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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

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Transforming growth factor-β (TGF-β) is a potential mediator of placental trophoblast functions, including differentiation, hormone production, endometrial invasion, and immunosuppression. Equilibrium binding and affinity-labeling assays were used to investigate the binding characteristics of TGF-β1 and TGF-β2 on an established human choriocarcinoma trophoblastic cell line (BeWo). The equilibrium binding experiments indicated that the BeWo cells exhibited similar average affinities and total number of binding sites for TGF-β1 and TGF-β2. The K_d values obtained from Scatchard analyses were ~65 pM for ^{125}I-TGF-β1 and ~40 pM for ^{125}I-TGF-β2, with 70 000 and 85 000 sites per cell, respectively. Competitive equilibrium binding experiments indicated that TGF-β1 and TGF-β2 were equipotent (apparent half maximal inhibition [IC_{50}] ~70 pM) and that all binding sites were capable of recognizing both isoforms. Affinity-labeling studies with ^{125}I-TGF-β1 and ^{125}I-TGF-β2 and the chemical cross-linking agent bis(sulfosuccinimidyl)suberate (BS^3) revealed a predominant type III/betaglycan receptor, a low level of apparently heterogeneous type I and II receptors and an additional novel 38-kDa TGF-β binding glycoprotein that was present both under reducing and nonreducing conditions on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Affinity-labeling saturation and competition studies indicated that the type III/betaglycan component appears to have a 7-fold higher capacity for TGF-β1 than for -β2 yet exhibits a 5- to 10-fold higher affinity for TGF-β2 than for -β1. The 38-kDa TGF-β binding component, an N-linked glycoprotein, exhibits a higher affinity for TGF-β2 than for -β1 that is strikingly similar to that of the type III/betaglycan receptor. This 38-kDa binding protein appears to be upregulated after methotrexate-induced differentiation of the BeWo cells.

INTRODUCTION

Transforming growth factor-β (TGF-β) is a multifunctional regulatory peptide that controls growth, differentiation, and extracellular matrix deposition (for reviews see Massagué, 1990; Roberts and Sporn, 1990). The human TGF-β family consists of three isoforms, TGF-β1, TGF-β2, and TGF-β3, which are highly homologous. Although the biological potencies of these isoforms are similar in many in vitro assays, 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.*, 1990, 1991; Schmid *et al.*, 1991; Paria *et al.*, 1992; Roberts and Sporn, 1992).

Affinity-labeling techniques with chemical cross-linking agents and ^{125}I-TGF-β initially revealed, on most cell types, three types of high affinity binding compo-

nents, known as the type I, II, and III (also known as betaglycan) receptors (for reviews see Segarini, 1991; Massagué, 1992). The type I and II components are glycoproteins and are thought to be true signaling receptors (Boyd and Massagué, 1989; Laiho *et al.*, 1990, 1991), whereas the type III/betaglycan component, a proteoglycan, exists in both membrane-anchored and soluble forms and is thought to serve nonsignaling roles (Andres *et al.*, 1989). Although on most cell types the type I and II TGF-β receptors show a higher affinity for TGF-β1 and TGF-β3 than for TGF-β2, subtypes that have high affinity for TGF-β2 also exist (Cheifetz *et al.*, 1990; Cheifetz and Massagué, 1991; Mitchell *et al.*, 1992). In general, the type III/betaglycan receptor exhibits equal affinity for TGF-β1 and TGF-β2 although type III/betaglycan subtypes having a higher affinity for TGF-β2 than for TGF-β1 have also been detected (Segarini *et al.*, 1987; Mitchell and O'Connor-McCourt, 1991a; Mitchell *et al.*, 1992). The type III/betaglycan and type II receptors have now been cloned and sequenced (Lopez-Casillas *et al.*, 1991; Wang *et al.*, 1991; Lin *et al.*, 1992). In addition, affinity-labeling and affinity chromatography studies have recently demonstrated some other TGF-β binding proteins that are distinct from the type I, II, and III/betaglycan receptors (MacKay *et al.*, 1990, 1992; Ichijo *et al.*, 1991; MacKay and Danielpour, 1991; O'Grady *et al.*, 1991a,b; Segarini *et al.*, 1992; Hannah, unpublished data), including some glycolipid-anchored binding proteins (Cheifetz and Massagué, 1991).

