Mv1Lu, were obtained from the American Tissue Culture Collection (Rockville, MD.). BeWo cells were maintained as adherent cultures in RPMI medium (Gibco/BRL, Toronto, Ont.) supplemented with 10% ($^{v}/_{v}$) fetal bovine serum (FBS) and 1% ($^{v}/_{v}$) sodium pyruvate stock solution (Gibco/BRL, Toronto, Ont.). Mv1Lu cells were also maintained as adherent cultures but were grown in DMEM (Gibco/BRL, Toronto, Ont.) supplemented with 10% ($^{v}/_{v}$) FBS. All cells were maintained at 37 C in 5% CO in humidified air. Stock cultures were grown in the absence of antibiotics, had their medium changed twice a week and were subcultured after dissociation with 0.25% trypsin-EDTA (Gibco/BRL, Toronto, Ont.). 24 hours before experiments cells were seeded in their normal growth medium at a density of 6 X 10^{5} cells/well in 24-well plastic tissue culture plates. For some experiments, cells were plated at a higher density 18 hours prior to experiments and some were plated at a lower density and allowed to reach confluency in 48 hours.

Iodinations:

Recombinant TGF-β1 was obtained from Bristol-Myers Squibb Pharmaceutical Research Institute, (Seattle, WA). TGF-β2, from porcine platelets, was purchased from R&D Systems (Minneapolis, MN.). Recombinant TGF-β2 was from Austral Biologicals (San Ramon, CA.). TGF-β1 and TGF-β2 were iodinated to high specific activity (1.8 - 2.7 μCi/pmol) by the chloramine-T method as described, (Frolik et al., 1984, Ruff & Rizzino, 1986) with modifications as follows. Either 2 or 5 μg of recombinant TGF-β1, or 2 μg of recombinant TGF-β2 diluted in 4 mM HCL or 1 μg of TGF β2 from porcine platelets (redissolved into 15 μl of 30% acetonitrile/0.1% trifluoroacetic acid since this is the chromatography solvent in which TGF-β2 elutes during its purification) was used. TGF-β was diluted with 10 μl of 1.0 M sodium phosphate, pH 7.5, and 1 mCi

$Na^{125}I$ (13 mCi of ^{125}I/μg iodine; Amersham International). To initiate the reaction, 5 μl of chloramine-T solution (50 μg/ml in 1.0 M sodium phosphate buffer, pH 7.5) was added, and the time was noted. Following this, a second (after 2 min), then a third (after 3.5 min) additional aliquot 5 μl of chloramine-T solution was added. The reaction was performed at room temperature with occasional agitation. One min after the final addition of chloramine-T solution, the reaction was terminated by adding: 20 μl of a saturated tyrosine solution (9 mg/ml) in 50 mM sodium phosphate buffer, pH 7.5 (PB); 200 μl 60 mM potassium iodide in PB; and 200 μl of ultrapure urea (1.2g/1 ml in 1 M HCl). The final mixture was passed over a PD-10 Sephadex G-25 column (Pharmacia LKB, Uppsala, Sweden) which was equilibrated and eluted with a solution of 4 mM HCl, 75 mM NaCl and 0.1% bovine serum albumin (BSA), in order to separate free $Na^{125}I$ that incorporated into protein. Typically 97 -99% of the iodinated peptides was precipitable by 20% trichloroacetic acid (TCA).

Equilibrium binding assays:

For equilibrium saturation studies, confluent monolayers in 24-well plates were washed three times with binding buffer (Dulbuecco's phosphate buffer solution, (D-PBS), pH 7.4 containing 0.1% BSA) over a period of 30 min at 4 °C. After washing, cells were incubated in 0.3 ml binding buffer containing 0 - 1000 pM ^{125}I-TGF-β1 or ^{125}I-TGF-β2. Nonspecific binding was determined in the presence of 100-fold excess of unlabeled TGF-β1 or TGF-β2 and duplicate wells were used for each condition. At the end of a 3 hour incubation, so that equilibrium could be reached, cells were washed quickly three times in 1 ml binding buffer. Preliminary time course experiments demonstrated that total ^{125}I-TGF-β binding to BeWo cells appeared to reach equilibrium within one hour (data not shown). The 3 hour incubation period was chosen based on previous studies, since there is little internalization or degradation of ligand for most cell types (Wakefield, 1987), and because a 3 hour period ensures that equilibrium has been reached

for all three major types of TGF-β receptors (Like & Massague, 1985). Bound counts were determined following incubation with 600 µl solubilization buffer, 1% (v/v) Triton-X-100, 10% (v/v) glycerol, 20 mM Hepes, pH 7.4, for 30 min. at 4 °C with gentle agitation (Wakefield et al, 1987). The standard error of duplicates were below 10% demonstrating tightness of data and permitting reliable analysis. Cell number was determined on wells treated identically up to the solubilization step by counting with a hemocytometer after trypsinization.

For equilibrium competition studies, confluent monolayers in 24-well plates were washed in binding buffer as described above and then incubated with 10 or 25 pM of ^{125}I-TGF-β1 or 25 pM ^{125}I-TGF-β2 together with varying concentrations, ranging from 0 to 5000 pM of unlabelled TGF-β1 or TGF-β2 for 3 hours at 4 °C. The cells were washed 3 times with 1 ml of binding buffer, to remove non-specifically bound TGF-β, and then bound counts were determined after incubating with solubilization buffer as above. Duplicate wells were used for each condition. Binding data were analyzed by the LIGAND program developed by Munson & Robard (1980).

Thymidine incorporation assay:

TGF-β1 and TGF-β2 effects on cellular proliferation were examined by measuring the amount of [methyl-^{3}H] thymidine incorporated by BeWo and Mv1Lu cells. Cells were plated in 24-well culture dishes, in their appropriate medium (RPMI for BeWo cells and DMEM for Mv1Lu cells) containing 10% FBS, at a density such that at the time of TGF-β addition the cells were approximately 60% confluent and still actively growing. After a 24 hour period, to permit the cells to settle and to adhere, the cells were washed twice with serum free medium (RPMI for BeWo and DMEM for Mv1Lu). Serum was excluded so as to avoid the effects of growth factors and hormones found in serum. Serum exclusion also prevents α_2M, a serum binding protein, from binding to TGF-β. α_2M has been shown to bind to and inactivate TGF-β (O'Connor-McCourt & Wakefield, 1987). The cells were then incubated for 24 hours at 37 °C and 5%

CO_2 with serum free medium in the absence or presence of various concentrations of TGF-β1 and TGF-β2 ranging from 1 - 1000 pM, in triplicate. At the end of this incubation period, cells were washed twice and the medium replaced with serum free medium and pulse-labelled with 5 μCi/ml [methyl-^{3}H] thymidine (Specific activity 85 Ci/mMol, Amersham International) for 3 hours at 37 °C. A PHD cell harvester (Cambridge Technology Inc., Watertown, MA.) was used to wash away the excess ^{3}H-thymidine and to harvest the cells onto glass filter fibers, (Skatron Inc., Sterling, VA.) and the radioactivity was measured by liquid scintillation counting with Universol (ICN Biomedicals, Irvine, CA.) scintillation cocktail. Nonspecific binding to glass filter fibers was determined by washing control wells, containing only medium and 5 μCi/ml [methyl-^{3}H] thymidine, through the harvester on to glass filter fibers. The amount of nonspecific binding of [methyl-^{3}H] thymidine determined in this manner was subtracted from the counts obtained from TGF-β treated wells, to obtain [methyl-^{3}H] thymidine labelled DNA specifically bound to glass filter fibers.

Gel electrophoresis and autoradiography:

Samples for fibronectin analysis were electrophoresed on 6% SDS-polyacrylamide gels. Electrophoresis was performed according to the conditions of Laemmli (Laemmli, 1970). Following electrophoresis, gels were stained with Coomassie Brilliant Blue, destained, dried, and exposed to Kodak Omat AR film (Eastman Kodak Co., Rochester, N.Y.) at -80 °C with use of DuPont Cronex Lightning Plus intensifying screens (E.I. DuPont De NeMons & Co., Wilmington DE.) for 2 - 4 days. ^{14}C-labelled molecular mass standards from Bethesda Research Laboratories (Gaithersburg, MD.), were used and include: myosin heavy chain, 200 kDa; phosphorylase, 97 kDa; bovine serum albumin, 68 kDa, ovalbumin, 43 kDa and carbonic anhydrase, 29 kDa.

Quantification of fibronectin synthesis:

A) Isolation of fibronectin by Gelatin:

Cultures of BeWo cells or Mv1Lu cells at 80% confluency, in 12-well plates were fed serum-free RPMI medium or DMEM medium respectively, supplemented with the various concentrations of TGF β1 or TGF-β2 ranging between 1 - 2000 pM. After a 24 hour incubation at 37 °C, 5% CO_2, cells were washed 2 times and replaced with serum-free, methionine-free DMEM medium (Gibco/BRL, Toronto, Ont.) containing ^{35}S-methionine-cysteine (Trans ^{35}S-Label, specific activity 1130 Ci/mMole, ICN Biomedicals, Irvine, CA.) at 100 µCi/ml and the appropriate concentration of TGF-β1 or TGF-β2. Control wells remained without TGF-β. After a 3 hour labelling period, fibronectin in both the conditioned medium and cell layer was extracted as described by Ignotz & Massague (1986) and Segarini et al., (1987), with some modifications. The cells were washed 2 times with 1 ml cold buffer containing 0.15 M NaCl, 25 mM Tris HCl, pH 7.4. The monolayers were extracted with 0.5 ml of buffer containing urea and protease inhibitors, (1 M urea, 1 mM Dithiothreitol (DTT), 10mM Tris-HCl, pH 7.4, 10 mM ethylenediaminetetraacetic acid (EDTA), and proteases inhibitors: 2 mM phenylmethylsulfonyl-fluoride (PMSF); 5 µg/ml leupeptin; 50 µg/ml benzamidine; 50 µg/ml soybean trypsin inhibitor (STI); 10 µg/ml apoprotinin), by rocking on ice for 5 min. The cells were then scraped up using a rubber policeman, transferred to microfuge tubes, and then vigorously vortexed for 5 min, to further facilitate extraction of extracellular matrix proteins. The cells were pelleted at 25,000 x g for 10 min and the supernatants retained for further analysis.

For the isolation of fibronectin, the supernatants were diluted with an equal volume of dH_2O and made to 0.5% (v/v) Triton-X-100. The conditioned medium was also made to 0.5% (v/v) Triton-X 100. 50 µl of a gelatin-Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden) suspension (1:1 in buffer containing 0.15 M NaCl and 25 mM Tris-HCl, pH 7.4) was added to each sample followed by an overnight incubation at 4 °C with gentle rocking. Samples were then centrifuged at 20,000 x g to recover the gelatin-Sepharose

beads. The beads were washed three times with buffer containing 0.5% (v/v) Triton-X-100, 0.15 M NaCl, 25 mM Tris-HCL, pH 7.4. Fibronectin was released by resuspending the beads in electrophoresis sample buffer (5% (v/v) β-mercaptoethanol, 1% SDS, 10% glycerol, 50 mM Tris-HCl, 0.0004% (w/v) bromophenol blue) and heating at 100 °C for 5 min. Finally the samples were centrifuged at 20,000 x g for 10 min and the supernatants were analyzed by SDS-PAGE.

B) Isolation of fibronectin by Immunoprecipitation:

Alternatively, fibronectin was isolated from the diluted urea extracts of BeWo cells by immunoprecipitation with rabbit anti-human polyclonal antibodies (Upstate Biotechnology Inc, Lake Placid, N.Y.). Samples were pre-cleared with $^1/_{10}$ volume of packed protein-A Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden). 10 µg of anti-fibronectin antibody was added to the samples and then incubated overnight at 4 °C. The immune complexes were recovered by addition of $^1/_{10}$ volume of packed protein-A Sepharose and incubated for 1 additional hour. The protein-A Sepharose beads were washed two times in buffer containing 0.5 M urea, 5 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.4 mM DTT, 0.2% (v/v) Triton-X-100, 1 M NaCl, 5 mM EGTA and protease inhibitors: 1 mM PMSF, 5 µg/ml leupeptin, 50 µg/ml benzamidine, 50 µg/ml STI and 10 µg/ml apoprotinin. Since alternating wash buffers from high salt to low salt may help in removing background proteins, the beads were then washed two more times with essentially the same buffer as above but containing 150 mM NaCl rather than 1 M NaCl. Each wash was separated by a 10 min incubation at 4 °C. Fibronectin was released by heating at 100 °C for 5 min in electrophoresis sample buffer under reducing conditions and analyzed by SDS-PAGE.

C) Western analysis of fibronectin:

For Western immunoblot analysis, BeWo cell monolayers treated with or without TGF-β1 were extracted as described with buffer

containing urea and protease inhibitors. After pelleting the cells by centrifugation, the extracted ECM proteins in the supernatant were separated by SDS-PAGE and transferred to a nitrocellulose sheet using transblot buffer containing 25 mM Tris HCl, pH 7.5, 192 mM Glycine, 20 % methanol, 0.1% SDS for 15 hours at 30 volts, then 1 hour at 100 volts. After the transfer was completed, the nitrocellulose sheet was washed twice with immunoblotting buffer containing 0.1% ($^{v}/_{v}$) Tween-20, 20 mM Tris HCl, 500 mM NaCl, and then blocked in immunoblotting buffer containing 3% powdered milk. The blot was then incubated with polyclonal anti-human fibronectin antibody (Upstate Biotechnology Inc., Lake Placid, N.Y.) in immunoblotting buffer followed by incubation with 20 µl ^{125}I-protein A (30 mCi/mg; Amersham International) in 50 ml immunoblotting buffer. The blot was washed 4 times after incubations with antibodies and ^{125}I-protein A and then subjected to autoradiography.

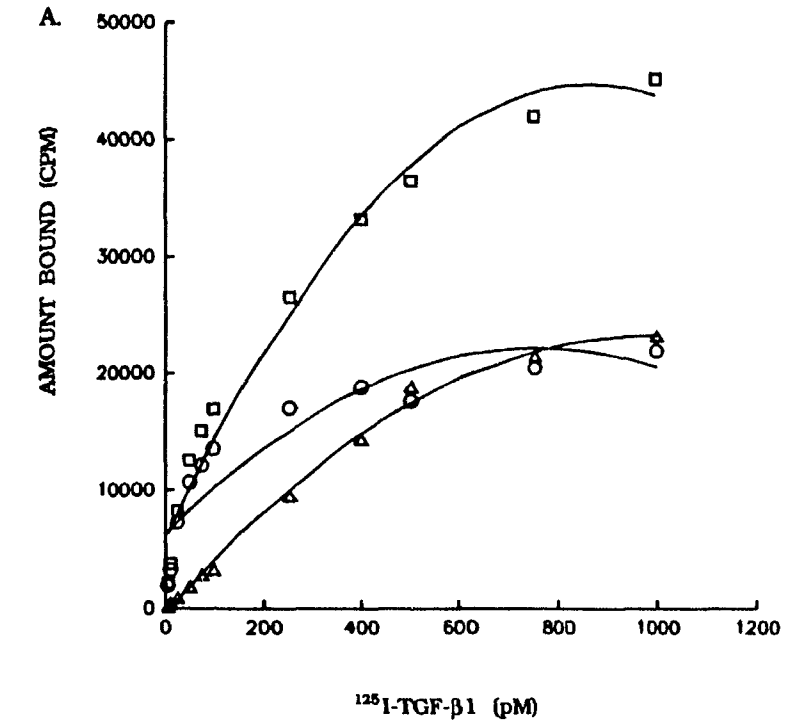
RESULTS

Radioligand Binding Assays

A. Equilibrium Saturation Experiments:

One of the criteria that must be fulfilled in ruling that a ligand binds to and exerts it actions via a receptor is saturability. To determine whether ^{125}I-labelled TGF-β1 binds saturably to BeWo cells in monolayer, equilibrium saturation binding experiments were performed. Figure 4A shows a typical binding isotherm where BeWo cells were incubated with increasing amounts of ^{125}I-TGF β1 in the presence and absence of unlabelled TGF-β1. Nonspecific binding was determined in the presence of 100-fold excess unlabelled TGF-β1. Specific binding was determined by subtracting nonspecific binding from total binding, obtained in the absence of unlabelled TGF-β1. BeWo cells show specific saturable binding with ^{125}I-TGF-β1 and specific binding was essentially saturated by 600 pM ^{125}I-TGF-β1. Transformation of the data by Scatchard analyses of the binding by the LIGAND programs developed by Munson and Robard (1980) gave a linear plot characteristic of a single high affinity binding site with an estimated number of binding sites (+/- s.e.m.) of 70,000 +/- 16,500 (n=3) and a dissociation constant (K_d, +/- s.e.m) of 66 +/- 3 pM (n=3)(Figure 4B). Relatively high background of nonspecific binding (10 to 30% of total CPM) which increased with increasing amount of ^{125}I-TGF-β1 was observed. The high nonspecific background obtained especially at higher concentrations of ^{125}I-TGF-β1 is typical in TGF-β studies (Wakefield, 1987). This situation makes it difficult to exclude the possible existence of additional lower affinity sites since specific binding is masked as background nonspecific binding increases.

Since trypsin dissociation of the cells and the time between plating and assay may affect the binding characteristics of TGF-β receptors, a set of experiments were performed with cells plated at


A.
AMOUNT BOUND (CPM)
50000
40000
30000
20000
10000
0
0
200
400
600
800
1000
1200
125I-TGF-β1 (pM)

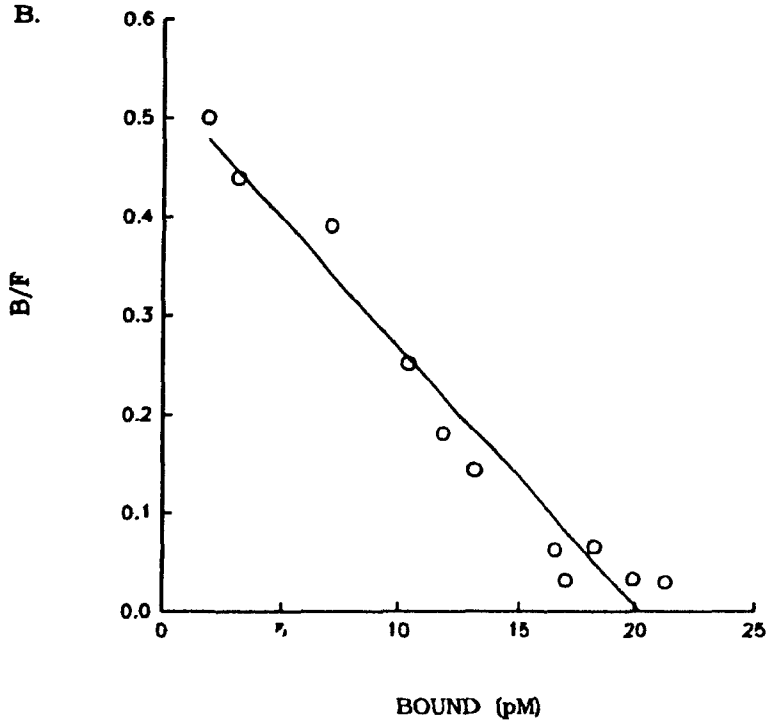

B.
B/F
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
5
10
15
20
25
BOUND (pM)

FIGURE 4. ^{125}I-TGF-β1 binds specifically and saturably to BeWo cell monolayer. Monolayers of BeWo cells were incubated with various concentrations (pM) of ^{125}I-labelled TGF-β1 (recombinant) at 4 °C for 3 h as described in materials and methods. (A) represents a saturation binding curve where ^{125}I-TGF-β1 specifically bound (open circles) is the difference between the total (open squares) and nonspecific (open triangles) binding. Each point is the mean of duplicate samples that generally differed by < 10%. (B) represents the respective data transformed and plotted by the method of Scatchard for the representative experiment. The binding parameters for the plot shown here are 44,317 binding sites/cell with K_D value of 37 pM. The specific activity of ^{125}I-TGF-β1 in this experiment was 1.5 μCi/pMole. The K_D values of ^{125}I-TGF-β1 binding to BeWo cell monolayers ranged in three separate experiments between 39 pM and 93 pM and the number of binding sites ranged between 45,000 and 100,000 per cell.