In previous studies we used an affinity-labeling approach to characterize the TGF-β receptors in human placental membrane preparations (Mitchell and O'Connor-McCourt, 1991a) and on primary placental cells (Mitchell *et al.*, 1992). We have described a type III/betaglycan receptor which has a higher affinity for TGF-β2 than for TGF-β1 that was detected in crude term placental membrane preparations and on primary placental trophoblast cells, but not on primary placental mesenchymal cells. In addition to the usual type I and II receptors, which have a higher affinity for TGF-β1 than for TGF-β2, these primary trophoblast cells also exhibited subtypes of the type I and II receptors with similar affinities for TGF-β1 and TGF-β2. Trophoblast cells are the epithelial-like covering of the chorionic villae at the fetal-maternal interface. These cells have crucial roles in pregnancy including nutrient/waste exchange, the physical attachment of the placenta, hormone production, and the regulation of the maternal immune response toward the fetus. The roles of growth factors in trophoblast function are poorly understood, although they are assumed to mediate their effects through various paracrine and autocrine pathways (for reviews see Pollard, 1988; Blay and Hollenberg, 1989; Ohlsson, 1989; Morrish, 1990). The activities of TGF-β, notably in extracellular matrix remodeling, differentiation, and immunosuppression, point to potential roles for TGF-β as a mediator of trophoblastic invasion of the uterus, trophoblast differentiation, and maintenance of pregnancy (Altman *et al.*, 1990; Clark *et al.*, 1990; 1991; Lala and Graham, 1990; Tamada *et al.*, 1990; Graham and Lala, 1991, 1992a,b; Morrish *et al.*, 1991; Ritvos and Eramaa, 1991; Graham *et al.*, 1992a,b; Lea *et al.*, 1992). Placenta and amniotic fluid are both relatively rich sources of TGF-β (Frolik *et al.*, 1983; Altman *et al.*, 1990), and the TGF-β2 isoform, in particular, has been implicated in placental function (Miller *et al.*, 1989; Altman *et al.*, 1990; Clark *et al.*, 1990; 1991; Lea *et al.*, 1992). Therefore, the characterization of trophoblast TGF-β receptors is an essential element for understanding the possible roles of the different TGF-β isoforms in placental trophoblast function. Our findings concerning the TGF-β receptors on primary trophoblast cells led us to examine the human choriocarcinoma trophoblastic cell line BeWo, which is a more uniform model than primary trophoblasts, to further study trophoblastic TGF-β receptors. BeWo cells are also an interesting differentiative model because, in response to methotrexate, these cells undergo a complex response that resembles the differentiation of cytotrophoblast into syncitiotrophoblast (Friedman and Skehan, 1979; Burres and Cass, 1987). In this study we have studied the binding of ^{125}I-TGF-β1 and ^{125}I-TGF-β2 to BeWo cells both by equilibrium binding studies and by affinity labeling. We have demonstrated that many of the characteristics of the TGF-β receptors that we had observed on primary trophoblast cells are retained in the BeWo trophoblast-like cell line. However, we also detected an additional novel TGF-β binding glycoprotein of 38 kDa on the BeWo cells that appeared to be upregulated upon methotrexate-induced differentiation of these cells.

These results have been presented in preliminary form at the Annual Meeting of the American Society for Cell Biology in Boston, MA, December, 1991 (Lee *et al.*, 1991; Mitchell and O'Connor-McCourt, 1991b).

MATERIALS AND METHODS

Cell Culture

The human trophoblastic cell lines BeWo, JEG-3, and JAR (originally derived from choriocarcinomas) were obtained from the American Tissue Culture Collection (Rockville, MD) and maintained as adherent cultures in RPMI medium supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 1% (vol/vol) sodium pyruvate solution (Gibco/BRL, Toronto, Ontario, Canada) at 37°C in 5% CO_2 in humidified air. The rat 1 cell line was obtained from Dr. David Stern (Yale University, New Haven, CT) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) FBS. Cultures were grown in the absence of antibiotics and were passaged by dissociation with 0.25% trypsin-EDTA (Gibco/BRL, Toronto, Ont., Canada). Typically, cells were dissociated with 0.25% trypsin-EDTA and plated at a density of 6×10^5 cells/well in 24-well plastic tissue culture plates, 1 d before binding experiments. Monolayers that were just confluent were used for both equilibrium binding

and affinity-labeling studies and contained ~9×10^5 cells/well. We found no difference in the number of receptors determined by Scatchard analysis, when the cells were trypsin dissociated, but plated at a lower density and allowed to reach confluency in 48 h rather than 24 h. Although we routinely used cultures of BeWo cells that were just confluent, we found no difference in the pattern of the affinity-labeled bands when we tested them at ~60%, 100%, or over confluency. JEG-3 and JAR cells required that the wells be coated with poly-L-lysine to remain adherent throughout the affinity-labeling procedure. The method of Friedman and Skehan (1979) was used for the induction of BeWo cell differentiation by methotrexate. Briefly, cells were plated at a density of 4×10^4 cells/well and then treated with 1 μM methotrexate in medium (as above) after 18–24 h when they had reached ~60% confluency. The medium containing methotrexate was changed daily until day 4 when the majority of the cells appeared to be multinucleate.

Iodination of TGF-β

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-βs were iodinated following the method of Frolik *et al.* (1984), which was modified by Ruff and Rizzino (1986) for the iodination of 1-μg TGF-β. The details were outlined previously by us (Mitchell and O'Connor-McCourt, 1991a). In this study, we iodinated either 1 μg of carrier-free porcine TGF-β2 or 2–5 μg of recombinant TGF-β1 or recombinant TGF-β2. The range for the specific activity was between 1.5–3.25 μCi/pmol (60–130 μCi/μg) as determined in ≥10 individual iodinations of each of these TGF-β preparations.

Equilibrium Binding Assays and Affinity Labeling of Cells in Monolayer

For both the equilibrium and affinity-labeling binding studies, cell monolayers (in 24-well plates) were washed three times with ice cold binding buffer [Dulbecco's-phosphate-buffered saline (D-PBS), pH 7.4, containing 0.1% (wt/vol) bovine serum albumin (BSA)] for a total of 30 min at 4°C. The monolayers were then incubated with either ^{125}I-TGF-β1 or ^{125}I-TGF-β2, in the absence or presence of unlabeled TGF-β1 or -β2 as indicated, in a final volume of 300 μL/well for 3 h at 4°C with continuous gentle agitation.

For equilibrium saturation binding assays, cells were washed as described above then incubated with varying concentrations of the iodinated TGF-β ligand ranging between 1 and 500 pM. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled TGF-β1 or TGF-β2, and duplicate wells were used for each condition. At the end of the incubation period, the cells were quickly washed three times with ice cold binding buffer, then bound counts were solubilized in 600 μL of 1% (vol/vol) Triton X-100, 10% (vol/vol) glycerol, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4 for 30 min at 4°C with gentle agitation (Wakefield *et al.*, 1987). Cell number was determined with a hemocytometer on wells treated as above except that trypsinization substituted for the solubilization step. Equilibrium competition studies were performed as above except that a constant amount of ^{125}I-TGF-β1 or ^{125}I-TGF-β2 was added together with varying concentrations (between 0 and 5000 pM) of unlabeled TGF-β1 or TGF-β2. Three different concentrations of radiotracer were tested (10, 25, and 50 pM) with no significant difference in the half maximal inhibition (IC_{50}) values being observed. Additionally, no significant difference in IC_{50} values were observed between competition experiments when either the recombinant or porcine platelet TGF-β2 was used.

The affinity-labeling studies were performed as outlined by us previously for primary placental cells (Mitchell *et al.*, 1992). Briefly, after incubation with the ^{125}I-TGF-β in the absence or presence of the unlabeled TGF-β as described above, monolayers were washed quickly three times with ice cold D-PBS pH 7.4, then incubated with 300 μL 1 mM BS^3 in D-PBS for 10 min at 4°C. The cross linking was stopped by the addition of 75 μL of 500 mM glycine and a further 5-min incubation. Cells were washed once with D-PBS and then incubated for 30 min at 4°C with 100 μL of solubilization buffer containing 1% (vol/vol) Triton X-100, 10% (vol/vol) glycerol, 1 mM EDTA, 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.4, and the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/mL aprotinin, 20 μg/mL leupeptin, 20 μg/mL soybean trypsin inhibitor, and 25 mM benzamidine. Solubilized material was recovered from each well with a pipette tip after placing the tissue culture plate at a slight angle. One-fifth volume of ×5 electrophoresis sample buffer (0.25 M Tris-HCl, pH 6.8, 5% [wt/vol] SDS, 10% β-mercaptoethanol, 50% [vol/vol] glycerol, 0.0004% [wt/vol] bromophenol blue) was added to each sample, followed by heating at 100°C for 5 min. SDS-PAGE, autoradiography, and densitometry were performed as previously described (Mitchell and O'Connor-McCourt, 1991a).

Enzymatic Deglycosylation of Affinity-Labeled BeWo Cells

The enzymatic removal of the glycosaminoglycan (GAG) chains with heparitinase (EC 4.2.2.8) (ICN Biomedicals, Cleveland, OH) and chondroitinase ABC (EC 4.2.2.4) (Seikagaku America, Rockville, MD) or the enzymatic removal of N-linked complex carbohydrate with endoglycosidase F (EC 3.2.1.96) were performed according to Cheifetz *et al.* (1988).