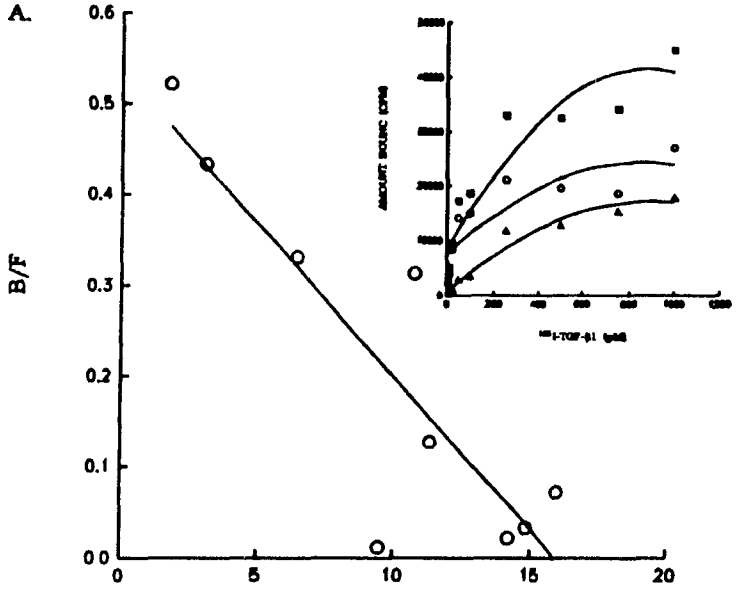
different times prior to assay. Cells trypsin dissociated, plated and allowed to reach confluency in 18 hours and 48 hours gave essentially identical results compared with those of 24 hours, (Figure 5A & 5B) i.e. no significant difference in the total number of receptors and dissociation constant (K_D) was found. TGF β1 bound to 81,987 sites per cell and 35,238 sites per cell with K_D of 37 pM and 32 pM for BeWo cells trypsinized 18 hours and 48 hours prior to assay respectively.

Equilibrium saturation studies of ^{125}I-labelled TGF-β2 binding to BeWo cells showed that ^{125}I-TGF-β2 binds specifically and saturably (Figure 6A) with an apparent K_D (+/- s.e.m) of 39 +/- 5 pM (n=3) and an estimated number of binding sites per BeWo cell (+/- s.e.m.) of 85,000 +/- 17,500 (n=3) (Figure 6B). In these experiments the background of nonspecific binding, which was slightly higher than that observed with TGF-β1, approached 50% of total CPM. These results indicate that the average binding affinities for TGF-β1 and TGF-β2 binding to BeWo cells are similar, the apparent K_D values averaging at approximately 50 pM for both TGF-β1 and TGF-β2. The total number of binding sites per BeWo cell are similar as well, averaging at approximately 75,000 sites per cell.

B. Equilibrium Competition Experiments:

Competition experiments were performed with ^{125}I-labelled TGF β1 or ^{125}I-labelled TGF-β2 in order to examine whether ^{125}I-TGF-β1 or ^{125}I-TGF-β2 were binding to the same or different sites on BeWo cells (Figure 7A and 7B). Unlabelled TGF-β1 and unlabelled TGF β2 were able to completely inhibit the binding of 25 pM ^{125}I TGF β1 and 50 pM ^{125}I-TGF-β2, indicating that there are no sites available to one isoform and not the other. The mean half maximal inhibition of binding (IC_{50} +/- s.e.m.), when ^{125}I-TGF β1 was used as the radiotracer, was 81 +/- 1.5 pM (n=3) with unlabelled TGF β1 and 68 +/- 2.5 pM (n=3) with unlabelled TGF-β2. Similarly, when ^{125}I-TGF-β2 was the radiotracer the IC_{50} values (+/- s.e.m) were 74 +/- 6.4

A.


B/F
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
5
10
15
20
BOUND (pM)

B.

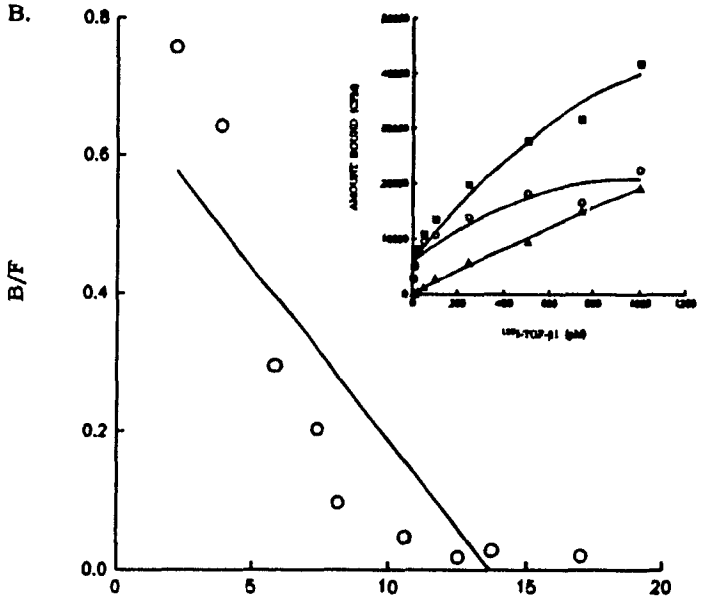
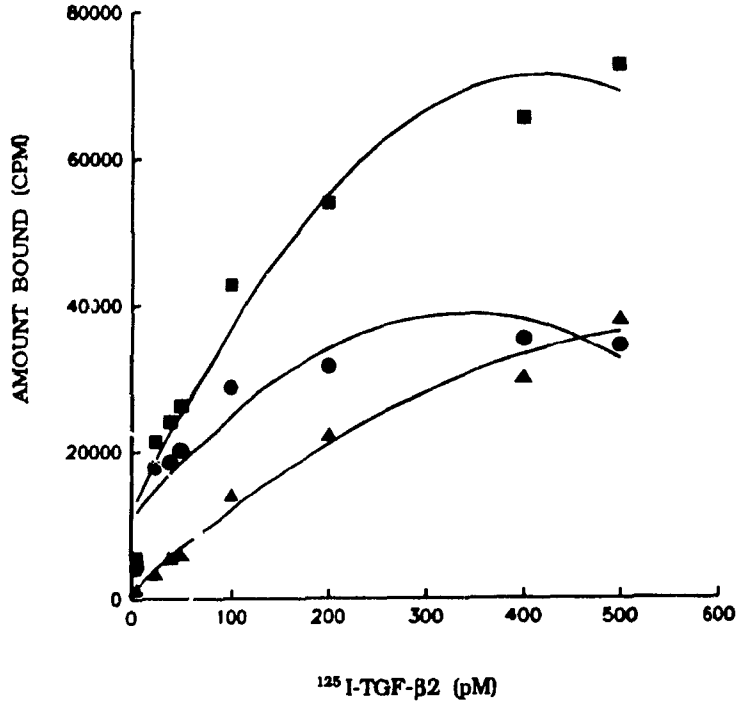

B/F
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
BOUND (pM)

FIGURE 5. TGF-β1 binds to BeWo cell monolayer plated 18 h and 48 h post-trypsin dissociation with similar binding parameters. (A) and (B) represent Scatchard plots of ^{125}I-TGF-β1 (recombinant) binding to BeWo cells trypsinized and plated 18 h and 48 h prior to incubation with various concentrations of ^{125}I-labelled TGF β1 at 4 °C for 3 h as described. The binding parameters for the plots shown here are 81,987 and 35,238 binding sites/cell with K_D values of 37 and 32 pM for ^{125}I-TGF-β1 binding to BeWo cells plated 18 and 48 h after trypsin dissociation respectively. The insets show saturation binding curves of the same data. The specific activity of ^{125}I-TGF-β1 in both experiments was 2 μCi/pMole.

A


80000
60000
40000
20000
0
AMOUNT BOUND (CPM)
0
100
200
300
400
500
600
125I-TGF-β2 (pM)

B.

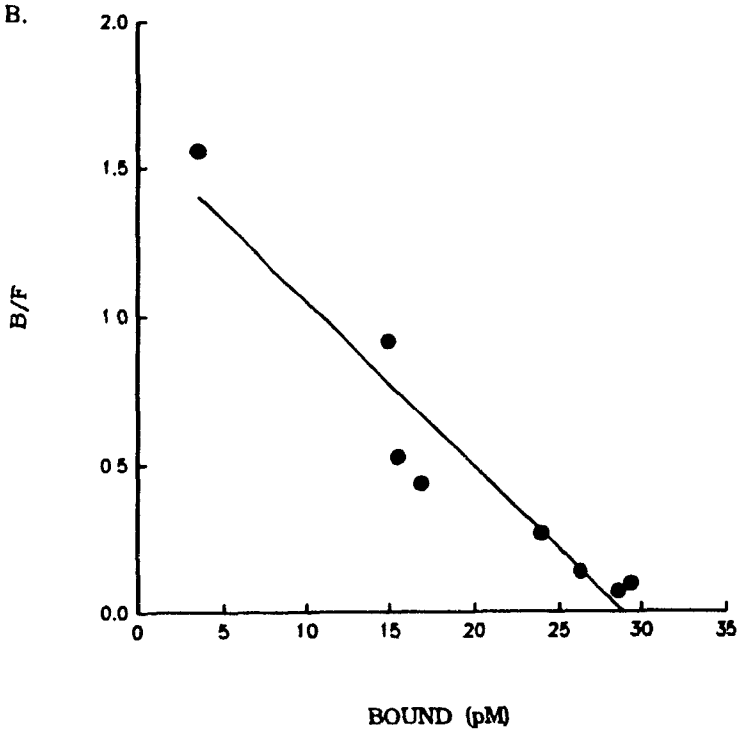
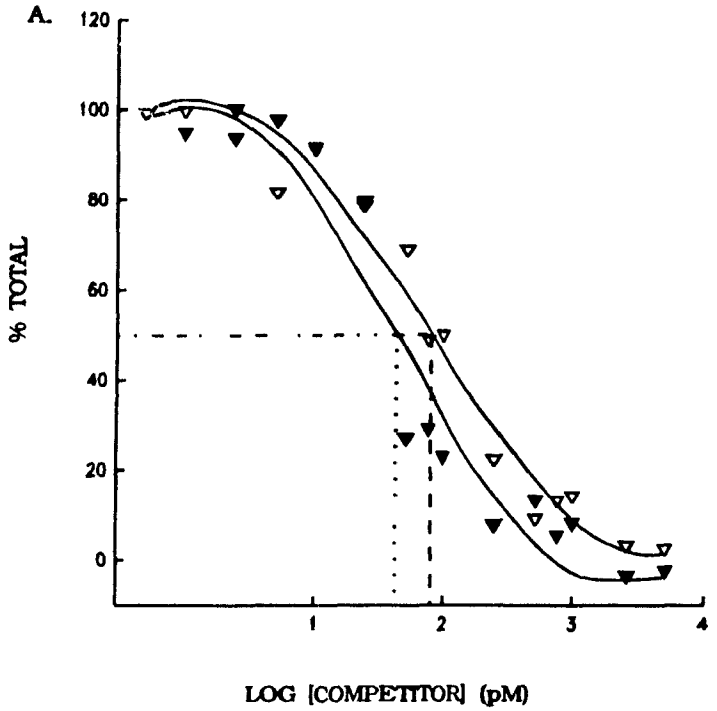

2.0
1.5
1.0
0.5
0.0
B/F
0
5
10
15
20
25
30
35
BOUND (pM)

FIGURE 6. ^{125}I-TGF-β2 binding to BeWo cell monolayer. Various concentrations of ^{125}I-TGF-β2 (recombinant) was incubated with BeWo cells at 4 °C for 3 h. (A) represents the saturation analysis of ^{125}I-TGF-β2 binding to BeWo cells and demonstrates that ^{125}I TGF β2 binds specifically and saturably. (B) Scatchard analysis of the binding data presented in (A) using the LIGAND program developed by Munson and Robard (1980). The binding parameter of ^{125}I-TGF β2 binding in this representative experiment are 82,873 binding sites/cell with K_D value of 35 pM. The specific activity of ^{125}I TGF-β2 in this assay was 2.7 μCi/pMole. The K_D values of ^{125}I-TGF-β2 binding to BeWo cell monolayers in three separate experiments ranged between 31 pM and 51 pM. The number of binding sites ranged between 82,000 and 120,000 per cell.


A.
120
100
80
60
40
20
0
% TOTAL
1
2
3
4
LOG [COMPETITOR] (pM)

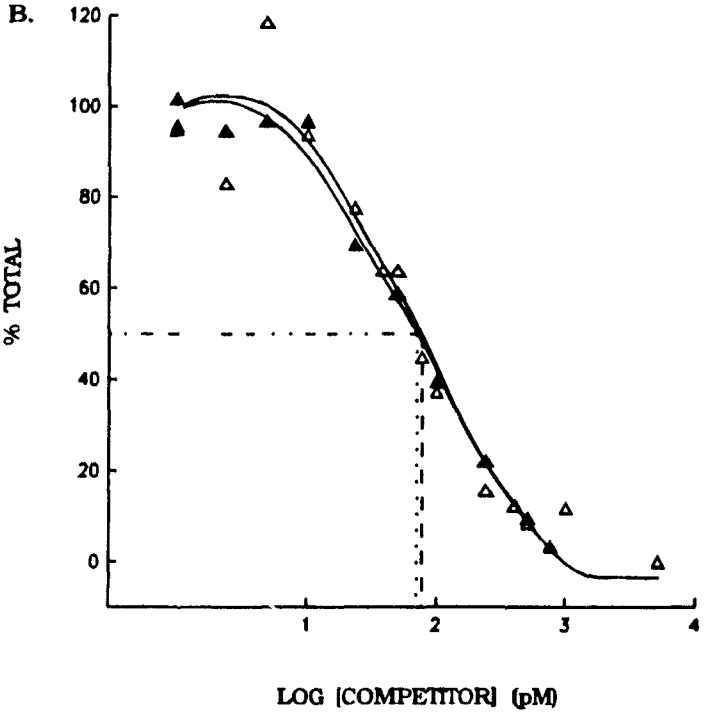

B.
120
100
80
60
40
20
0
% TOTAL
1
2
3
4
LOG [COMPETITOR] (pM)

FIGURE 7. Representative competition experiments of ^{125}I-TGF-β1 or ^{125}I-TGF-β2 binding to BeWo cell monolayer. Confluent monolayers of BeWo cells were incubated for 3 h at 4 °C in the presence of 25 pM ^{125}I-TGF-β1 (recombinant) (A) or 50 pM ^{125}I-TGF-β2 (porcine) (B) with the indicated concentrations of unlabelled TGF β1 (recombinant) (open triangles) or unlabelled TGF-β2 (porcine) (closed triangles). Specifically bound radiolabel was determined for each sample and expressed as a percent of the amount specifically bound in samples without unlabelled TGF-β. Duplicate wells were used for each condition and each value represents the mean. The IC_{50} of TGF β1 and TGF-β2 competing for ^{125}I-TGF-β1 binding from the representative experiment presented in panel A was 80 and 71 pM respectively. The IC_{50} of TGF-β1 and TGF-β2 competing for ^{125}I-TGF-β2 binding from the representative experiment presented in panel B was 74 and 71 pM respectively. The IC_{50} values ranged in three separate experiments between 79-84 pM for TGF-β1 and 63-71 for TGF-β2 displacement of ^{125}I-TGF-β1 and between 63 - 88 pM for TGF-β1 and 59 - 84 pM for TGF-β2 displacement of ^{125}I-TGF-β2. The specific activities of both ^{125}I-TGF-β1 (recombinant) and ^{125}I-TGF-β2 (porcine) in these representative experiments was 2.0 μCi/pMole.

pM (n=3) and 71 +/- 4.7 pM (n=3) for unlabelled TGF-β1 and TGF-β2 respectively.

As a general rule, for equilibrium competition binding studies, the concentration of radioligand present should be at least one fourth or less than that of the concentration of unlabelled ligand giving 50% displacement. As the concentration of radiotracer is increased, the experimentally observed IC_{50} will be an overestimation of the actual IC_{50} (Goldstein et al , 1974). Considering that the 25 pM of TGF-β1 and the 50 pM of radiotracer used in the above competition experiments are more than one fourth of the observed IC_{50} values, further experiments were performed with 10 pM ^{125}I-TGF-β1 as radiotracer (Figure 8). Unlabelled TGF-β1 and unlabelled TGF β2 were able to compete for 10 pM ^{125}I-TGF-β1 binding to BeWo cells to the same extent as when 25 pM of radiotracer was used. The IC_{50} values of TGF-β1 and TGF-β2 competing for 10 pM of ^{125}I-TGF-β1 binding were 68 pM and 84 pM respectively, similar to those observed above. It was not possible to reduce the concentration of radiotracer further since a sufficient amount of specific radioactivity is required to enable minute amounts of binding to be measured.