RESULTS

BeWo Cells Show Specific Saturable Binding with ^{125}I-TGF-β1 and ^{125}I-TGF-β2

BeWo cells that were incubated with increasing concentrations of ^{125}I-TGF-β1 or ^{125}I-TGF-β2 showed specific saturable binding, and Scatchard analysis of the binding by the LIGAND program developed by Munson and Rodbard (1980) gave linear plots for both isoforms (Figure 1). The apparent dissociation constants for TGF-β1 and TGF-β2 binding were 37 and 35 pM, respectively, in the experiments shown and ranged in three separate experiments from 37 to 93 pM for TGF-β1 and from 29 to 51 pM for TGF-β2. The estimated number of binding sites per BeWo cell in the experiments shown were 44 000 for TGF-β1 and 83 000 for TGF-β2 and ranged in three experiments from 44 000 to 101 000 and from 49 000 to 122 000, respectively. This determination places the BeWo cell line among the richest sources of TGF-β receptors, comparable to the Swiss mouse 3T3 and rat-1 cell lines (Wakefield *et al.*, 1987). The results indicate that BeWo cells exhibit similar average affinities and total number of binding sites for TGF-β1 and TGF-β2.

Competition of TGF-β1 and TGF-β2 for Specific Binding Sites on BeWo Cells

To determine whether TGF-β1 and TGF-β2 bind to the same sites on BeWo cells, competition experiments with either ^{125}I-TGF-β1 or ^{125}I-TGF-β2 and unlabeled TGF-

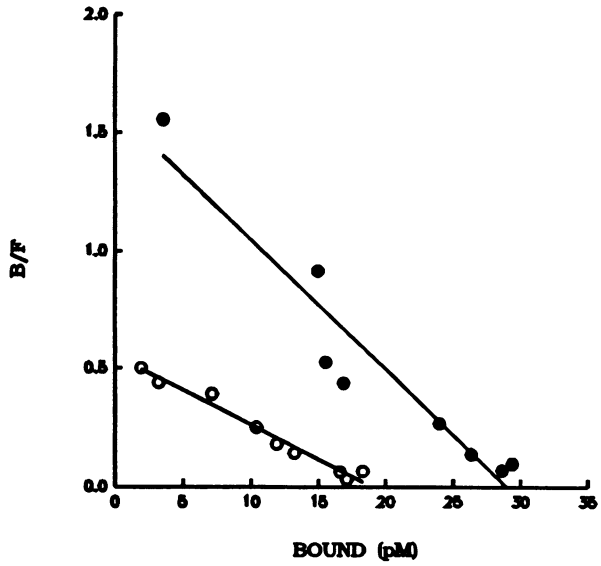

Figure 1. TGF-β1 and TGF-β2 bind specifically and saturably to BeWo cells. Monolayers of BeWo cells were incubated with increasing concentrations of ^{125}I-TGF-β1 or ^{125}I-TGF-β2 as described in MATERIALS AND METHODS. Shown are the Scatchard plots derived from a representative saturation binding curve with ^{125}I-labeled recombinant TGF-β1 (○) or ^{125}I-labeled recombinant TGF-β2 (●). Specific activities of ^{125}I-TGF-β1 and ^{125}I-TGF-β2 in these experiments were 2.0 and 2.7 μCi/pmol, respectively.

β1 or TGF-β2 were performed (Figure 2, A and B). Complete inhibition of ^{125}I-TGF-β1 and ^{125}I-TGF-β2 binding was observed in the presence of unlabeled TGF-β1 or TGF-β2 indicating that there were no sites uniquely available to one isoform. In the representative experiments shown, the IC_{50} of binding of ^{125}I-TGF-β1 was 80 pM with unlabeled TGF-β1 and 71 pM with unlabeled TGF-β2. Similarly, when ^{125}I-TGF-β2 was used as the radiotracer, the IC_{50} values were 71 and 70 pM for TGF-β1 and TGF-β2, respectively. These IC_{50} values are in good agreement with the range of average affinities determined by Scatchard analysis (Figure 1) (i.e., 35–90 pM).

Equilibrium binding assays provide quantitative results with respect to the total number of receptors present and the average affinity of these receptors. However, when the affinities of separate structural classes of receptors are similar, or the relative abundance of different structural classes and subtypes masks their different affinities, these classes and/or subtypes cannot be distinguished by Scatchard analysis. Affinity-labeling techniques have an advantage over equilibrium binding studies because the structural classes of binding components can be analyzed independently as labeled bands on SDS-PAGE.

Affinity-Labeling Saturation Studies on BeWo Cells

Confluent monolayers of BeWo cells were incubated with increasing concentrations of ^{125}I-TGF-β1 or ^{125}I-TGF-β2. Bound ligand was covalently cross-linked to the binding proteins with BS^3 and the Triton-soluble membrane extracts were reduced and analyzed by SDS-PAGE and autoradiography. As illustrated in Figure 3A, two predominant ^{125}I-TGF-β1 affinity-labeled bands and several less intensely labeled bands were observed. The most predominant high molecular mass band is characteristic of the type III/betaglycan receptor. The second most intensely labeled band represents a novel 50-kDa complex, designated here as 'N'. This novel component has an apparent molecular mass of 38 kDa after subtraction of the TGF-β monomer and was seen not only on BeWo cells but also on two other human choriocarcinoma cell lines, JEG-3 and JAR (Figure 4). Several ^{125}I-TGF-β1 labeled bands in the molecular mass range of the type I and II receptors were also observed. The level of these receptors relative to the level of the type III/betaglycan receptor was very low, similar to our findings on primary trophoblast cells (Mitchell *et al.*, 1992). Moreover, the BeWo, JEG-3, and JAR type I and II TGF-β receptor bands were much more heterogeneous than the type I and II bands observed on most other cell types (Figure 4). For comparison, we have shown the rat 1 cell line in which the type I and II TGF-β receptors each appear as more discrete bands (Figure 4). Interestingly, the JEG-3 and JAR cells displayed more of the type II receptor and less of the type I receptor than the BeWo cells (Figure 4). When ^{125}I-TGF-β2 was used to label the TGF-β binding components on BeWo cells, the type III/betaglycan receptor and the 38-kDa TGF-β binding component were readily observed (Figure 3C). However, the type I and II receptors were much less readily detected with ^{125}I-TGF-β2 as compared with ^{125}I-TGF-β1, which is as expected for the usual type I and II receptors that have a much higher affinity for TGF-β1 than for -β2. An additional specifically labeled band of ~150 kDa was detected only when ^{125}I-TGF-β2 served as the radiolabel, however it was not consistently observed (Figure 3C).

The saturation curves for the type III/betaglycan and 38-kDa binding components that are shown in Figure 3, B and D were derived by densitometric analysis of the autoradiograms shown in Figure 3, A and C. The autoradiograms shown were exposed for 11 d, and the specific activities of ^{125}I-TGF-β1 and ^{125}I-TGF-β2 were 3.5 and 2.4 μCi/pmole, respectively. To avoid overexposure and consequently nonlinear response of the X-ray film, a 1-d exposure of the ^{125}I-TGF-β1 experiment was scanned for densitometry. The slightly higher specific activity of TGF-β1 does not account for the much higher intensity of ^{125}I-TGF-β1 labeling observed for the type III/betaglycan receptor. When the intensity of labeling by ^{125}I-TGF-β1 is adjusted by multiplying by a factor of 11 (for exposure time) and a factor of 2.4/3.5 (for specific activity), we estimated that the type III/betaglycan receptor appears to have an approximately