C. TGF-β2 from porcine platelets binding vs. recombinant TGF-β2 binding:

Initially when these studies were begun only TGF-β2 from porcine platelets was available. More recently, recombinant TGF-β2 was available for use. To verify if TGF-β2 from porcine platelets has the same binding characteristics as recombinant TGF-β2, competition experiments using recombinant TGF-β2 as the radiolabelled ligand and as the unlabelled competitor, were performed (Figure 9A & 9B). No difference between recombinant TGF-β2's and porcine TGF-β2's ability to compete for the indicated radiolabelled ligand's binding was found. The IC_{50} of recombinant TGF β2 and TGF-β1 competing for ^{125}I-TGF-β1 was 66 pM and 67 pM respectively. Moreover, TGF-β1 was able to compete for recombinant

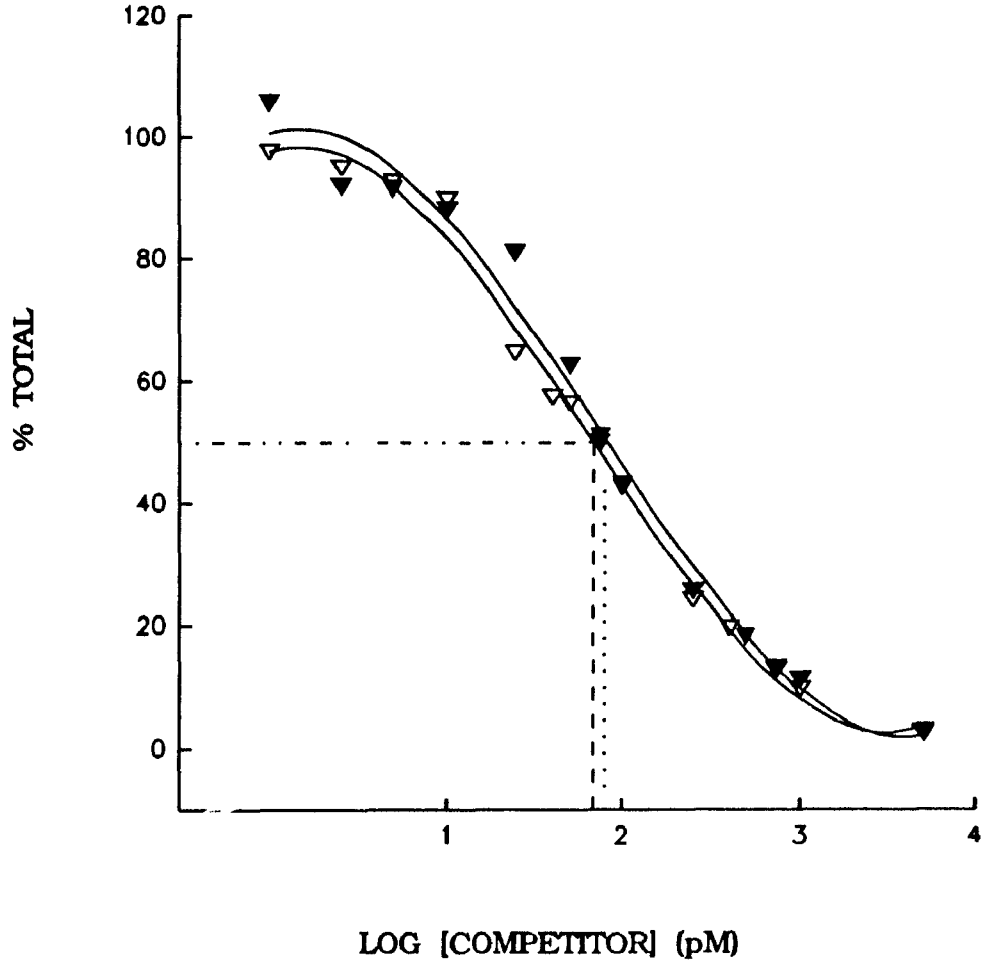
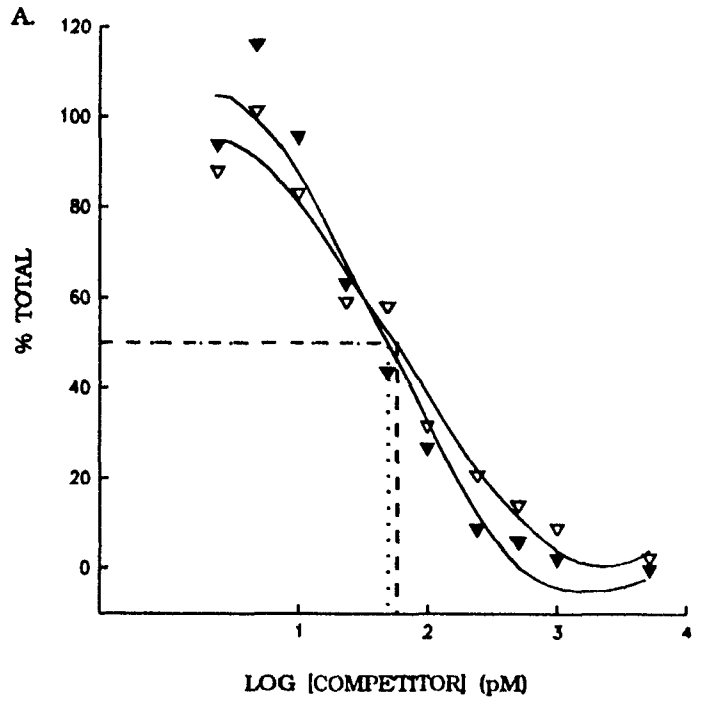

120
100
80
60
40
20
0
% TOTAL
1
2
3
4
LOG [COMPETITOR] (pM)

FIGURE 8. TGF-β1 and TGF-β2 competition of 10 pM ^{125}I-TGF-β1 binding. Confluent monolayers of BeWo cells were incubated at 4 C for 3 h with 10 pM ^{125}I-TGF-β1 in the presence of various concentrations of TGF-β1 (open triangles) or TGF-β2 (closed triangles). The results are plotted as the percentage of binding relative to the radioactivity bound to cells incubated with ^{125}I-TGF-β1. Duplicate wells were used for each condition and each value represents the mean. The IC_{50} of TGF-β1 and TGF-β2 competing for 10 pM ^{125}I-TGF-β1 in this experiment was 68 and 84 pM respectively. The specific activity of ^{125}I-TGF-β1 in this experiment was 1.5 μCi/pMole.


A.
120
100
80
60
40
20
0
% TOTAL
1
2
3
4
LOG [COMPETITOR] (pM)

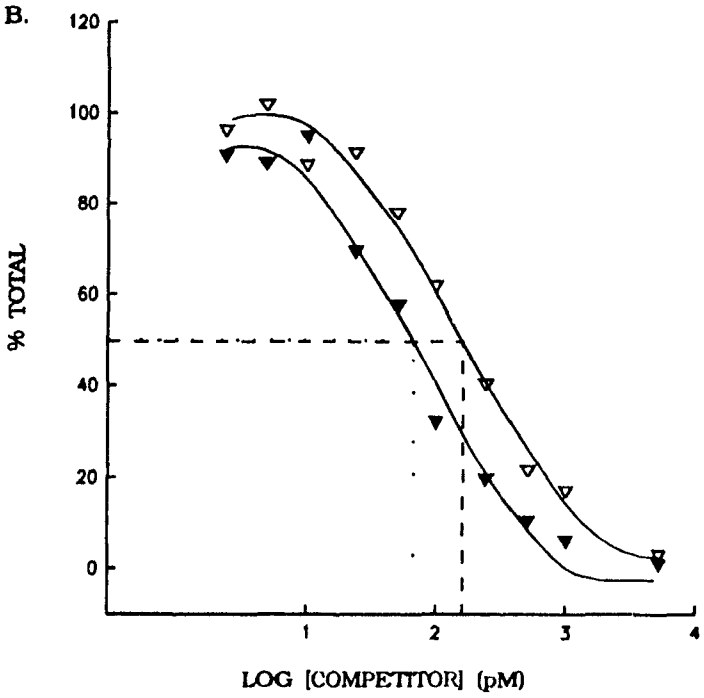

B.
120
100
80
60
40
20
0
% TOTAL
1
2
3
4
LOG [COMPETITOR] (pM)

FIGURE 9. Competition experiment with recombinant TGF-β2. Confluent monolayers of BeWo cells were incubated with recombinant ^{125}I-TGF-β1 (A) or with recombinant ^{125}I-TGF-β2 (B) in the presence of indicated concentrations of unlabelled recombinant TGF-β1 (open triangles) or unlabelled recombinant TGF-β2 (closed triangles). The IC_{50} of TGF-β1 and TGF-β2 competing for ^{125}I-TGF-β1 binding in panel A was 67 pM and 66 pM respectively. The IC_{50} of TGF-β1 and TGF-β2 competing for ^{125}I-TGF-β2 binding shown in panel B was 112 pM and 58 pM respectively. The specific activity of ^{125}I-TGF-β1 and ^{125}I-TGF-β2 in these experiments were 2.5 μCi/pMole and 2.3 μCi/pMole respectively.

^{125}I-TGF-β2 binding in a fashion similar to its competition of porcine TGF-β2 with an IC_{50} of 112 pM. The IC_{50} for recombinant TGF-β2 competition for recombinant ^{125}I-TGF-β2 was 58 pM. Since the IC_{50} value of the competing ligand is a reasonable first approximation of it's apparent equilibrium dissociation binding constant (K_i; Bernett, 1978), it is reasonable to assume that recombinant TGF-β2 and purified porcine TGF-β2 have the same binding characteristics. These competition studies suggest that overall, the population of TGF-β receptors have similar average affinities for both TGF-β1 and TGF-β2, and that the two TGF-β isoforms bind to the same population of TGF-β receptors on BeWo cells.

Functional Assays

A. Proliferation Assays:

An understanding of a ligand-receptor system requires studies of the ligand binding characteristics of the receptor(s) together with studies on the functional response(s). Radioligand binding assays are performed to determine whether a particular ligand binds to a receptor specifically and saturably. Radioligand binding assays also determine receptor binding characteristics, such as its equilibrium dissociation constant for a particular ligand and its number in a given source. However, the demonstration that a ligand binds to a cell with a high affinity and a limited capacity does not absolutely infer that it exerts its effect through a receptor, and a function must be designated as well. With a functional assay, dose/response curves for different agonists or isoforms may be obtained, permitting the differentiation of each agonist's efficacy and potency to elicit a response. Often functional assays complement binding assays in that correlations between binding affinities and pharmacological efficacy and potency can be inferred.

The assay of choice to date for monitoring the purification of

TGF-β present in conditioned medium or cellular extracts has been a well-established ^{3}H-Thymidine incorporation assay which measures the inhibition of Mink Lung Epithelial (Mv1Lu) cell growth by TGF- (Ikeda et al., 1987). ^{3}H-Thymidine incorporation assays measure the proliferative activity of cells. When cells divide and proliferate they must replicate and synthesize DNA. Thymidine is the pyrimidine nucleotide that is exclusively incorporated into DNA. The replication of DNA occurs only in synthetic (S) phase of interphase in the cell cycle.

Figure 10 shows control experiments with Mv1Lu cells treated with either TGF-β1 TGF-β2 for 24 hours which demonstrate that both TGF-β isoforms inhibit growth to the same extent as observed previously (Cheifetz et al., 1987). However, ^{3}H-Thymidine incorporation experiments with BeWo cells showed that there was no significant effect of up to 1000 pM TGF-β1 or TGF-β2 on the growth of these cells (Figure 10). Cells must be treated with TGF-β before they enter S phase in order to see an effect because in general, the inhibitory effect of TGF-β1 on the cell cycle results in delayed progression of cells from G1 to S phase as discussed previously. From experience with passaging BeWo cells, it is estimated that these cells double within 16 hours, indicating that a 24 hour treatment period is adequate time for all cells to complete the cell cycle and thus pass the S phase at least once. Preliminary experiments with shorter TGF-β treatment periods, 6 and 12 hours, also showed no effect on BeWo growth (data not shown).

B. Fibronectin Deposition:

Since, both TGF-β isoforms bind to BeWo cells, further studies were performed in order to try and find a functional assay that may aid in differentiating the biological responses to TGF-β1 and TGF-β2 in this cellular model. The increase in fibronectin expression upon TGF β treatment has also been used as a functional assay for TGF-β in some primary cultures and established cell lines, (Ignotz & Massague, 1986 & 1987,; Segarini et al., 1989; Shi et al., 1990).

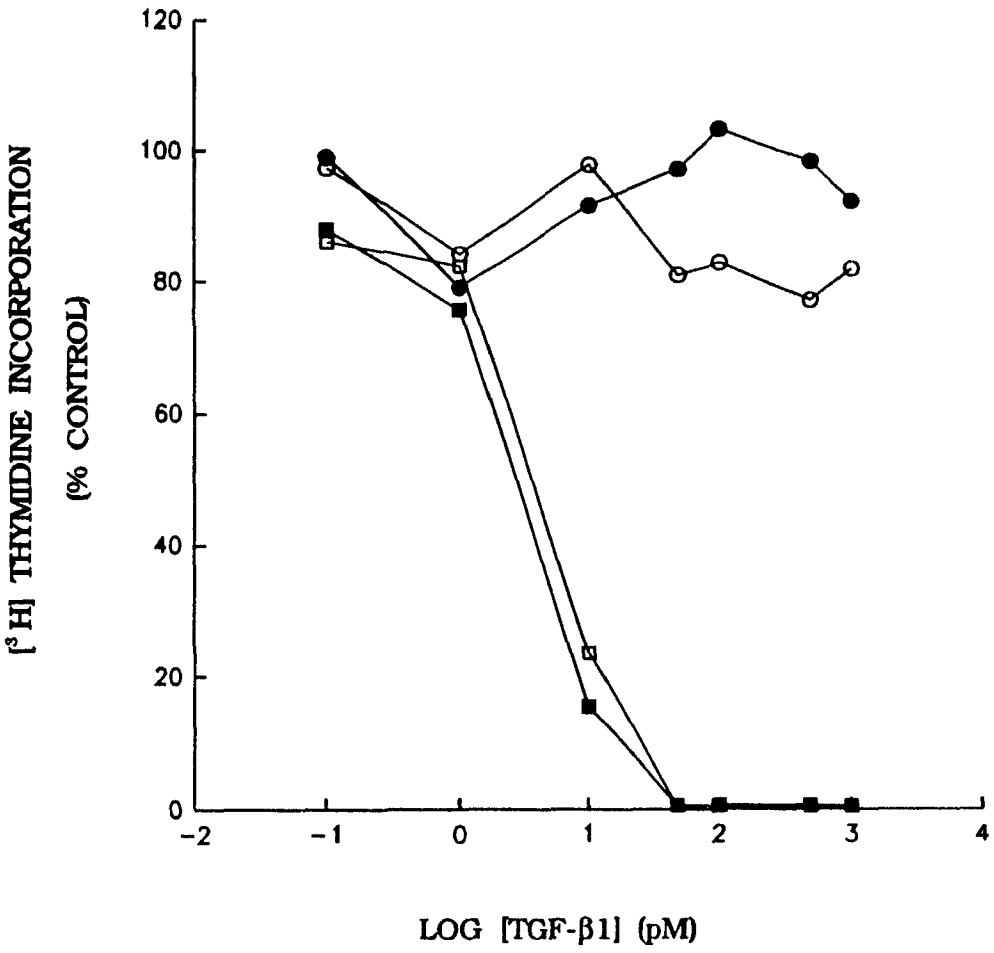

[^{3}H] THYMIDINE INCORPORATION
(% CONTROL)
120
100
80
60
40
20
0
-2
-1
0
1
2
3
4
LOG [TGF-β1] (pM)

FIGURE 10. The effect of TGF-β1 or TGF-β2 on ^{3}H-Thymidine incorporation in BeWo cells or Mv1Lu cells. Monolayers of BeWo cells (Circles) or Mv1Lu cells (Squares) at 60% confluency in 24 well plates were treated with serum-free medium containing varying concentrations (pM) of TGF-β1 (open symbols) or TGF-β2 (closed symbols). After 24 h the cells were pulsed with ^{3}H-Thymidine for 3 h. Cells were trypsinized and collected onto glass fiber filters and the incorporation of radioactivity determined by liquid scintillation counting as described in materials and methods. The results are expressed as the percentage of incorporation of ^{3}H-Thymidine in control cells incubated without TGF-β1 or TGF-β2. Data are the average of triplicate determinations.

To examine whether or not TGF-β1 or TGF-β2 has any effect on fibronectin synthesis in BeWo cells, two strategies were taken. The first involves the ability of gelatin to specifically bind to fibronectin (Engvall and Ruoslahti, 1977). Ignotz and Massague (1986) as well as Segarini et al. (1989) have already applied this technique in order to isolate newly synthesized fibronectin in TGF β studies. The second strategy involves isolating fibronectin by immunoprecipitation with a polyclonal anti-human fibronectin antibody.

After an induction period of 24 hours with increasing concentrations of TGF-β1 or TGF-β2 (0 - 2000 pM), BeWo cells were labelled with ^{35}S Trans-label™ for 3 hours as described in materials and methods. Crude urea extracts of the monolayers were subjected to 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions, (Figure 11A). The crude urea extracts show various protein bands that may include components of the extracellular matrix. No band in particular was significantly affected by 50 or 500 pM TGF-β1 treatment. The urea extracts were then subjected to gelatin-Sepharose affinity chromatography to concentrate newly synthesized fibronectin and to see if its expression responded in a dose-dependent manner to TGF β1. SDS PAGE revealed that the gelatin-Sepharose affinity chromatography procedure was unable to clearly distinguish any particular protein band as fibronectin (Figure 11B), which would be expected to migrate as a 220 kDa band under reducing conditions, (Yamada & Olden, 1978).

These results indicate that the measurement of fibronectin synthesis, in BeWo cells, by its isolation with gelatin Sepharose may not be a sensitive enough assay. However, the positive control of Mv1Lu cells treated and assayed identically showed that TGF β1 (Figure 12A) and TGF-β2 (Figure 12B) induced increased fibronectin synthesis. Mv1Lu cells have previously been shown to respond to TGF-β1 treatment with increased fibronectin synthesis as measured

TGF-β_1 (pM)

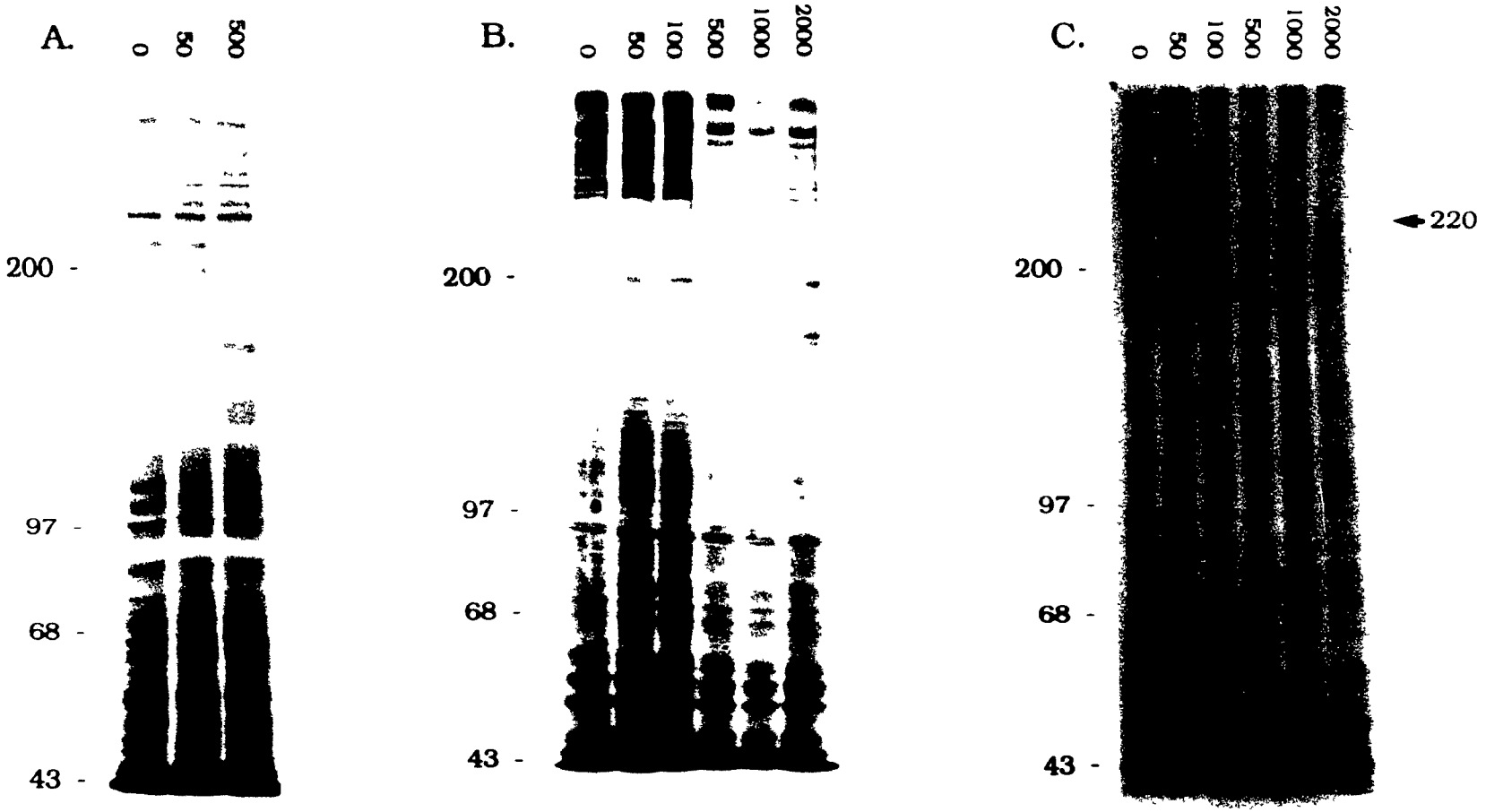

A.
0
50
500
200
97
68
43
B.
0
50
100
500
1000
2000
200
97
68
43
C.
0
50
100
500
1000
2000
220
200
97
68
43

FIGURE 11. Effect of TGF-β1 on fibronectin from BeWo cells. Monolayers of BeWo cells at 80% confluency in 12-well plates were treated with serum-free medium containing the indicated concentrations (pM) of TGF-β1. After 24 h the medium was replaced with methionine-free medium containing Trans ^{35}S-label™ and the appropriate concentration (pM) of TGF-β1. After 3 hours the medium was collected and monolayers were extracted with urea containing buffer to extract extracellular matrix proteins as described in materials and methods. The extracted material was subjected to gelatin-Sepharose affinity chromatography or immunoprecipitated using an anti-human fibronectin polyclonal antibody. Shown are: the urea extracts containing total extracellular matrix proteins (A); the eluates from the gelatin-Sepharose affinity chromatography (B); and the anti-fibronectin immunoprecipitates (C); that were subjected to SDS-polyacrylamide gel electrophoresis on 6% gels under reducing conditions and autoradiography. Arrow shows the position of purified human fibronectin migrating in the same gels and detected by coomassie blue staining. The position and the molecular mass (kDa) of ^{14}C-labeled protein standards in the gels are indicated. The exposure times shown are 1 day, 4 days and 3 days for panel A, B, and C respectively.

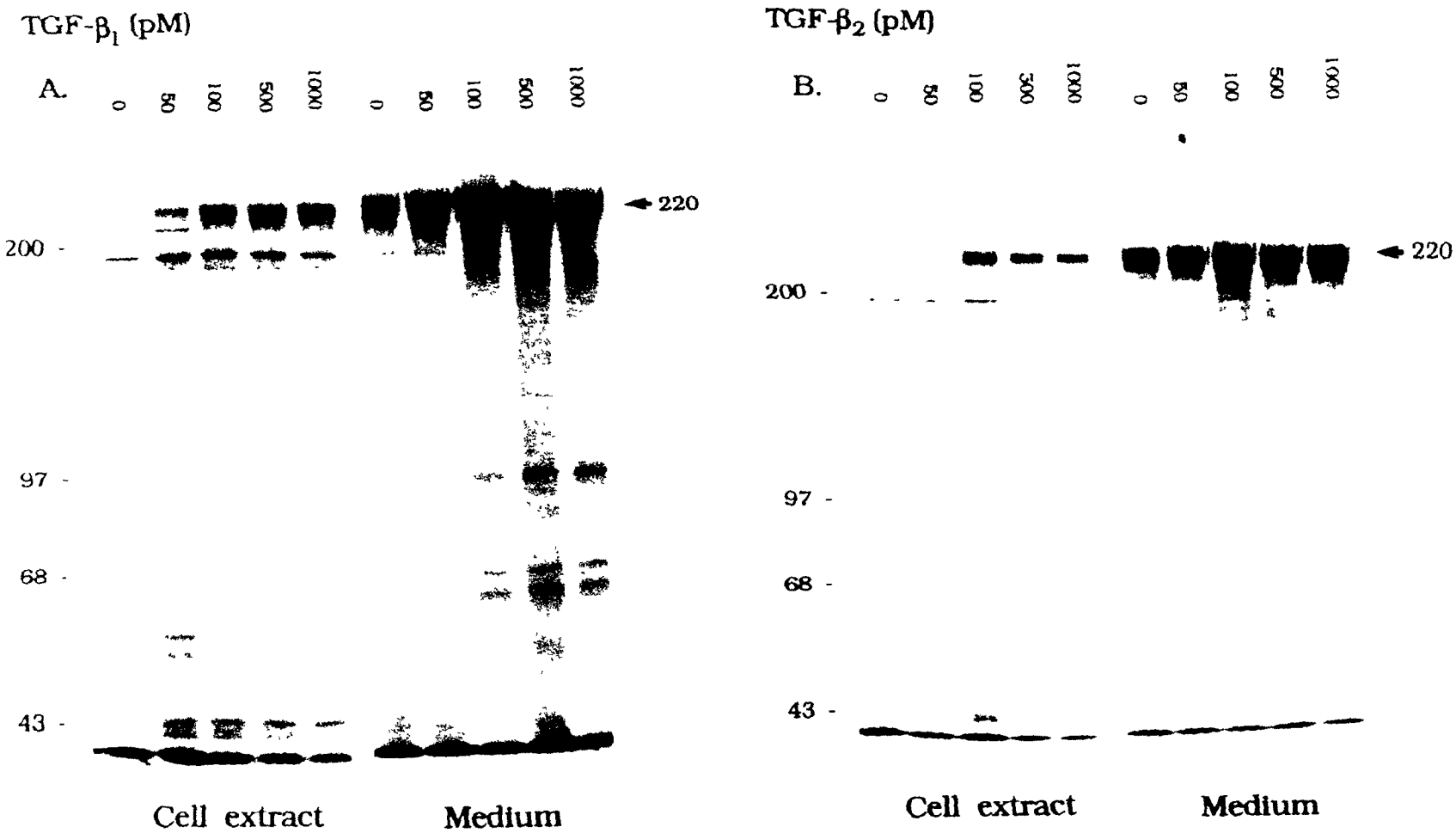

TGF-β1 (pM)
A.
0
50
100
500
1000
0
50
100
500
1000
220
200
97
68
43
Cell extract
Medium
TGF-β2 (pM)
B.
0
50
100
300
1000
0
50
100
500
1000
220
200
97
68
43
Cell extract
Medium

FIGURE 12. Effect of TGF-β1 or TGF-β2 on fibronectin from Mv1Lu cells. Monolayers of Mv1Lu cells at 80% confluency were treated with the indicated concentrations (pM) of TGF-β1 (A) or TGF-β2 (B), labelled with Trans ^{35}S-Label™ and extracted with urea-containing buffer. The urea extracts as well as conditioned medium were subjected to gelatin-Sepharose affinity chromatography and analyzed by SDS-PAGE and autoradiography. The exposure time for the autoradiograms shown here was 3 days.

by concentrating fibronectin with gelatin-Sepharose chromatography (Ignotz & Massague, 1986). Figure 12 also shows that the medium of MvlLu cells contain a large amount of newly synthesized fibronectin that increases in response to TGF-β1 (Figure 12A) or TGF-β2 (Figure 12B).

The alternative method chosen to try to measure fibronectin deposition in BeWo cells is an immunoprecipitation of the urea extracts with a polyclonal antibody against human fibronectin. However, immunoprecipitation with this antibody (Figure 11C) did not significantly reduce the background of ^{35}S-labelled proteins compared with the experiment shown in figure 11B. Several steps were taken in order to try and minimize the number of nonspecific protein bands, none were effective. These included steps, such as: spinning the cellular extract for 30 min prior to adding the antibody; spinning the antibody-antigen binding reaction before adding protein A-Sepharose, in order to remove aggregates and polymerizing proteins; allowing the immune complexes to incubate 10 min between washes; and transferring the protein-A-Sepharose beads to fresh tubes prior to eluting the antibody-antigen complex (data not shown). Thus, this anti-human fibronectin antibody did not appear to be particularly useful for fibronectin immunoprecipitation experiments. One band of approximately 220 kDa that co-migrated in the gels with purified human fibronectin was observed (Figure 11C). However, since the 220 kDa protein band was very minor and flanked by other bands of similar molecular mass, analysis by densitometry could not be interpreted reliably. Nevertheless, as measured by eye, the 220 kDa band did not show any change with increasing concentrations of TGF-β1.

Similar experiments with TGF-β2 treatment were performed and the results of their gelatin-Sepharose isolation and immunoprecipitation of fibronectin are shown in Figure 13. Again no band in particular could be demonstrated to be fibronectin from the gelatin-Sepharose eluates (Figure 13A) and the 220 kDa band

TGF-β_2 (pM)

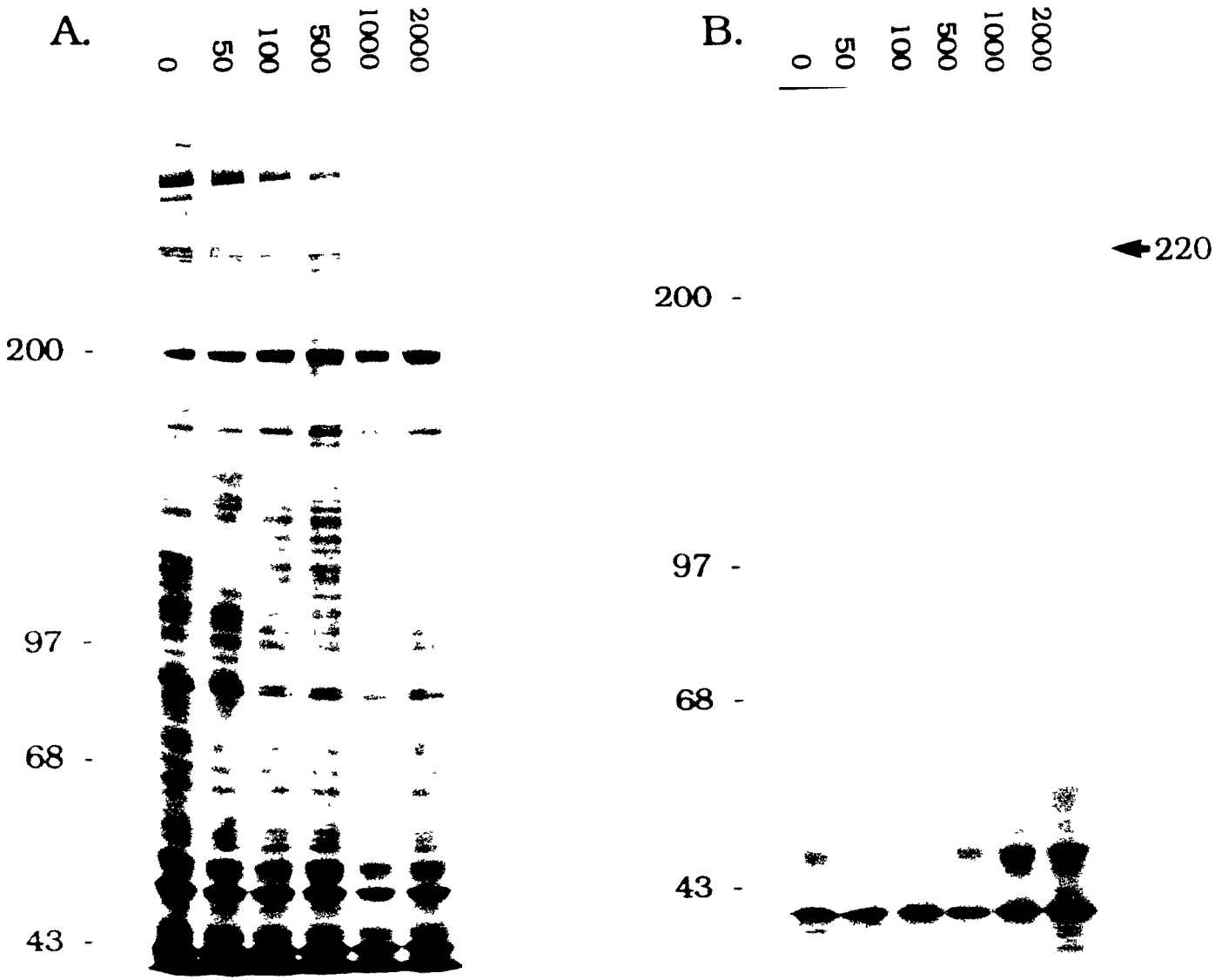

A.
0
50
100
500
1000
2000
200
97
68
43
B.
0
50
100
500
1000
2000
220
200
97
68
43

FIGURE 13. Effect of TGF-β2 on fibronectin from BeWo cells. Monolayers of BeWo cells at 80% confluency were treated with varying concentrations (pM) of TGF-β2 labeled with Trans S-Label™ and extracted with urea-containing buffer. The extracts were subjected to gelatin-Sepharose affinity chromatography (A) or immunoprecipitated with anti-human fibronectin polyclonal antibody (B) as in figure 10. The eluates were subjected to SDS-PAGE and autoradiography. The exposure time for both the autoradiograms shown here was 3 days.

from immunoprecipitation experiments did not increase or decrease in response to TGF-β2 treatment (Figure 13B).

The level of background proteins was higher in the gelatin-Sepharose eluates of BeWo cells' urea extracts (figure 11 & 13) than in those of Mv1Lu cells (figure 12). The experimental treatment of both types of cells were identical with autoradiographic exposure times ranging from 2 to 4 days. The higher level of background proteins in BeWo cells may be due to gelatin-Sepharose's affinity for proteins other then fibronectin found in BeWo cells but not in Mv1Lu cells. Proteolytic activity from BeWo cells which degrades fibronectin and producing fragments to which gelatin-Sepharose binds could, in part, explain the high level of background protein found in the urea extracts of BeWo cells. However, several protease inhibitors were included as noted in
materials and methods and there are also background proteins in the BeWo extracts with higher molecular mass than fibronectin. Time course experiments performed in order to determine fibronectin degradation with time are described later. The 220 kDa band found from immunoprecipitating the urea extracts of BeWo cell monolayers was minor, suggesting that only a minute amount (if any) of newly synthesized fibronectin was being incorporated into the extracellular matrix. Furthermore, gelatin-Sepharose eluates of conditioned medium of Mv1Lu cells treated with TGF-β1 or TGF β2 shows a large increase in newly synthesized fibronectin, (Figure 12A & B). Thus, the medium of BeWo cells treated with or without TGF-β1 was examined by gelatin Sepharose affinity chromatography and immunoprecipitation to see whether BeWo cells were secreting newly synthesized soluble fibronectin into the medium in response to TGF-β1. The immunoprecipitates of BeWo conditioned medium in the presence and absence of increasing concentrations of TGF β1 show that there is no protein in the medium which is recognized by the anti-human fibronectin antibody, (Figure 14A). Gelatin Sepharose eluates of BeWo conditioned medium showed only one band

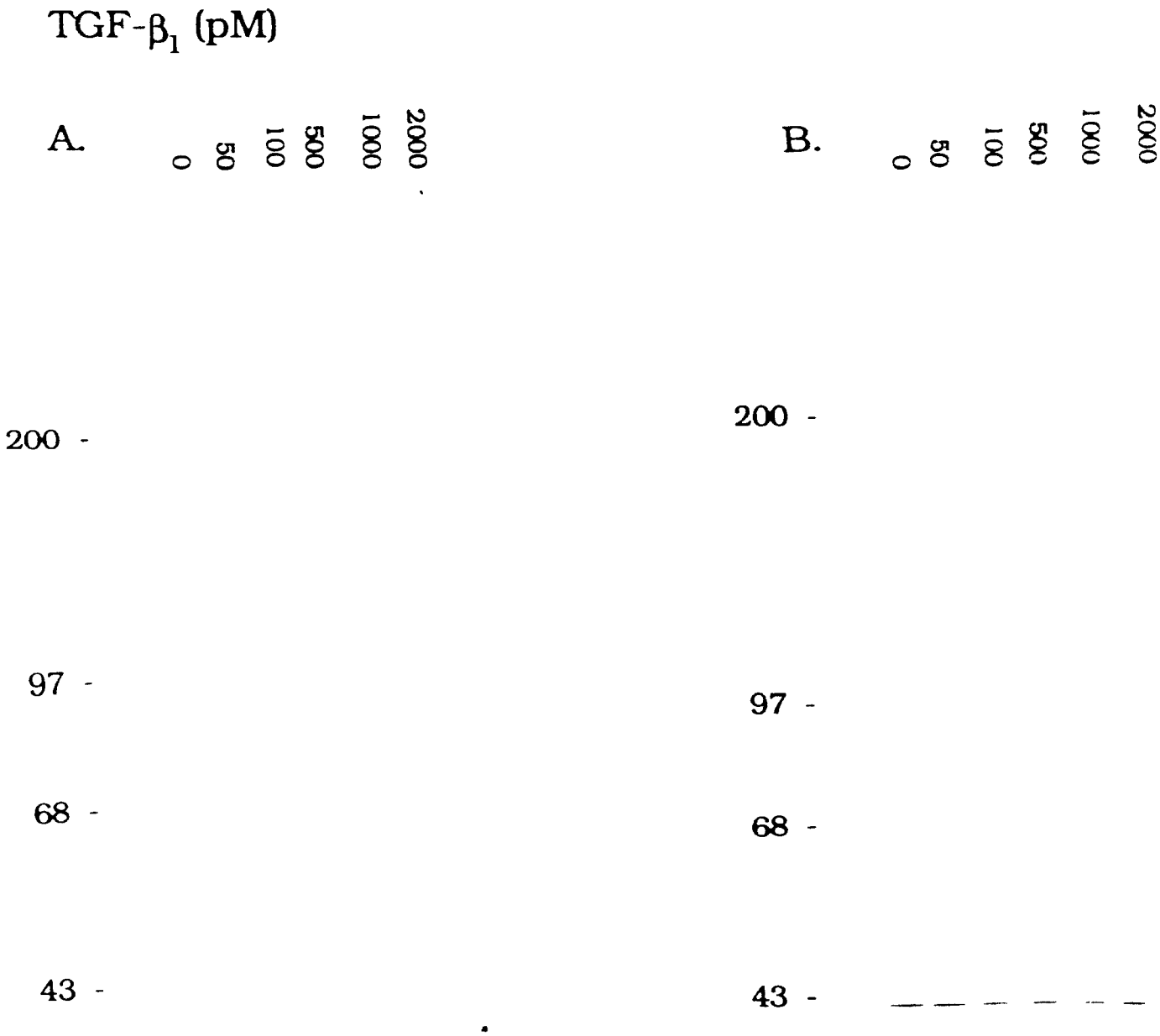

TGF-β_1 (pM)
A.
0
50
100
500
1000
2000
200
97
68
43
B.
0
50
100
500
1000
2000
200
97
68
43

FIGURE 14. Effect of TGF-β1 on fibronectin from conditioned medium of BeWo cells. Monolayers of BeWo cells at 80% confluency were treated with the indicated concentrations (pM) of TGF-β1 for 24 h. After labelling for 3 h with Trans S-Label the medium was collected and either immunoprecipitation with anti-human fibronectin polyclonal antibody (A) or affinity purified with gelatin-Sepharose (B), followed by analysis by SDS-PAGE and autoradiography. The exposure time shown are 4 days and 3 days for panels A and B respectively.

which migrated with a molecular mass of 68 kDa, which was not affected by the presence of TGF-β (Figure 14B). The identity of this protein band is unknown. Therefore, the minute amount of fibronectin observed from cell extracts is not a result of soluble fibronectin being secreted into the medium and not being incorporated into the extracellular matrix.