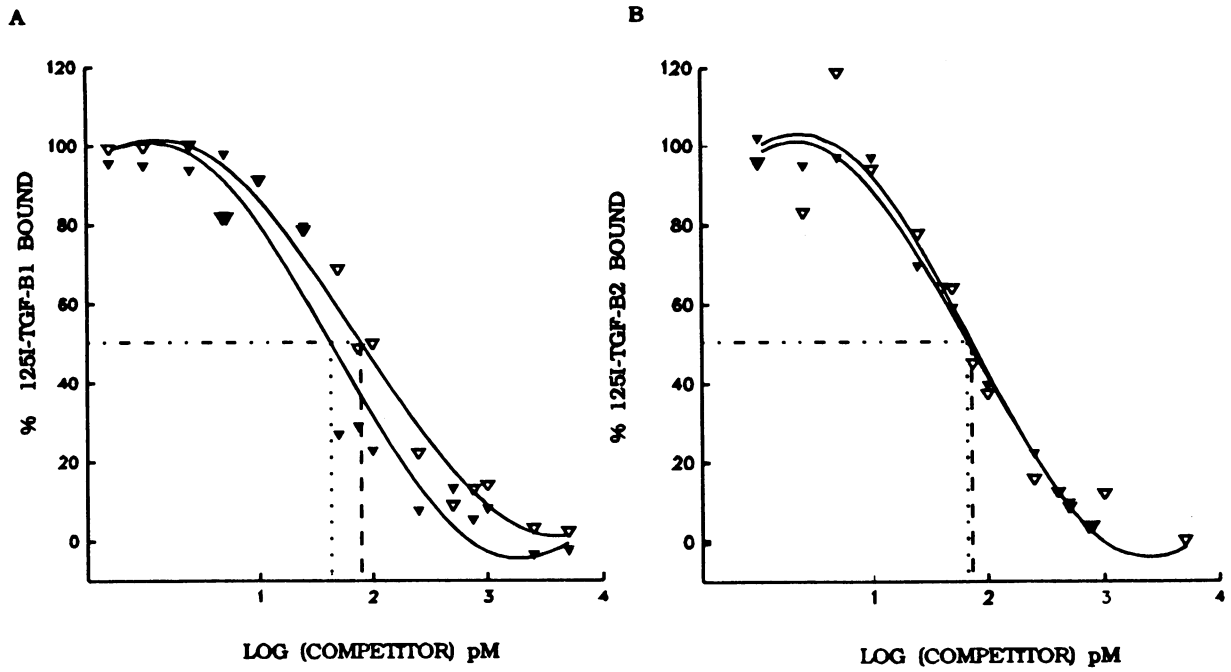

Figure 2. Competition of ^{125}I-TGF-β1 or ^{125}I-TGF-β2 binding to BeWo cells. Monolayers of BeWo cells were incubated with 25 pM ^{125}I-TGF-β1 (A) or 50 pM ^{125}I-TGF-β2 (B) in the absence or presence of unlabeled TGF-β1 (▽) or TGF-β2 (▼) as described in MATERIALS AND METHODS. Specific activities of both ^{125}I-TGF-β1 (recombinant) and ^{125}I-TGF-β2 (porcine platelet) in these experiments was 2.0 μCi/pmol. Comparable results were obtained when recombinant ^{125}I-TGF-β2 was used as the radiotracer. IC_{50} values ranged in 3 separate experiments between 79–84 pM for TGF-β1 and 63–71 pM 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.

sevenfold higher capacity for TGF-β1 than for TGF-β2, whereas the capacity for TGF-β1 versus -β2 of the 38-kDa component differs by only twofold. A similar difference in TGF-β1 versus -β2 saturation labeling intensities of the type III/betaglycan receptor was observed with primary trophoblast cells (Mitchell *et al.*, 1992). The simplest interpretations of this result are either that BeWo cells have two distinct type III/betaglycan subtypes (one which binds TGF-β1 exclusively) or that a high degree of cooperativity occurs with TGF-β1 binding but not with TGF-β2 binding. The possibility of disproportional cross-linking effects are presented in the DISCUSSION.

Affinity-Labeling Competition Studies with BeWo Cells

Whereas information concerning the relative capacity of TGF-β binding components for TGF-β isoforms is best obtained from affinity-labeling saturation studies, affinity-labeling competition studies can better infer the relative affinities of the individual receptors and their subtypes for the TGF-β isoforms. BeWo cells were affinity-labeled with ^{125}I-TGF-β1 or ^{125}I-TGF-β2 in the absence or presence of various concentrations of unlabeled TGF-β1 or TGF-β2. As illustrated by the autoradiograms shown in Figure 5, A and C, the type III/betaglycan TGF-β receptor and the 38-kDa TGF-β binding component exhibit a 2- to 5-fold higher affinity for TGF-β2 than for TGF-β1 when ^{125}I-TGF-β1 is used as the radiotracer and an almost 10-fold higher affinity for TGF-β2 as compared with TGF-β1 when ^{125}I-TGF-β2 is used as the radiotracer. From the densitometrically derived competition curves (Figure 5, B and D), we estimate the IC_{50} of ^{125}I-TGF-β1 binding by TGF-β1 at 300 pM and by TGF-β2 at 90 pM for the type III/betaglycan TGF-β receptor. Likewise, the IC_{50} of ^{125}I-TGF-β1 binding to the 38-kDa TGF-β binding component by TGF-β1 was estimated at 200 pM and by TGF-β2 at 90 pM. Similarly, when ^{125}I-TGF-β2 was the radiotracer, the IC_{50} values were estimated at 1 nM with TGF-β1 and ~150 pM with TGF-β2 for both the type III/betaglycan receptor and the 38-kDa TGF-β binding component. These results indicate that both the type III/betaglycan receptor and the 38-kDa component exhibit a ~ 5- to 10-fold higher affinity for TGF-β2 as compared with TGF-β1. Thus the type III/betaglycan TGF-β receptor on BeWo cells exhibits the same preferential affinity for TGF-β2 as we had previously observed with human placental membrane preparations (Mitchell and O'Connor-McCourt, 1991a) and on human primary trophoblast cells (Mitchell *et al.*, 1992).

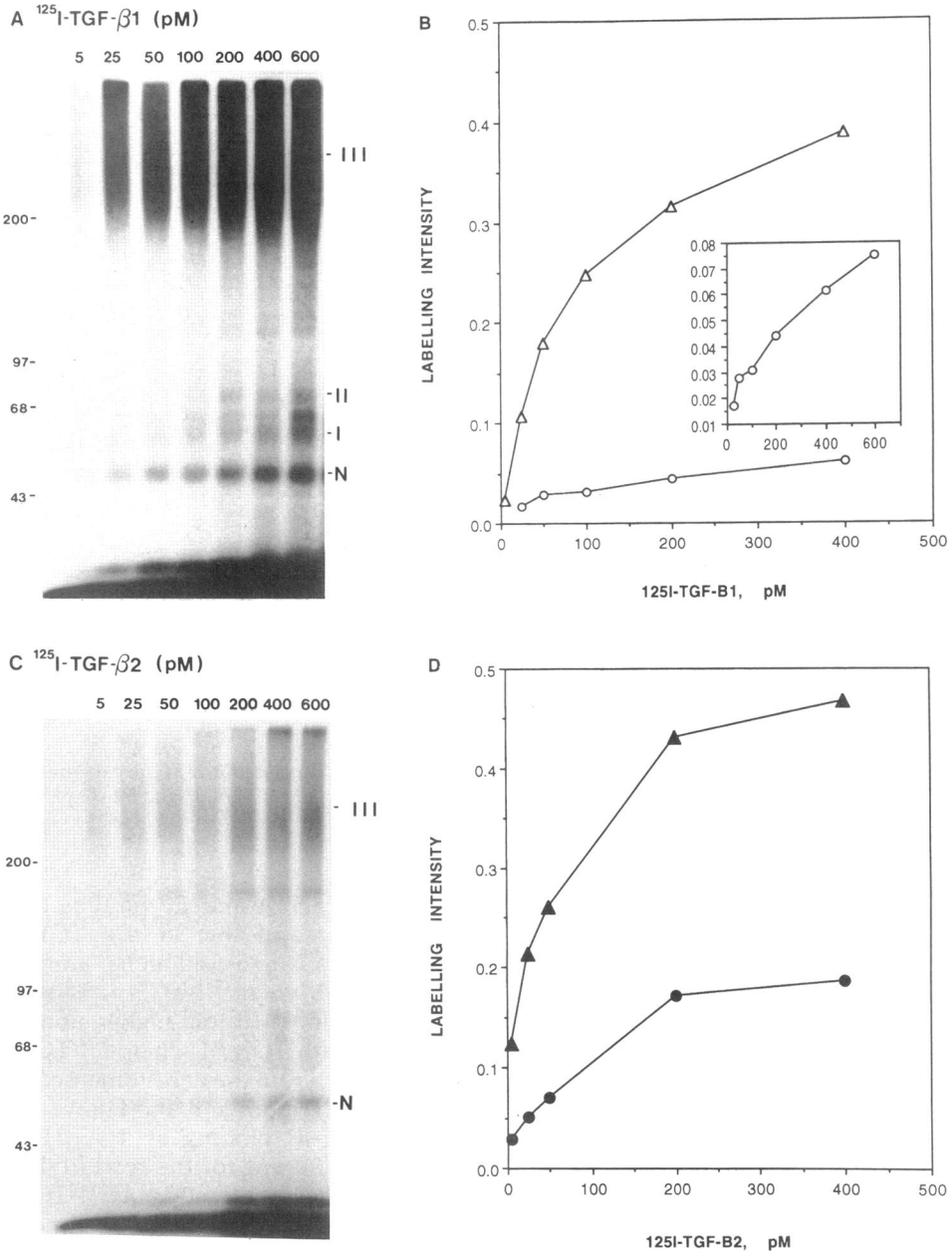

Figure 3. Affinity-labeled receptor saturation patterns for TGF-β1 and TGF-β2 on BeWo choriocarcinoma cells. Cells were affinity-labeled with increasing concentrations (pM) of ^{125}I-TGF-β1 (A and B) or ^{125}I-TGF-β2 (C and D) as indicated. Autoradiograms shown (A and C) are of Triton X-100 extracts of affinity-labeled cells that were treated with sample buffer in the presence of reducing agent and electrophoresed on 3–10% linear gradient polyacrylamide gels. The type III/betaglycan receptor and the novel 38-kDa TGF-β binding component (designated here as 'N') are the predominantly labeled bands with both ^{125}I-TGF-β1 and ^{125}I-TGF-β2. The relatively low level of the heterogeneous type I and II TGF-β receptors are more clearly seen when labeled with ^{125}I-TGF-β1 than with ^{125}I-TGF-β2 in this autoradiographic exposure time of 11 days. The specific activities of ^{125}I-TGF-β1 (recombinant) and ^{125}I-TGF-β2 (recombinant) in this experiment were 3.5 and 2.4 μCi/pmol, respectively. Molecular mass markers are indicated in kilodaltons. Saturation curves (B and D) were derived by densitometric scanning of a 1 d autoradiographic exposure of the ^{125}I-TGF-β1 experiment and the 11 day autoradiographic exposure of the ^{125}I-TGF-β2 experiment. Shown are the saturation curves for type III/betaglycan (△) and the 'N' novel 38-kDa (○) receptors with ^{125}I-TGF-β1 (○, △) and with ^{125}I-TGF-β2 (●, ▲).

At the low picomolar concentration of radiotracer used in this experiment (50 pM), there appears to be the same amount of maximal displacement seen for the type III/betaglycan component with either unlabeled TGF-β1 or TGF-β2 (Figure 5, A and C). This indicates that all of the radiotracer is bound to a type III/betaglycan subtype(s) that binds both TGF-β1 and TGF-β2 and that none of the radiotracer is bound to a subtype(s) that exclusively binds either TGF-β1 or TGF-β2. Similar observations were made when a higher concentration of TGF-β was used as the radiotracer (200 pM). These results indicate that BeWo cells did not appear to have a type III/betaglycan receptor subtype that binds TGF-β1 exclusively, which is