Further examination of the urea extracts from BeWo cells treated with or without TGF-β1 were conducted, by preliminary Western immunoblotting experiments, in order to rule out inadequate specificity and sensitivity of the two other methods (Figure 15). Western immunoblotting with the polyclonal anti-human fibronectin antibody is able to detect the total amount of fibronectin present, in comparison to the two other methods which measured newly synthesized fibronectin by incorporation of ^{35}S Trans-Label™. Western immunoblot analysis confirmed that the amount of fibronectin in the extracellular matrix of BeWo cells is insufficient to measure. Immunoblots of urea extracts of BeWo cells treated with or without TGF-β1 demonstrated no visible protein bands yet as little as 5 μg of purified human fibronectin was readily detected as a broad band of 220 kDa. It should be noted that the immunoblot in Figure 15 is a three hour autoradiographic exposure. A longer exposure (16 hours) showed no BeWo fibronectin protein bands either (data not shown).

The process of extracellular matrix remodelling involves laying down new extracellular matrix proteins and the degradation of such components. This phenomenon of synthesis and degradation of extracellular matrix proteins, including fibronectin, implies that a net amount of deposited protein can be measured within a certain time frame and this amount may change with time. Taking this into consideration, a preliminary time course experiment was performed (Figure 16). BeWo cells treated with 500 pM of TGF-β1, were immunoprecipitated with anti-human fibronectin antibody at the indicated times, 4, 8, 12, and 24 hours. At all time points tested

A.

TGF-β1 (pM)

0 50 500 1000

B.

FIBRONECTIN (μg)

5 10 25 50

FIGJRE 15. Effect of TGF-β1 on the total accumulation of fibronectin in BeWo cell layers. BeWo cell monolayers at 80% confluency were treated with the indicated concentrations (pM) of TGF β1 for 24 h and extracted with urea-containing buffer. The solubilized material was subjected to 6.0 % SDS-PAGE under reducing conditions, followed by electroblot transfer onto nitrocellulose. Panel A shows the result when the blotted nitrocellulose sheet was then sequentially incubated with an anti-human fibronectin polyclonal antibody and ^{125}I-protein A followed by autoradiography. Panel B shows a control experiment with different amounts (μg) of purified human fibronectin. The exposure time of these autoradiograms was 3 h; no bands were observed following a longer exposure time.

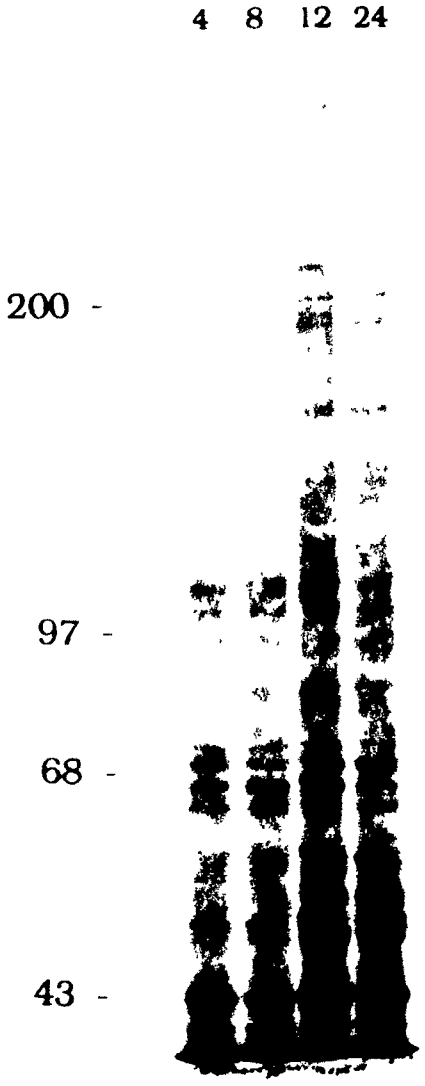

Hours
4 8 12 24
200 -
97 -
68 -
43 -

FIGURE 16. Immunoprecipitation of metabolically labelled BeWo cells after treatment with TGF-β1. Time course study. BeWo cells at 80% confluency were incubated with 500 pM of TGF-β1 for the indicated periods of time and metabolically labelled with Trans S-label™ for 3 h. Cells were then extracted with urea-containing buffer, immunoprecipitated by anti-human fibronectin polyclonal antibody and analyzed by SDS-PAGE and autoradiography. The exposure time of the autoradiogram shown here was 2 days.

no significant increase or decrease in any of the protein bands wasdemonstrated ruling out the possibility that within a 24 hour window, fibronectin had been degraded so that it could not be measured. All together these results indicate that neither TGF-β1 nor TGF-β2 have a net effect on fibronectin expression in BeWo cells.

DISCUSSION

TGF-β is synthesized by almost every cell and virtually all cells express TGF-β receptors. TGF-β has been shown to play a role in important physiological processes such as cellular proliferation, cellular differentiation and extracellular matrix remodelling (for reviews see Roberts & Sporn, 1990; Massague, 1990). The functions of placental trophoblast cells which are crucial to the development and the survival of the growing fetus include: placental implantation into the uterine epithelium; fetal/maternal exchange; hormone production; and the regulation of the maternal immune response. TGF-β may play a role in controlling any one of these trophoblastic functions. The functional role(s) of different mammalian TGF-β isoforms, i.e. TGF-β1, TGF-β2 and TGF-β3, along with their mechanisms of action, and their regulation are pertinent determinants to the understanding of trophoblast and placental function.

Radioligand binding assays:

In this study TGF-β receptors on BeWo cells, an established human choriocarcinoma trophoblast-like cell line, have been characterized using equilibrium binding assays. Scatchard analysis of equilibrium saturation binding assays of ^{125}I-TGF-β1 and ^{125}I-TGF-β2 binding revealed that these two forms of TGF-β bind to BeWo cells in a specific and saturable manner with similar average affinities (K_D values of approximately 50 Pm) and similar capacities (approximately 75,000 sites per cell). These results places the BeWo cell line among the richest sources of TGF-β receptors comparable to values obtained with the Swiss mouse 3T3 and Rat-1 cell lines (Wakefield et al., 1987). A linear plot characteristic of a single high affinity site was obtained for TGF-β1 and TGF-β2. Furthermore, equilibrium competition assays demonstrated that ^{125}I-TGF-β1 and ^{125}I-TGF-β2 bind to the same population of TGF-β receptors on BeWo cells with similar IC_{50} values of approximately 70 Pm being obtained.

A straight line Scatchard plot is usually obtained from TGF-β radioligand binding studies. A survey by Wakefield et al., (1987) of ^{125}I-TGF-β1 binding to 35 different cell types, transformed and non-transformed, also presented linear plots following Scatchard analyses. However, receptor profiles determined by affinity-labelling with iodinated TGF-β1 of most of these cells revealed that they demonstrate the three different structural classes of TGF-β binding proteins i.e. Type I, Type II and Betaglycan (Massague et al., 1990). Recent affinity-labelling experiments performed in our laboratory demonstrate that four distinct TGF-β binding proteins are expressed on BeWo cells (Mitchell et al., 1992 b). These studies indicated that the predominant Betaglycan on BeWo cells exhibits a (5 - 10 fold) higher affinity for TGF β2 than for TGF-β1 yet exhibits an approximately 7 fold higher overall capacity for TGF-β1. Since BeWo cells express a predominant Betaglycan component and very low levels of the Type I and Type II binding components, it was anticipated that the equilibrium binding experiments should reflect the Betaglycan component, i.e. a somewhat higher overall capacity for TGF β1 and a somewhat higher average affinity for TGF-β2 than for TGF β1. Obviously, this was not the case. There are discrepancies between the affinity labelling results and the equilibrium binding assay results on BeWo cells as described here as well as in other systems (Wakefield, 1987; Segarini, 1987). On BeWo cells these include: a linear Scatchard plot, indicative of a single binding site for both TGF β1 and TGF-β2 versus four different structural classes of TGF β binding proteins as demonstrated by affinity labelling receptor profiles; similar binding affinities and capacities for TGF β1 and TGF-β2 binding to BeWo cells as demonstrated by radioligand binding assays versus differential binding of TGF β1 and TGF β2 in terms of affinity and capacity for the individual TGF β binding components as shown by affinity-labelling experiments.

Factors which may be involved in the presentation of a straight line Scatchard plot when there are several affinity

labelled TGF-β binding components, as well as those which may account for discrepancies between estimates of binding affinity and binding capacity from the two different assays are discussed below. First of all, the different parameters measured by these two methods, i.e. equilibrium binding and affinity-labelling assays, should be noted. Equilibrium binding assays provide quantitative results with respect to the total number of receptors and the overall affinity of the population of receptors present. In contrast, affinity-labelling studies demonstrate which binding components are present and give only a qualitative indication of the relative affinity of each binding protein for a particular ligand in relation to another because the efficiency of cross-linking is relatively low. The most obvious reason why TGF-β1 and TGF-β2 present a linear Scatchard plot is that the analytic techniques employed to process radioligand binding data require computer programs which utilize in someway a non-linear least square curve fitting technique thus, it is likely that receptors with similar enough affinity for a particular ligand would not be differentiated. Secondly, the determination of nonspecific binding is crucial to proper Scatchard analysis, and TGF-β having a high isoelectric point (Pi - 9) (Roberts & Sporn , 1990) is considered a sticky molecule. As was noted in Results, the non-specific binding was up to 30% (TGF-β1) and 50% (TGF-β2) at high concentrations of ligand added. TGF-β may bind to non-receptor (non specific) sites with high affinity that can mask the binding to specific receptor sites, and thereby cloud the mathematical analyses and lead to misinterpretation of binding data. For example, this would mean that the Scatchard plot as a whole would be shifted towards the right, thereby increasing B_{max} (total number of binding sites) and overestimate capacity. On the other hand, the chemical cross-linking efficiency between ligand and binding protein is low and may vary between ligand isoforms and receptor species. Recently, Attisano et al., (1992) have shown that the different number of lysine groups available in the recombinantly expressed activin binding proteins, ActR-IIB and ActR-II appear to

contribute to the different cross-linking efficiencies in affinity-labelling studies with ^{125}I-activin A. There are also a different number of lysines between TGF-β1 and TGF-β2 which may contribute to different cross-linking efficiencies between the two TGF-β isoforms. Moreover, there are different lysine substitutions between TGF-β1 and TGF-β2 within a region that was recently implicated in the different specific activity of TGF β1 and TGF β2 (Qian et al., 1992), suggesting the possibility of different cross-linking efficiencies existing between TGF-β1 and TGF β2.

Competition experiments provide an alternative method in which to estimate a ligand's affinity for its binding site(s). Competition experiments may also be used to determine whether different ligands are binding to the same set of receptors or not. The advantage of competition curve experiments is that they require a minimal amount of radioligand and thus provide a clearer indication of non-specific binding at high concentration of competitor (Kermode, 1989). Whereas saturation experiments are more likely to introduce greater non-specific binding at higher concentrations especially in a case such as TGF β, which is a very basic protein and is very sticky. The IC_{50} values obtained are considered a reasonable first approximation of the K_D value of the competing ligand (Bernett, 1978). In this study there was good agreement between the K_D values obtained by Scatchard analysis (i.e. ranging from 39 to 93 for TGF-β1 and 31 to 51 for TGF-β2) and the IC_{50} value obtained by equilibrium competition studies (i.e. ranging from 63 to 84 for TGF-β1 and 59 to 88 for TGF β2).

Alternatively, experiments designed to measure the dissociation rate of a ligand for its receptor(s) may help in distinguishing differences in TGF-β1 and TGF-β2 affinity for TGF β receptors on BeWo cells (Titeler, 1989). Such experiments would involve measuring the amount of time required for an excess of unlabelled TGF-β to compete off saturated and equilibrated ^{125}I TGF β from BeWo cells. This experiment may also aid in distinguishing

the different TGF-β receptors exhibited by affinity-labelling on BeWo cells which were not detected by either equilibrium saturation or competition studies, and should be considered for future studies.

Following the elucidation of Betaglycan's and Type II receptor's sequences by cDNA cloning (Wang et al., 1991; Lopez-Casillas et al., 1991; Lin et al., 1992) the isolation of other TGF-β receptors is sure to follow. The availability of recombinantly-expressed, isolated form of the receptors will permit more detailed studies. The complexity of non-specific non-receptor binding as well as the presence of other distinct binding proteins, as discussed above, could then be excluded such that the determination of the actual binding affinity of each individual TGF-β receptor for each different TGF-β isoforms could then be accomplished. For example, muscarinic receptors can be expressed in an heterologous expression system, reconstituted into liposomes and studied individually (Haga et al., 1986). Furthermore, the availability of TGF-β receptor sequences will permit the generation of antibodies directed to specific sequences and the examination of their structure/function relationships by site directed mutation studies, such as those described for the EGF receptor (Brown et al., unpublished work)

Functional assays:

As shown by both equilibrium binding and affinity-labelling studies, TGF-β1 and TGF-β2 bind specifically to BeWo cells. However, these assays are unable to demonstrate whether a functional response is elicited upon binding of the ligand. Thus, it is important to ascertain the function of TGF-β in BeWo cells. TGF-β1 has been shown to abrogate primary trophoblast invasiveness, affect their growth, differentiation and hormone production (Graham et al., 1992; Graham & Lala, 1992; Morrish et al., 1991). The differential binding affinities of TGF-β Type I, Type II and Betaglycan receptors for TGF-β1, and -β2 in primary trophoblast cells are retained in the BeWo trophoblastic cell line (Mitchell et

al., 1992a; Mitchell et al., 1992b). Thus, certain functional differences between TGF-β1 and TGF-β2 may be exhibited in BeWo cells and could lead to a pharmacological correlation between biological potency and binding affinity for TGF-β receptors for the different TGF-β isoforms.

A. Effect of TGF-β on BeWo cell proliferation:

TGF-β is a multifunctional polypeptide, but its most recognized function is its ability to regulate cell proliferation. Although TGF-β has been shown to both induce and inhibit cell growth, TGF-β is described as the most potent polypeptide growth inhibitor (for review see Moses et al., 1990; Sporn et al., 1986). TGF-β's ability to inhibit cell growth has been observed in cells of many lineages, both normal and transformed, and leads to a complete growth arrest in certain cell lines including Mv1Lu mink lung epithelial cells (Ikeda et al., 1987). In this study, ^{3}H Thymidine assays were use to measure TGF β effects on BeWo growth. The results revealed that neither TGF-β1 nor TGF β2, when tested at concentrations up to 1000 fm, had any effect on the proliferation of BeWo cells. Positive controls, measuring the response of Mv1Lu cells to either TGF-β1 or TGF-β2, showed that both isoforms were able to totally abolish ^{3}H-thymidine incorporation.

Although TGF-β1 has been shown to inhibit the growth of primary trophoblast in a dose dependent manner (Graham et al. 1992), TGF-β effects have been shown to depend on cell type, and BeWo cells which are trophoblast-like differ from primary trophoblast cells in that they are derived from a human choriocarcinoma. Many cultured malignant or transformed cells have been shown to have lost sensitivity to regulation of growth by TGF β. Rat-liver epithelial cells are normally growth inhibited by TGF-β but when transformed by carcinogens (McMahon et al., 1986) or oncogenes (Huggett et al., 1990; Houck et al., 1989) they become unresponsive to TGF-β's antiproliferative actions. TGF β may not affect BeWo cell growth because its action on BeWo cells involves

other cellular functions. Alternatively, BeWo cells being derived from a choriocarcinoma may have lost their ability to respond to TGF-β's antiproliferative action either as a result of its tumorigenic transformation or during its history as a cell line. A recent study by Geiser et al. (1992), with a human bladder carcinoma cell line and a human colon adenocarcinoma cell line showed that inhibition of growth by TGF-β can be restored by fusing these two nonresponsive human carcinoma cell lines. In was observed that preceding fusion the individual cell lines displayed a high level of Betaglycan, and low levels of Type I and Type II receptors. Following fusion the resulting non-tumorigenic hybrids displayed a large increase in the level of Type II and a small increase in the level of the Type I components suggesting that a critical threshold of functional Type II receptors may be required for signalling inhibition of growth. Affinity-labelling studies show that BeWo cells display Betaglycan predominantly and very low levels of Type I and Type II receptors (Mitchell et al., 1992b). Therefore it is reasonable to postulate that this low level of Type II receptors is below a certain threshold needed for TGF-β's growth inhibitory response. Subsequently, other TGF-β functions were considered for study in this cellular model.

B. Effect of TGF-β on BeWo cell fibronectin synthesis:

The regulation of the accumulation and maintenance of the extracellular matrix is of great significance in wound healing, invasiveness and fibrotic conditions (for review see Sporn & Roberts, 1989). Many extracellular matrix genes have been shown to be modulated by TGF-β. The synthesis of fibronectin, an extracellular matrix protein, was measured in order to see whether or not TGF-β1 or TGF-β2 were able to modulate its expression in BeWo cells. Fibronectin is a very large glycoprotein of 440,000 daltons, it has two identical subunits covalently linked by a single disulfide bond (Yamada & Olden, 1978). The release of fibronectin by the cell contributes to the deposition of extracellular matrices such as the basement membrane. Many cells

synthesize fibronectin, including primitive mesenchymal cells, astroglia, fibroblasts and some epithelial cells (Hynes, 1976).

Two separate methods, both involving metabolic labelling of cells with ^{35}S trans-label™, were used to measure fibronectin deposition in BeWo cells treated with or without TGF β1 or TGF β2. The first method, which depends on fibronectin binding to gelatin Sepharose was unable to detect any newly synthesized fibronectin in extracellular matrix extracts of TGF-β treated BeWo cells. However, control experiments with MvlLu cells, previously shown to increase its fibronectin synthesis in response to TGF β1 (Ignotz & Massague, 1986), demonstrated a large increase in newly synthesized fibronectin.

The second method used to measure fibronectin synthesis, involved immunoprecipitating fibronectin with a polyclonal anti human fibronectin antibody. The resulting autoradiograms displayed a large number of nonspecific protein bands, in addition to a minor 220 kDa band which corresponds to the migration of purified human fibronectin. Even with several preclearing and wash steps the background noise was not reduced. Since the antibody used in these immunoprecipitation experiments has been established as appropriate for Western immunoblotting analyses (Upstate Biotechnology Inc., 1992) , thus able to recognize denatured fibronectin, it may not have been the appropriate choice for immunoprecipitation experiments. Western immunoblotting was subsequently performed in order to determine if this anti-human fibronectin antibody was unable to efficiently immunoprecipitate fibronectin yet able to recognize fibronectin in a Western immunoblot. Results showed that the antibody was able to recognize purified human fibronectin but did not detect any fibronectin in BeWo cellular extracts.

Together, the results from measuring fibronectin synthesis by gelatin-Sepharose, immunoprecipitation techniques and total fibronectin Western immunoblot analysis all suggest that there is

relatively little detectable fibronectin in BeWo extracellular matrix extracts. Furthermore, it does not appear that fibronectin synthesis in BeWo cells is inducible by TGF-β treatment.

Gelatin-Sepharose and immunoprecipitation isolation of fibronectin from conditioned medium of TGF-β treated BeWo cells indicated that there was no newly synthesized soluble fibronectin in the medium. However, gelatin-Sepharose isolation of fibronectin from conditioned medium of Mv1Lu cells showed not only a large amount of newly synthesized fibronectin in the absence of TGF-β1 treatment but an increase in response to TGF-β. These results further support the interpretation that BeWo cells express very low levels of fibronectin.

Although many types of cells do synthesize fibronectin there are several types of transformed cells that do not (Hynes, 1976). The results obtained in this study indicate that TGF-β1 and TGF-β2 do not induce fibronectin synthesis or degradation in BeWo cells. This may be because BeWo, a cell line derived from a choriocarcinoma, do not synthesize fibronectin, or do not synthesize enough fibronectin so as to permit its detection at the protein level. Furthermore, human placental fibronectin has an additional polylactosamine carbohydrate which somewhat decreases its binding affinity for gelatin (Zhu & Laine, 1985), this may be contributing to the inability to detect BeWo expression of fibronectin at the protein level by affinity chromatography with gelatin/Sepharose. Alternatively, it is possible that BeWo cells cannot respond to TGF-β as discussed previously with respect to the proliferation studies. Studies with mutant cells deficient in either or both Type I and Type II receptors show that these two receptors are involved in signalling TGF-β's actions (Laiho et al. 1990). The results of Geiser et al (1992) suggest that the Type II receptor is responsible for mediating TGF-β's growth inhibitory function, in bladder and colon cell lines, yet it is not required to mediate TGF-β's gene activation function. They found that cells

with very low number of the Type II receptor were unresponsive to TGF-β's antiproliferative effects but responded with the activation of fibronectin and plasminogen activator inhibitor-1 genes. TGF-β action may involve several different signalling pathways which can allow some responses, such as gene activation, in the absence of others, such as growth inhibition. Thus, the resulting response depends on which receptor is present and how many are available to initiate a signal. BeWo cells express very low levels of both the Type I and Type II TGF-β receptors (Mitchell et al., 1992 b). It is possible that there may not be enough of these two receptors types in BeWo cells to initiate activation of the fibronectin gene by TGF-β. Alternatively, BeWo cells which express all three TGF β receptors (albeit low levels of Type I and Type II receptors) may be similar to S mutants (Boyd & Massague, 1989) and unable to respond to TGF-β because of some fault in their TGF-β signalling mechanism.

In this study, fibronectin synthesis was examined at the protein level. It would be worthwhile to determine whether BeWo cells express message for fibronectin and if the level of message changes in response to TGF-β by Northern blot analysis. A study of fibronectin mRNA expression in BeWo cells, as conducted in bovine adenocarcinoma cells (Shi et al., 1990), would be useful.

SUMMARY

These studies indicate that there is no difference in the binding parameters, equilibrium dissociation constant (K_D) and maximum binding sites (B_{max}) between TGF-β1 and TGF-β2 for binding to BeWo cells as measured by equilibrium saturation and competition experiments. The number of binding sites determined per BeWo cell places the BeWo cell line among the richest cell lines in terms TGF-β receptors. However, affinity labelling studies in this laboratory indicate that the different structural classes of TGF-β receptors on BeWo cells exhibit differential binding between TGF-β1 and TGF-β2. Thus, in an attempt to differentiate the response to these two TGF-β isoforms by way of biological potency, two different TGF-β functions were studied, modulation of growth and regulation of fibronectin synthesis. Neither TGF-β1 nor TGF-β2 had any affect on the growth and/or fibronectin synthesis in BeWo cells. Future studies should be aimed toward finding a functional response to TGF-β in this model system.

FUTURE DIRECTIONS

Proteases and protease inhibitors play an important role in regulating the accumulation and degradation of the extracellular matrix. The modulation of these enzymes are involved in trophoblastic and tumorigenic invasion. Graham et al. (1992) have shown that TGF-β blocks trophoblast invasion with implications of TGF-β indirectly controlling TIMP (tissue inhibitor of metalloproteinases) levels in these cells. It has also been demonstrated that cultured human trophoblast secrete urokinase type plasminogen activator (u-PA) with proteolytic activity and that is thought to play a role in the degradation of the extracellular matrix (Queenan et al. 1987). Plasminogen activator inhibitors (PAI) inactivate the proteolytic activity of plasminogen activators. PAI type I (PAI-1) has been detected in extravillous trophoblast (Yeh & Kurman, 1989). TGF-β has been shown to increase the expression of PAI-1 in a human fibrosarcoma cell line (Laiho et al., 1987) and in rat osteoblast-like cells (Allan et al., 1991). TGF-β has also been shown to increase PA expression in human synovial fibroblasts (Hamilton et al., 1991). If TGF-β can inhibit the invasiveness of trophoblast cells by controlling TIMP as the results of Graham et al., (1992) suggest, TGF β may also control the expression of either PA or PAI 1 in these cells as well. It would be interesting to see whether TGF-β1 or TGF β2 has any effect on the expression of these proteases and protease inhibitors in BeWo cells, both at the MRNA and protein levels. Several other groups have already measured PA (Hamilton et al., 1991) and PAI 1 (Laiho et al., 1987 and Allan et al., 1991) at the MRNA level by Northern Analysis.

At the protein level the activity of PA and PAI-1 has been measured by zymographic analysis. (Laiho et al., 1987). The establishment of a zymography assay was attempted in order to measure the PA activity in the conditioned medium of BeWo cells treated with or without TGF-β1 and TGF-β2. Zymography involves

using SDS-PAGE gels containing gelatin and purified plasminogen, (Heussen & Dowdle, 1980). Electrophoresis separates proteins in the medium according to their molecular mass. Washing out the SDS with Triton-X-100 renatures the enzymes allowing PA to activate plasminogen to plasmin which will then degrade the gelatin revealing a clear zone at the appropriate molecular mass. Similarly, the activity of other proteases such as metalloproteinases and cysteine proteases can be visualized. Several attempts to optimize this technique in the laboratory failed to produce consistent results and therefore the use of this assay to measure the effects of TGF-β on proteolytic activity in BeWo cells was discontinued. Nevertheless future studies should be directed towards examining the effects of TGF β on the activity of PA and other extracelluar matrix degrading enzymes in BeWo cells by zymography. The modulation of the expression of extracellular matrix degrading enzymes by TGF-β should be studied at the mRNA by Northern analysis as well.

The effect of TGF-β on the differentiation of trophoblast to syncytiotrophoblast has been examined in two laboratories (Graham et al., 1992; Morrish et al., 1991). However, whereas Morrish et al., (1991) reported the inhibition of trophoblast differentiation to syncytiotrophoblast by TGF-β1, Graham et al., (1992) reported the stimulation of trophoblast differentiation by TGF β . Both of these groups defined differentiation of trophoblast cells to syncytiotrophoblast cells as the formation of multinucleated cells as observed by light microscopy. However, there has been recent debate as to whether the observation of multinucleated cells by light microscopy is enough to demonstrate a true syncytia (Aplin 1991). BeWo cells have been shown to undergo a complex response to methotrexate that resembles the formation of syncytiotrophoblast from mononuclear trophoblast (Friedman & Skehan ,1979; Burres & Cass, 1987). It would be interesting to study TGF β's ability to modulate differentiation in BeWo cells, taking care that the formation of multinucleated cells be demonstrated as a true

syncytia by electron microscopy or microinjection of fluorescent dyes (Aplin, 1991).

Another interesting aspect of BeWo cells in respect to TGF-β is that BeWo cells are polarized cells where they display structural and biochemical differences between their apical and basolateral surfaces. It was recently shown that BeWo cells can be cultured on permeable filter supports which direct independent access to the apical and basolateral domains, (Cerneus & van der Ende,1991) thus enabling TGF-β receptors to be studied from the two sides of the cell.

BeWo cells are a interesting model system in which to study TGF-β receptors and functions, moreover they are a more uniform model than primary trophoblast. It is important to find a function for TGF-β in BeWo cells but it is also important to determine whether TGF-β functions in a similar fashion in these cells as compared with primary trophoblasts, which should more closely represent the <u>in vivo</u> state. Thus, future studies should involve continued characterization of the binding properties and the functional responses to TGF-β1, TGF-β2, and TGF-β3 in primary trophoblast as well.

REFERENCES

Allan E. H., Zeheb R., Gelehrter T. D., Heaton J. H., Fukumoto S., Yee J. A. and Martin T. J. (1991) Transforming growth factor beta inhibits plasminogen activator (PA) activity and stimulates production of urokinase-type PA, PA inhibitor 1 mRNA, and protein in rat osteoblast-like cells. J. Cell. Phys. 149:34-43.

Altman, D. J., Schneider, S. L., Thompson, D. A., Cheng, H. L., and Tomasi, T. B. (1990) A transforming growth factor β2 (TGF-β2)-like immunosuppressive factor in amniotic fluid and localization of TGF-β2 in the pregnant uterus. J Exp. Med., 172:1391-1401.

Andres J. L., Stanley K., Cheifetz S., Massague J. (1989) Membrane-anchored and soluble forms of betaglycan, a polymorphic proteoglycan that binds transforming growth factor-β. J. Cell Biol. 109:3137-3145.

Anzano M. A., Roberts A. B., Meyers C. A., Komoriya A., Lamb L. C., Smith J. M., Sporn M. B. (1982) Cancer Res. 42:4776-4778.

Aplin, J. D. (1991) Implantation, trophoblast differentiation and hoemochorial placentation: mechanistic evidence in vivo and in vitro. J. Cell Sci, 99:681-692.

Arey, L. B. Developmental Anatomy, 7th Ed., W.B. Saunders Co., Philadelphia, 1965 pp.146-148.

Assoian R. K., Komoriya A., Meyers C. A., Miller D. M. Sporn M. B. (19:681-692.83) Transforming growth factor-β in human platelets. J. Biol. Chem. 258: 7155-7160.

Attisano, L., Wrana, J. L., Cheifetz, S. and Massague, J. (1992) Novel activin receptors: distinct genes and alternative mRNA splicing generate a repetoire of serine/threonine kinase receptors. Cell, 68:97-108.

Barnard J. A., Beauchamp R. D., Coffey R. J., Moses H. L. (1989) Regulation of intestinal epithelial cell growth by transforming growth factor-beta. Proc. Natl. Acad. Sci. USA 86:1578 1582.

Bassols, A., and Massague, J. (1988) Transforming growth factor-β regulates the expression and structure of extracellular matrix chondoitin/dermatan sulfate proteoglycans. J. Biol. chem. 263:3039-3045.

Barton, d. E., Foellmer, B. E., Du, J., Tamm, J., Derynck, R., Franke, U. (1988) Chromosomal locations of TGF-βs 2 and 3 in the mouse and human. Oncogene Res. 3:323031

Billington, W. D. (1971) Biology of the trophoblast. Adv. Reprod.

Physiol. 5:27-66.

Blay, J., Hollenberg, M. D. (1989) the nature and function of polypeptide growth factor receptors in human placenta. J. Devel. Phy. 12:237-248.

Boerner, P., Resnick, R J., and Racker, E. (1985) Stimulation of glycolysis and amino acid uptake in NRK-49F cells by transforming grwoth factor beta and epidermal growth factor. Proc. Natl. Acad. Sci. USA., 82:1350-1353.

Boyd, F. T. and Massague, J. (1989) Transforming growth factor-β inhibition of epithelial cell proliferation linked to the expression of a 530kD membrane receptor. J. Biol. Chem. 264:2272-2278.

Boyd, J. D., and Hamilton, W. J. (1970) The Human Placenta, pp. 137. Cambridge: W. Heffer & Sons Ltd.

Bulmer, J. N., and Johnson, P. M. (1985) Antigen expression by trophoblast populations in the human placenta and their possible immunobiological relevance. Placenta, 6:127-140.

Burnett, J. P. Jr. (1978) Methods in binding studies. Neurotransmitter Receptor Binding, H. I Yamamura et al., Eds., Raven Press, New York.

Burres, N. S. and Cass, C. E. (1987) Inhibition of methotrexate-induced differentiation of cultured human choriocarcinoma (BeWo) cells by thymidine. Cancer Res., 47:5059-5064.

Centrella M., McCarthy T. L. and Canalis E. (1987) Transforming growth factor β is a bifunctional regulator of replication and collagen synthesis in osteoblast-enriched cell cultures from fetal rat bone J. Biol. Chem. 262:2869-2874.

Cerneus, D. P. and van der ende, A. (1991) Apical and basolateral transferrin receptors in polarized BeWo cells recycle through separate endosomes. J. Cell Biol. 114:1149-1158.

Chambard J. C. and Pouyssegur J. (1988) TGF-β inhibits growth factor induced DNA synthesis in hamster fibroblasts without affecting the early mitogenic events. J. Cell Physiol 135:101-107.

Cheifetz S., Andres J. L. and Massague J. (1988 A) The transforming growth factor-β receptor type III is a membrane proteoglycan. Domain structure of the receptor. J. Biol. Chem. 263:16984-16991.

Cheifetz S., and Massague J. (1991) Isoform-specific transforming growth factor-β binding proteins with membrane attachments

sensitive to phosphatidylinositol-specific phospholipbase C. J. Biol. Chem. 266:20767-20772.

Cheifetz S., Weatherbee J. A., Tsang M. L. S., Anderson J. K., Mole J. E., Lucas R. and Massague J. (1987) The transforming growth factor-β system, a complex pattern of cross reactive ligands and receptor. Cell. 48:409-415.

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

Cheifetz S., and Massague J. (1989) Transforming growth factor-β (TGF-β) receptor proteoglycan. Cell surface expression and ligand binding in the absence of glycosaminoglycan chains. J. Biol. Chem. 264:12025-12028.

Cheifetz S., Bassols A., Stanley K., Ohta M., Greenberger J. and Massague J. (1988 B) Heterodimeric transforming growth factor β. Biological properties and interaction with three types of cell surface receptors. J. Biol. Chem. 263:10983 10789.

Cheifetz S., Ling N., Guillemin R. and Massague J. (1988 C) A surface component on GH, pituitary cells that recognizes transforming growth factor-β activin and inhibin. J. Biol. Chem. 263:17225-17228.

Chen, J.-K., Hoshi, H. and McKeehan, W. L. (1987) Transforming growth factor-β specifically stimulates synthesis of proteoglycan in human arterial smooth muscle cells. Proc Natl. Acad, Sci. USA., 84:5287-5291.

Chiang C. P. and Nilsen-Hamilton M. (1986) Opposite and selective effects of epidermal growth factor-β on the production of secreted proteins by murine 3Y3 cells and human fibroblasts. J. Biol. Chem. 261:10478-81.

Clark, D. A., Flanders. K. C., Banwatt, D., Millar-Book, W., Manuel, J., Stedronska-Clark, J. and Rowleyh, B. (1990) Murine pregnancy decidua produces a unique immunosuppressive molecule related to transforming growth factor β2. J. Immunol., 144:3008 3014.

Clark, D. A., Lea, R. G., Podor, T., Daya, S., Banwatt, D., and Harley, C. (1991) Cytokines determine the success of failure of pregnancy. ann N. Y. Acad. Sci. 626:524-536.

Daopin, S., Piez, K. A., Ogawa, Y., Davies, D. R. (1992) Crystal sturcture of transforming growth factor-β1: An unusual fold for the superfamily. Science, 257:369-373.

Deal, C. L., and Guyda, H. J. (1983) Insulin receptors of human term placental cell and choriocarcinoma (JEG-3) cells: Characteristics and regulation. Endocrinology, 112:1512-1523.

de Martin R., Haendler B., Hofer-Warbinek R., Gaugitsch H. and Wrann M. (1987) Complementary DNA for human glioblastoma-derived T cell suppressor factor, a novel member of the transforming growth factor-β gene family. EMBO J. 6:3673-3677.

De Larco J. E., and Todaro G. J., (1978) Growth factors from murine sarcoma virus transformed cells. Proc. Natl. Acad. Sci USA 75:4001-4005.

Derynk R., Jarrett J. A., Chen, E. Y., Eaton D. H. and Bell J. R. (1985) Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. Nature, 316: 701-705.

Derynk R., Rhee L., Chen E. Y. and Van Tilburg A. (1986) Intron-exon structure of human transforming growth factor-β precursor gene. Nucleic Acids Res. 15:3188-3189.

Derynk, R. (1987) Structure of transforming growth factors -α and β and thier precursors. Oncogenes, Genes, and Growth Factors, Guroff, G. Eds., John Wiley & Sons, New York. pp 133-163.

Diaz, A., Varga, J. and Jimenez, S. A. (1989) Transforming growth factor-β stimulation of lung fibroblast prostaglandin E_2 production. J. Biol. Chem. 264:11554-11557.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M.D. (1984) Close similarity of epidermal growth factor receptor and v-erb B oncogene protein sequences. Nature 307:521-527.

Dungy, L. J., Siddiqu, T. A. and Khan S. (1991) Transforming growth factor-β1 expression during placental development. Am. J. Obstet. Gynecol. 165:853-857.

Edwards D. R., Murphy G., Reynolds J. J., Whitman S. E. and Doherty A. J. P. (1987) Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. EMBO J. 6:1899-1904.

Engvall, E. and Ruoslahti, E. (1977) Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. Int. J. Cancer 20:1-5.

Fanger, B. O., Wakefield, L. M. and Sporn, M. B. (1986) Structure and properties of the cellular receptor for transforming growth-factor type-beta. Biochemistry 25:3083-3091.

Fine, L.F., Holley R. W., Nasri H. and Badie-Dezfooly B. (1985) BSC-1 growth inhibitor transforms a mitogenic stimulus into a hpertrophic stimulus for renal proximal tubular cells: Relationship to Na'/H+ antiport activity. Proc. Natl. Acad. Sci USA. 82:6163-6166.

Fischer, S. J., Cui, T., Zhang, L., Hartman, L., Grahl, K., Guo Yang, Z., Tarpey, J. and Damsky, C. (1989) Adhesive and degradative properties of human placental cytotrophoblast cells in vitro. J. Cell Biol. 109:891-902.

Florini J. R., Roberts A. B., Ewton D. Z., Falen S. L., Flanders K. C., Sporn M. B. (1986) Transforming growth factor β. A very potent inhibitor of myoblast differentiation, identical to the differentiation inhibitor secreted by Buffalo rat liver cells. J.Biol. Chem. 261:16509-16513

Friedman, S. and Skehan, P. (1979) Morphological differentiation of human choriocarcinoma cells induced by methotrexate. Cancer Res., 39:1960-1967.

Frolik C. A., Dart L. L., Meyers C. A., Smith D. M. and Sporn M. B. (1983) Purification and initial characterization of a type β transforming growth factor from human placenta. Proc. Natl. Acad. Sci. USA. 80:3676-3680.

Frolik C. A., Wakefield L. M., Smith D. M. and Sporn M. B. (1984) Characterization of a membrane receptor for transforming growth factor-β in normal rat kidney fibroblasts. J. Biol. Chem. 259:10995-11000.

Fujii, D., Brissenden, J. E., Derynck, R. and Franke, U. (1986) Transforming grwoth factor-β gene maps to human chromosome 19 long arm and to mouse chromosome 7. Somat. Cell Mol. Genet. 12:281-288.

Geiser, A. G., Burmester, J. K., Webbink, R., Roberts, A. B. and Sporn, M. B. (1992) Inhibition of growth by transforming growth factor-β following fusion of two non-responsive human carcinoma cell lines. J. Biol. Chem. 267:2588-2593.

Gentry, L. E., Webb, N. R., Lim, G. J., Brunner, A. M., Ranchalis, J. E., Twardzik, D. R., Liobin, M. H., Marquardt, H. and Purchio, A. F. (1987) Type I transforming growth factor beta: Amplified expression and secretion of mature and precursor polypeptides in chinese hamster ovary cells. Molec. Cell Biol., 7:3419-3427.

Georgi, L. L., Albert, P. S. and Riddle, D. L. (1990) daf 1, a C. Elegans gene controlling dauer larva development, encodes a novel receptor protein kinase. Ce.., 61:635-645.

Glass, R. H., Aggeler, J., Spindle, A., Pedersen, R A. and Web Z. (1983) Degradation of extracellular matrix by mouse trophoblast outgrowths: a model for implantation. J. Cell. Biol. 96:1108-1116.

Goldstein, L. D., Reynolds, C.P. and Perez-Polo, J. R. (1978) Isolation of human nerve growth factor from placental tissue. Neurochemical Research. 3:175-183.

Goldstein, A., Lewis, A. and Sumner, K. S. (1974) Principles of Drug Action; the Basis of Pharmacology, pp 82-105..

Gospodarowicz, D., Cheng, J., Lui, G. M., Fujii, D. K., Baird, A. and Bohlen, P. (1985) Fibroblast growth factor in the human placenta. Biochem Biophys Res Comm 128:554-562.

Goustin, A. S., Betsholtz, C., Pfeifer-Ohlsson, S., Persson, H., Rydnert, J., Bywater, M., Holmgren, G., Heldin, C. H., Westermark, B. and Ohlsson, R. (1985) Coexpression of the *sis* and *myc* proto oncogenes in developing human placenta suggests autocrine control of trophoblast growh. Cell, 41:301-312.

Graham, C. H., Lysiak, J. L., McCrae, K. R. and Lala, P. K. (1992) Localization of transforming growth factor β (TGF-β) at the human fetal-maternal interface: role on tropholbast growth and differentiation. Biol Reprod. 46:561-572.

Graham, C. H., and Lala, P. K. (1992) Mechanisms of placental invasion of the uterus and their control. Biochemistry and Cell Biology. ****in press****

Graycar J. L., Miller D. A., Arrick B. A., Lyons R. M., Moses H. L. and Derynck R. (1989) Human transforming groth factor-β3: recombinant expression, purification, and biological activities in comparison with transforming growth factors-β1 and-β2. Mol. Endocrinol. 3:1977-1986.

Haga, K , Haga, T. and Ichiyama, A. (1986) Reconstitution of the muscarinic acetylcholine receptor. J. Biol. Chem. 261:10133-10140.

Hamilton J. A., Piccoli D. S., Leizer T., Butluer D. M., Croatto M. and Royston K. M. (1991) Transforming growth factor β stimulates urokinase-type plasminogen activator and DNA synthesis, but not prostaglandin E production, in human synovial fibroblasts. Proc. Natl. Acad. Sci. USA 88:7180-7184.

Hanks S. K., Armour R., Baldwin J. H., Maldonado F., Spiess J. and Holley R. W. (1988) Amino acid sequence of the BSC-1 cell growth inhibitor (polyergin) deduced from the nucleotide sequence of the cDNA. Proc. Natl. Acad. Sci. USA. 85:79-83.

Hannah. R., Philip, A., O'Connor-McCourt, M. D. (1992) Novel TGF-β binding proteins from rat lung. ****Submitted****

Heimark R. L., Twardzik D. R. and Schwartz S. M., (1986) Inhibition of endothelial cell regeneration by type beta transforming growth factor from platelets. Science 233:1078-1080.

Heussen, C. and Dowdle, E. B. (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Anal. Biochem. 102:196-202.

Hill D. J., Strain A. J., Elstow S. F., Swenne I. and Milner R. D. G., (1986) Bifunctional action of transforming growth factor β on DNA synthesis in early passage human fetal fibroblasts. J. Cell. Physiol. 128:322-328.

Hock, R. A., Nexo, E. and Hollenberg, M. D. (1979) Isolation of the human placenta receptor for epidermal growth factor urogastrone. Nature, 277:403-405.

Hock, r. A., Nexo, E., Hollenberg, M. D. (1980) Solubilization and isolation of the human placenta receptor for epidermal growth factor-urogastrone. J. Biol. Chem., 255:10737-10743.

Houck, K. A., Michalopoulos, G. K. and Strom, S. C. (1989) Introduction of a Ha-ras oncogene into rat liver epithelial cells and parenchymal hepatocytes confers resistance to the growth inhibitory effects of TGF-β. Oncogene, 4:19-25.

Huggett, A. C., Hampton, L. L., Ford, C. P., Writhe, P. J. and Thorgensson, S. S. (1990) Altered responsiveness of rat liver epithelial cells to transforming growth factor β1 following their trasformation with v-raf. Cancer Res., 50:7468-7475.

Hynes, R. O. (1976) Cell surface proteins and malignant transformation. Biochem. Biophys, Acta. 458:73 107.

Ichjo, H., Ronnstrand, L., Miyagawa, K., Ohashi, H., Heldin, C. H., Miyazona, K. (1991) Purification of Transforming Growth Factor-β1 binding proteins from porcine uterus membranes. J. Biol. Chem. 266:22459-22464.

Ignotz R. A. and Massague J. (1985) Type β transforming growth factor controls the adipogenic differentiation of 3T3 fibroblasts. Proc. Natl. Acad. Sci. USA 82:8530 8534.

Ignotz R. A., and Massague J. (1986) Transforming grwoth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J. Biol. Chem. 261:4337-4345.

Ignotz P. A., and Massague J. (1987) Cell adhesion receptors as targets for transforming growth factor-β action. Cell, 51:189-197.

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

Inman, W. H., and Colowick, S. P. (1985) Stimulation of glucose uptake by transforming growth factor-β: Evidence for the requirement of epidermal growth factor receptor activation. Proc. Natl. Acad. Sci. USA., 82:1346-1349.

Jakowlew S. B., Dillard P. J., Sporn M. B. and Roberts A. B. (1988) Complementary deoxyribonucleic acid cloning of a messenger ribonucleic acid encoding tranforming growth factor beta 4 from chichen embryo chondrocytes. Mol. Endocrinol., 2:1186 1195.

Jennings J. C., Mohan S., Linkhart T. A., Widstrom R. and Baylink D. J. (1988) Comparison of the biological actions of TGF Beta-1 and TGF-Beta-2: differential activity in endothelial cells. J. Cell. Physiol., 137:167-172.

Jetten A. M., Shirley J. E. and Stoner G. (1986) Regulation of proliferation and differentiation of respiratory tract epithelial by TGF β Exp. Cell Res. 167:539-549.

Kehrl J. H., Roberts A. B., Wakefield L. M., Jakowlew S. B., Sporn M. B. and Fauci A. S. (1986 a) Transforming growth factor-β is an important immunomodulatory protein for human B lymphocytes. J. Immunol., 137:3855-3860.

Kehrl J. H., Wakefield L.M., Roberts, A. B., Jakowlew S. B., Alvarez-Mon M., (1986 b) Production of transforming growth factor-beta by human T lymphocytes and its potential role in the regulation of T cell growth. J. Exp. Med., 163:1037-1050.

Kerr L. D., Olashaw N. E. and Matrisian L. M. (1988) Transforming growth factor β1 and cAMP inhibit transcription of epidermal growth factor- and oncogene-induced transin RNA. J. Biol. Chem. 263:16999-17005.

Kelly, D., Campbell, W. J., Piesman, J., and Rizzino, A. (1990) Regulation and expression of transforming growth factor type-β during early mammalian development. Cytotechnology, 4:227-242.

Kermode, J. C. (1989) The curvilinear Scatchard plot, experimental artifact or receptor heterogeneity? 38:2053-2060.

Kim, S.-J., Glick, A., Sporn, M. B. and Roberts, A. B. (1989)

Characterization of thr promoter region of the human transforming growth factor-β1 gene. J. Biol. Chem., 264:402-408.

Kirby, D. R. S. (1960) The development of mouse eggs beneath the kidney capsule. Nature, 187:707-708.

Kirby, D. R. S. (1963a) The development of mouse blastocysts transplanted to the spleen. J. Reprod. Fertil., 5:1-12.

Kirby, D. R. S. (1963b) The development of the mouse blastocyst transplanted tothe cryptorchid and scrotal testis. J. Anat., 97:119-130.

Kliman, H. J. and Feinberg, R. F. (1990) Human trophoblast - extracellular matrix (ECM) interactions in vitroL ECM thickness modulates morphology and proteolytic activity. Proc. Natl. Acad. Sci. USA., 87:3057-3061.

Kovats, S., Main, E. K., Librach, C., stubblebine, M., Fisher, S. J., and DeMars, R. (1990) A class I antigen, HLA G, expressed in human trophoblasts. Science, 248:220-223.

Kurokowa M., Lynch K. and Podolsky D. K. (1987) Effects of growth factors on an intestinal epithelial cell line : TGF β inhibits proliferation and stimulates differentiation. Biochem. Biophys. Res. Commun. 142:775-782.

Kubota S., Fridman R. and Yamada Y. (1991) Transforming growth factor-β suppresses the invasiveness of human fibrosarcoma cells in vitro by increasing expression of tissue inhibitor of metalloproteinases. Biochem. Biophys. Res. Comm. 176:129-136.

Laemmli, U.K. (1970) cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 277:690-685.

Lafyatis.R., Lechleider, R., Kim, S.-J., Jakowlew, S., Roberts, A. B., and Sporn, M. B. (1990) Structural and functional characterization of the transforming growth factor β3 promoter. J. Biol. Chem., 265:19128-19136

Lai, W. H., and Guyda, H. J. (1984) Characterization and regulation of epidermal grwoth factor receptors in human placental cell cultures. J. Clin. Endocrinol. Metab. 58:344-352.

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

Laiho, M., Weis, F. M. B., Boud, F. T., Ignotz, R. A. and Massague, J. (1991) Responsiveness to transforming growth factor β (TGF-β) restored by genetic complementation between cells

defective in TGF-β receptors I and II. J. Biol. Chem. 226:9108-9112.

Laiho M., Saksela O., Keski-Oja J. (1986) Transforming growth factor β alters plasminogen activator activity in human skin fibroblasts. EExp. Cell Res. 164:399-407.

Laiho M., Saksela O. and Keski-Oja J. (1987) Transforming growth factor β induction of type 1 plasminogen activator inhibitor. Pericellular deposition and sensitivity to exogenous urokinase. J. Biol. Chem. 262:17467-17474.

Lala, P. K., and Graham, C. H. (1990) Mechanisms of trophoblast invasiveness and their control: The role of proteases and protease inhibitors. Cancer Metastasis Rev., 9:369-379.

LeBon, T. R., Jacobs, S., Cuatrecasas, P., Kathuria, S. and Fujita Yamaguchi, Y. (1986) Purification of insulin-like growth factor I receptor from human placental membranes. J. Biol. Chem. 261:7685-7689.

Leof, E. B., Proper, J. A., Goustin, A. S., Shipley, G. D., diCorleto, P. E., and Moses, H. L. (1986) Induction of c-*sis* mRNA and activity similar to platelet-derived growth factor by transforming growth factor β: A proposed model for indirect mitogenesis involving autocrine activity. Proc. Natl. Acad. Sci. USA., 83:2453-2457.

Librach, C. L., Werb, Z., Fitzgerald, M. L., Chiu, K., Corwin, N. M., Esteves, R. A., Grobelny, D., Galardy, R., Damsky, C.H. and Fisher, S. J. (1991) 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. J. Cell Biol. 113:437-449.

Like, B., and Massague, J. (1986) The anti-proliferative effect of type β transforming growth factor occurs at a level distal from receptors for growth-activating factors. J. Biol. chem. 261:13426-13429.

Lin, H.Y., Wang, X.-F., Ng-Eaton, E., Weinberg, R. A. and Lodish, H. F. (1992) Expression cloning of the TGF-β type II receptor a functional transmembrane serine/threonine kinase. Cell68:1-20.

Lin P., Liu C., Tsao M. S. and Grisham J. W., (1987) Inhibition of proliferation of cultured rat liver epithelial cells at specific cell cycle stages by transforming growth factor-β. Biochem. Biophys. Res. Commun. 143:26-30.

Liu J. M. and Davidson J. M. (1988) The elastogenic effect of recombinant transforming growth factor-beta on porcine aortic smooth muscle cells. Biochem. Biophys. Res. Comm. 154:895-901.

Lopez-Casillas, F., Cheifetz, S., Doody, J., Andres, J. L., Lane,

W. S. and Massague, J. (1991) Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF β receptor system. Cell 67:785-795.

Lund L. R., Riccio A., Andreasen P. A., Nielsen L. S. and Kristensen P. (1987) Transforming growth factor-β is a strong and fast acting positive regulator of the level of type 1 plasminogen activator mRNA in WI-38 human lung fibroblasts. EMBO J. 6:1291 1286.

Lyons R. M., and Moses H. L. (1990) Transforming growth factors and the regulation of cell proliferation. Eur. J. Biochem. 187:467-473.

Lyon R. M., Keski-Oja J. and Moses H. L. (1988) Proteolytic activation of latent transfoming growth factor-β from fibroblast conditioned medium. J. Cell Biol. 106:1659 1665.

MacKay, K., Robbins, A. R. Bruce, M. D., Danielpour, D. (1990) Identification of disulfide linked transforming grwoth factor β1 specific binding proteins in rat glomeruli. J. Biol. Chem. 265:9351-9356.

MacKay, K., Danielpour, D., Miller, D., Border, W., Robbins, A. R. (1992) The 260 kDa transforming growth factor (TGF β) β binding protein in rat glomeruli is a complex comprised of 170 and 85- kDa TGF-β binding proteins. J. Biol. Chem. 267:11449 11454.

MacKay, K. and Danielpour, D. (1991) Novel 150 and 180 glycoproteins that bind transforming growth factor (TGF) β1 but not TGF-β2 are present in several cell lines. J. Biol. Chem. 266:9907-9911.

Madri J. A., Pratt B. M., Tucker A. M. (1988) Phenotypic modulation of endothelial cells by transforming growth factor β depends upon the composition and arganization of the extracellular matrix. J. Cell Biol. 106:1375-1384.

Maly, P. and Luthi, C. (1986) Purification of the typeI insulin like grwoth factor receptor from human placenta. Biochem. Biophys. Res. Comm. 137:695-701.

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

Massague, J. (1985) Subunit structure of a high-affinity receptor for type β-transforming growth factor. J. Biol. Chem. 260:7059 7066.

Massague, J., Like, B. (1985) Cellular receptors for type beta transforming growth factor. J. Biol. Chem. 260:2636-2645.

Massague J. (1987) The TGF-β family - grwoth and differentiation factors. Cell. 49:437-438.

Massague J., Cheifets S., Boyd F. T., Andres J. L. (1990) TGF-β receptors and TGF-β binding proteoglycans: recent progress in identifying their functional properties. Ann. N.Y. Acad. Sci., 593:59-72.

Massague J. (1992) The transforming growth factor -β family. Annu. Rev. Cell Biol. 6:597-641.

Massague J., Cheifetz S., Endo T. and Nadal-Ginard B. (1986) Type β transforming growth factor is an inhibitor of myogenic differentiation. Proc. Natl. Acad. Sci. USA 83:8206-8210.

Massague J. (1992) Receptors for the TGF-β family. Cell 69:1067-1070.

Masui T., Wakefield L. M. Lechner J. F., LaVeck M. A., Sporn M. B.and Harris C. C. (1986) Type β transforming growth factor is the primary differentiation inducing serum factor for normal human bronchial pithelial cells. Proc. Natl. Acad. Sci. USA. 83:2438-2442.

Mathews, L. S. and Vale, W. W. (1991) Expression cloning of an activin receptor, a predicted transmembrane seine kinase. Cell, 65:973-982.

Matrisian L. M., Leroy P., Ruhlmann C., Gesnel M. C. and Breathnack R. (1986) Isolation of the oncogene and epidermal growth factor-induced transin gene: complex control in rat fibroblasts. Mol. Cell. Biol. 6:1679-1686.

McMahon, J. B., Richards, W. L., del Campo, A. A., Song, M.-K. H. and Thorgeirsson, S. S. (1986) Differential effects of transforming growth factor-β on proliferation of normal and malignant rat liver epithelial cells in culture. Cancer Res., 46:4665-4671.

Mignatti, P., Robbins, E., and Rifkin, D.B. (1986) Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade. Cell, 47:487-498.

Miller, D. A., Lee, A., Pelton, R. W., Chen, E. Y., Moses, H. L., and Derynck, R. (1989) Murine transforming growth factor-β2 cDNA sequence and expression in adult tissues and embryos. Mol. Endocrinol., 3:1108-1114.

Mitchell E. J., Fitz-Gibbon L., O'Connor-McCourt M. D. (1992 a) Subtypes of betaglycan and of type I and Type II transforming growth factor-β (TGF-β) receptors with different affinities for TGF-β1 and TGF-β2 are exhibited by human placental trophoblast

cells. J. Cell. Phys. 150:334-343.

Mitchell, E. J., Lee, K., O'Connor-McCourt, M. D. (1992 b) Characterization of transforming growth factor-β (TGF-β) receptors on BeWo choriocarcinoma cells including the identification of a novel 38 kDa TGF-β binding glycoprotein. Mol. Biol. Cell 3:1295-1307.

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

Miyazono K., Hellman U., Wernstedt C. and Heldin C. H. (1988) Latent molecular weight complex of transforming growth factor-β1. J. biol. Chem. 263:6407-6415.

Morales, T. I., and Roberts, A. B., (1988) transforming growth factor-β regulates the metabolism of proteoglycans in bovine cartilage organ cultures. J. Biol. Chem. 263:12828-12831.

Morrish, D. W., Bhardwaj, D., and Paras, M. T. (1991) Transforming growth factor β1 inhibits placental differentiation and human chorionic gonadotropin and human placental lactogen secretion. Endocrinology, 129:22-36.

Moses, H. L., Yang, E. Y and Pietenpol, J. A. (1990) TGF-β stimulation and inhibition of cell proliferation: New mechanistic insights. Cell,63:245-247.

Mulder, K. M., Levine, A. E., Hernandez, X., McKnight, M. K., Brattain, D. E., and Brattain, M. G. (1988) Modulation of c-*myc* by transforming growth factor-β in colon carcinoma cells. Biochem. Biophys. Res commun. 150:711-716.

Muldoon, L. L., Rodland, K. D. and Magun, B. E. (1988) Transforming growth factor β and epidermal growth factor alter calcium influx and phosphatidylinositol turnover in Rat-1 fibroblasts. J. Biol. Chem. 263:18834-18841.

Munson, P. J. and Rodbard, D. (1980) LIGAND: A versatile computerized approach for characterization of ligand-binding systems. Anal. biochem. 107:220-239.

Noda M., Yoon K., Prince C. W., Butler W. T. and Rodan G. A. (1988) Transcriptional regulation of osteopontin production in rat osteosarcoma cells by type β transforming growth factor. J. Biol. Chem. 263:13916-13921.

Noma, T., Glick, A. B., Geiser, A. G., O'Reilly, M. A., Miller, J., Roberts, A. B. and Sporn, M. B. (1991) Molecular cloning and structure of the human transforming growth factor-β2 gene promoter. Growth Factors, 4:247255.

O'Connor-McCourt, M. D., and Hollenberg, M. D. (1983) Receptors, acceptors, and the action of polypeptide hormones: illustrative studies with epidermal growth factor (urogastrone). Can J. Biochem. Cell Biol., 61:670-682.

O'Connor-McCourt, M. D., and Wakefield, L. M. (1987) Latent transforming growth factor-β in serum. J. Biol. Chem. 262:14090-14099.

O'Grady P., Kuo M. D. Baldassare J. J., Huand S. S. and Huand J. S. (1991 a) Purification of a new type high molecular weight receptor (type V receptor of transforming growth factor β (TGF-β) from bovine liver. Identification of the type V TGF-β receptor in cultured cells. J. Biol. Chem. 266:8583-8589.

O'Grady P., Huand S. S. and Huand J. S. (1991 b) Expression of a new type high molecular weight receptor (type V receptor) of transforming growth factor β in normal and transformed cells. Biochem. Biophy. Res. Comm. 179:378-385.

Ohta M., Greenberger J. S., Anklesaria P., Bassols A. and Massague J. (1987) Two forms of transforming growth factor-β distinguished by multipotential haematopoietic progenitor cells. Nature 329: 539 541.

Ohtsuki, M. and Massague, J. (1992) Evidence for the involvement of protein kinase activity in TGF-β signal transduction. Mol. Cell Biol. 12:261-265.

Olson E. N. Stenberg E., Hu J. S., Spizz G. and Wilcox C. (1986) Regulation of myogenic differentiation by type beta transforming growth factor. J. Cell Biol. 103:1799-1805.

Ottmann O. G. and Pelus L. M. (1988) Differential proliferative effects of transforming growth factor-β on human hematopoietic progenitor cells. J. Immunol. 140:2661-2665.

Panigel, M. (1986) in Clinics in Obstetrics and Gynaecology, The Human Placenta. Chard, T. Eds., W.B Saunders Co., London. pp.422-428.

Paria B. C., Jones K. L., Flanders K. C. and Dey S. K., (1992) Localization and binding of transforming growth factor-β isoforms in mouse preimplantation embryos and in delayed and activated blastocysts. Dev. Biol. 151:91-104.

Pearson C. A., Pearson D., Shibahara S., Hofsteenge J. and Chiquet-Ehrismann R. (1988) Tenascin: cDNA cloning and induction by TGF-β. EMBO J. 7:2677-2981.

Pelton, R. W., Dickinson, M. E., Moses, H. L. and Hogan, B. L. M., (1992) In situ hybidization analysis of TGF-β3 RNA expression

during mouse development: comaparative studies with TGF-β1 and TGF-β2. Development 110:609-620.

Pelton, R. W., Saxena, B., Jones, M., Moses, H. L. and Gold, L. I. (1991) Immunohistochemical localization of TGF-β1, TGF-β2 and TGF-β3 in the mouse embryo: expression patterns suggest multiple roles during embryonic development. J. Cell. Biol. 115:1091 1105.

Pelton, R. W., Nomura, S., Moses, H. L., and Hogan, B. L. M. (1989) Expression of transforming growth factor β-2 RNA during murine embryogenesis. Deveolpment, 106:759-768.

Penttinen R. P., Kobayashi S. and Bornstein P. (1988) Transforming growth factor β increases mRNA for matrix proteins both in the presence and in the absence in mRNA stability. Proc. Natl. Acad. Sci. USA 85:1105-1108.

Pfeilschifter J. (1990) in Growth Factors, Differentiation Factors and Cytokines. Habenicht, A. Eds. Springer-Verlag, Berlin, pp. 56-64.

Pollard, J. W. (1990) Regulation of polypeptide growth factor syntheisi and growth factor-related gene expression in the rat and mouse uterus before and after implantation. J. Reprod. Fertil., 88:721-731.

Qian S. w., Burmester J. K., Merwin J. R., Madri J. A., Sporn M. B., and Roberts A. B. (1992) Identification of a structural domain that distinguishes the actions of the type 1 and 2 isoforms of transforming grwoth factor β on endothelial cells. Proc. Natl. Acad. Sci. USA 89:6290-6294.

Queenan, J. T., Kao, L.-C., Arboleda, C. E., Ulloa-Aguirre, A., Golos, T. G., Cines, D. B. and Strauss, J. F. (1987) Regulation of urokinase-type plasminogen activator production by cultured human cytotrophoblasts. J. Biol. Chem. 262:10903-10906.

Redini F., Lafuma C., Pujol J. P., Robert L. and Hornebeck W. (1988) Effect of cytokines and growth factors on the expression of elastase activity by human synoviocytes, dermal fibroblasts and rabbit articular chondrocytes. Biochem. Biophys. Res. Comm. 155:786-793.

Redman, C. W. G. (1986) Immunology of the placenta. In, Clinics in Obstetrics and Gynaecology, The Human Placenta. Chard, T., Eds, W. B. Saunders Co., London.

Ristow H. J. (1986) BSC-1 growth inhibitor type-β transforming growth factor is a strong inhibitor of thymnocyte proliferation. Proc. Natl. Acad. Sci. USA. 83:5531-5534.

Roberts, A. B., and Sporn, M. B. (1990) The transforming grwoth

factor-betas. In Handbook of Experimental Pharmacology. Peptide Growth Factors and Their Receptors (Sporn, M. B. and Roberts, A. B., eds.)Springer-Velag, Heidelberg, 95:419-472.

Roberts A. B., Anzano M. A., Lamb L. C., Smith J. M., Sporn M. B. (1981) New class of transforming growth factors potentiated by epidermal growth factor: Isolation from non-neoplastic tissues. Proc. Natl. Acad. Sci. USA. 78:5339-5343.

Roberts A. B., Anzano M. A., Lamb L. C., Smith J. M., Frolik C. A., Marquardt H., Todaro G. J., Sporn M. B. (1982) Isolation from sarcoma cells of novel transforming growth factors potentiated EGF. Nature (Lond) 295: 417-419.

Roberts A. B., Anzano M. A., Meyers C. a., Wideman J., Blacher R., Pan Y. C. E., Stein S., Lehrman S. R., Smith J. M., Lamb L. C., Sporn M. B. (1983) Purification and properties of a type β transforming groth factor from bovine kidney. Biochemistry 22: 5692 5698.

Roberts A. B., Anzano M. A., Wakefield L. M., Roche N. S., Stern D. F., Sporn M. B. (1985) Type-β transforming growth factor: A bifunctional regulator of cellular growth. Proc. Natl. Acad. Sci USA. 82:119-123.

Roberts A. B., Sporn M. B., Assosian R. K., Smith J. M., Roche N. S., (1986) Transforming growth factor type-β: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc. Natl. Acad. Sci. USA 83: 4167-4171.

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

Rosen D. M., Stempien S. A., Thompson A. Y., Seyedin P. R. (1988) Transforming grwoth factor-beta modulates the expression of osteoblast and chondroblast phenotypes in vitro. J. Cell. Physiol. 134:337-346.

Rosa F., Roberts A. B., Danielpour D., Dart L. L., Sporn M. B., David I. B. (1988) Mesoderm induction in amphibians: the role of TGF-β2-like factors. Science 236:783-786.

Ruff, E. and Rizzino, A. (1986) Biochem. Biophys. Res. commun. 138:714-719.

Russell W. E., Coffey R. J., Ouellette A. J., Moses H. L. (1988) Transformining growth factor beta reversibley inhibits the early proliferative response to partial hepatectomy in the rat. Proc Natl. Acad. Sci. USA 85:5126-5130.

Schlunegger, M. P. and Grutter, M. G. (1992) An unusual feature

revealed by the crystal structure at 2.2 A resolution of human transforming growth factor-β2. Nature, 358:430-434.

Shi, d. L., Savona, C., Chambaz, E. M. and Feige, J. J. (1990) Stimulation of fibronectin production by TGF-β1 is independent of effects on cell proliferation: The example of bine adrenocortical cells. J. Cell. Physiol. 145:60-68.

Schmid, P., Cox, D., Bilbe, Gl., Maier, R., McMaster, G. K. (1991) Differential expression of TGF-β1, TGF-β2 and β3 genes during mouse embryogenesis. Development 111:117-130.

Segarini, P. R., Rosen, D. M., Seyedin, S. M. (1989) Binding of transforming growth factor-β to cell surface proteins varies with cell type. Mol. Endocrinol. 3:261-272.

Segarini P. R. (1991) A system of transforming growth factor-β receptors. Am. J. Respir. Cell Mol. Biol. 4:395-396.

Segarini, P. R., Ziman, J. M., Kane, C.J. M., Dasch, J. R. (1992) Two novel patterns of transforming grwoth factor β (TGF β) binding to cell surface proteins are dependent upon binding of TGF-β1 and indicate a mechanism of positive cooperativity. J. Biol. Chem. 267:1048-1053.

Segarini P. R., Roberts A. B., Rosen D. M., Seyedin S. M. (1987) Membrane binding characteristics of two forms of transforming growth factor-β. J. Biol. Chem. 262:1465514662.

Segarini P., Seyedin S. M. (1988) The high molecular weight receptor to transforming growth factor-β contains glycosaminoglycan chains. J. Biol. Chem. 263:8366-8370.

Segarini P. R., Ziman J. M., Kane C. J. M., Dasch J. R. (1992) Two novel patterns of transforming growth factor β (TGF-β) binding to cell surface proteins are dependent upon the binding of TGF-β1 and indicate a mechanism of positive cooperativity. J. Biol. Chem. 267:1048-1053.

Seyedin S. M., Thomas T. C., Thompson A. Y., Rosen D. M., Piez K. A. (1985) Purification and characterization of two cartilage-inducing factors from bovine demineralized bone. Proc. Natl. Acad. Sci. USA. 82:2267-2271.

Seyedin S. M., Segarini P. R., Rosen D. M., Thompson A. Y., Bentz H. Graycar J. (1987) Cartilage-inducing factor-B is a unique protein structurally and functionally related to transforming growth factor-β. J. Biol. Chem. 262;1946-1949.

Shi, D. L., Savona, C., Chambaz, E. M. and feige, J. J.(1990) Stimulation of fibronectin production by TGF-β1 is independent of effects on cell proliferation: The example of bine adrenocortical

cells. J. Cell. Physiol. 145:60-68.

Shipley G. D., Pittelkow M. R., Wille J. J. Jr., Scott R. E., Moses H. L. (1986) Reversible inhibition of normal human prokeratinocyte proliferation by type β transforming grwoth factor-growth inhibition in serum-free medium Cancer Res. 46:2068-2071.

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

Smeland E. B., Blomhoff H. K., Holte H., Ruud E., Beiske K., Funderud S., Godal T., Ohlsson R. (1987) Transforming growth factor type β (TGF-β) inhibits G1 to S trasition, but not activation of human B Lymphocytes. Expt. Cell Res. 171:213-222.

Speigel, A. M. (1987) Signal transduction by guanine nucleotide binding proteins. Mol. Cell Endocrinol., 49:1-16.

Spence, A. P. and Mason, E. B. (1987) Human anatomy and physiology, 3rd ed., The Benjamin/Cummings Publishing Company, Inc. Menlo Park, CA.

Sporn & Todaro (1980) N. Eng. J. Med. P.878-880.

Sporn, M. B., Roberts, A. B., Wakefield. L. M. and Assoian, R. K. (1986) Transforming growth factor-β: Biological function and chemical structure. Science, 233:532-534.

Sporn, M. B., Roberts, A. B. (1989) Transforming growth factor-β, multiple actions and potential clinical applications. JAMA.,262:938-941.

Strickland, S., Reich, E., and Sherman, M. I. (1976) Plasminogen activator in early embryogenesis: enzyme production by trophoblast and parietal endoderm. Cell 9:231-240.

Tamada, H., McMaster, M. T., flanders, K. C., Andrews, G. K., and Dey, S. K. (1990) Cell type-specific expression of transforming growth factor-β1 in mouse uterus during the preiimplantation period. Mol. Endocrinol., 4:965-972.

Tashjian, A. H., Voelkel, E. F., Lazzaro, B., singer, F. R., and Roberts, A. B. (1985) α and β human transforming growth factors stimulate prostaglandin production and bone resorption in cultured mouse calvaria. Proc. Natl. Acad. Sci. USA., 82:4535-4538.

ten Dijke, P., van Kessel, A. H. M. G. Transforming grwoth factor type-β 3 maps to human chromosome 14, region q23-q24. Oncogeme 3:721-724.

Titeler, M. (1989) Receptor binding theory and methodology. In, Receptor Pharmacology and Function. Williams, M., Glennon, R. A., Timmermans, P. B. M. W. M., Eds,Marcel Dekker, Inc. N.Y., N. Y.

Tsunawaki S., Sporn M. B., Ding A., Nathan C. (1988) Deactivation of macrophages by transforming growth factor-β. Nature 334:260 262.

Upstate Biotechnology, Inc. (1992) Catalog and Protocols, Lake Placid, New York.

Wakefield L. M., Smith D. M., Flanders K. C., Sporn M. B. (1988) Latent transforming growth factor-β from human platelets. J. Biol. Chem. 263:7646-7654.

Wakefield L. M. Smith D. M., Masui T., Harris C. C., Sporn M. B. (1987) Distribution and modulation of the cellular receptor for transforming growth factor-beta. J. Cell Biol. 105:965-975.

Wakefield, L. M. (1987) An assay for type-β transforming growth factor receptor. Method in Enzymology, 146:167-173.

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

Wice, B., Menton, d., Geuze, H. and Schwartz, A. L. (1990) Modulators of cyclic AMP metabolism induce syncytiotrophoblast formation in vitro. Expl. Cell Res., 186:306-316.

Wrann M., Bodmer S., de Martin R., siepl C., Hofer-Warbinek R., (1987) T Cell Suppressor factor from human glioblastoma cells is a 12.5 kd protein closely related to tranforming growth factor-β. EMBO J. 6:1633-1636.

Wrana J. L., Sodek J., Ber R. L., Bellows C. G., (1986) The effects of platelet-derived transforming growth factor β on normal human diploid gingival fibroblasts. Eur. J. Biochem. 159:69-76.

Wu, M. C., and Fischer, R. A. (1980) Granulocyte and macrophage colony-stimulating gactor from human placenta conditioned medium. Biochemistry. 19:3846-3850.

Wynn, R. M. Principles of placentation and early human placental development. In The Placenta and its Maternal Supply Line, Effects of Irsufficiency on the Fetus. Gruenwald, Eds., University Park Press, Baltimore,1975 pp. 19 33.

van der Ende, A., du Maine, A., Schwartz, A. L., and Strous, G. J. (1990) Modulation of transferrin receptor activity and recycling after induced differentiation of BeWo choriocarcinoma

cells. Biochrm. J., 270:451-457.

van der Ende, A., A. du Maine, A. L. Schwartz, and G. J. Strous. (1989) Biochem. J. 259:685-

van der Ende, A., du Maine, A., Simmons, C. F., Schwartz, A. L., and Strous, G. J. (1987) Iron metabolism in BeWo chrion carcinomas cells. J. Biol. Chem. 262:8910-8916.

Varga J., Rosenbloom J., Jimenez S. A. (1987) Transforming growth factor β (TGF-β) causes a persistent increase in steady-state amounts of type I and type II collagen and fibronectin mRNAs in normal human dermal fibroblasts. Biochem. J. 247: 597-604.

Yagel, S., Parhar, R. S., Jeffrey, J. J., and Lala, P. K. (1988) Normal nonmestatic human trophoblast cells shore in vitro invasive properties of malignant cells. J. Ce.. Physiol. 136:455-462.

Yamadea, K. M. and Olden, K. (1978) Fibronectin-adhesive glycoproteins of cell surface and blood. Nature, 275:179-184.

Yeh, I.-T. and Kurman R. J. (1989) Functional and morphologic expressions of trophoblast. Laboratory Invest., 61:1-4.

ACKNOWLEDGEMENTS

First and formost I would like to show my appreciation to my research supervisor, Dr. E. J. Mitchell for her guidance, advise and for making this all possible. Thank you.

I would also like to take this opportunity to thank all my friends at the BRI, especially Dr. Rita Hannah, and my advisor, Dr. Dusica Maysinger, for their support, encouragement and helpful discussions.

Very special thanks to my parents and family for their patience and support throughout my thesis work.

Thanks are also extended to Les Fonds pour la Formation de Chercheurs et l'aide a la Recherche (FCAR) and Les Fonds de la Recherche en Sante du Quebec (FRSQ) for financial support throught my studies.

Last but not least, I am indebted, to Jean Labrecque for his help, his patience, tolerance and whole-hearted support throughout all of this.