one of the possible interpretations for its apparently different capacities for TGF-β1 and -β2 as shown on Figure 3, A and C. Another approach, which we took to test this hypothesis, was a TGF-β2 presaturation experiment. BeWo cells were preincubated for 1 h at 4°C with unlabeled TGF-β2 at a near saturating concentration of 250 pM (as determined in studies illustrated by Figure 3C). After this incubation, 50 pM ^{125}I-TGF-

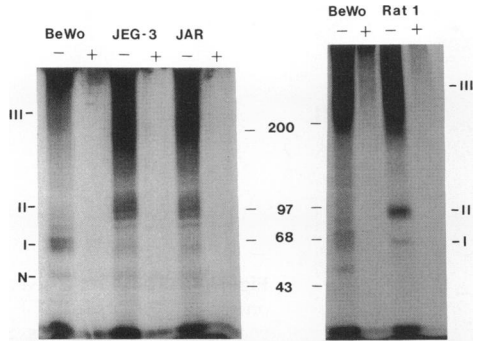

Figure 4. Affinity-labeling receptor patterns on BeWo, JEG, and JAR choriocarcinoma cell lines and on the rat 1 fibroblast cell line. Shown are autoradiograms of polyacrylamide gels of Triton X-100 extracts of cells affinity-labeled with 50 pM ^{125}I-TGF-β1 in the absence (−) or presence (+) of excess unlabeled TGF-β under identical experimental conditions. The 50-kDa affinity-labeled complex (N) is observed on the choriocarcinoma cell lines but not on the rat 1 cell line. The discrete type I and II bands are seen on the rat 1 cells in contrast to the heterogeneous type I and II bands on the choriocarcinoma cells.

β1 was added, and the experiment continued in the usual manner. The results showed that essentially no binding of the ^{125}I-TGF-β1 radiotracer to the type III/betaglycan receptor was observed. This indicates that all of the type III/betaglycan binding sites were blocked by the saturating concentration of TGF-β2 and argues against the existence of a subtype that binds TGF-β1 exclusively. In addition, the experiment presented in Figure 6 shows that type III/betaglycan core proteins of the same molecular mass (~115 kDa) appeared after heparitinase and chondroitinase treatment of Triton X-100 extracts of BeWo cells affinity-labeled with either ^{125}I-TGF-β1 or ^{125}I-TGF-β2. This result indicates that if type III/betaglycan subtypes with distinct binding properties for TGF-β1 and -β2 exist, then these subtypes have core proteins that cannot be distinguished by SDS-PAGE.

Because of the heterogeneity in molecular mass of the BeWo type I and II receptors, as well as their very low level, it was difficult to reliably quantify their binding parameters with TGF-β1 and TGF-β2, even after

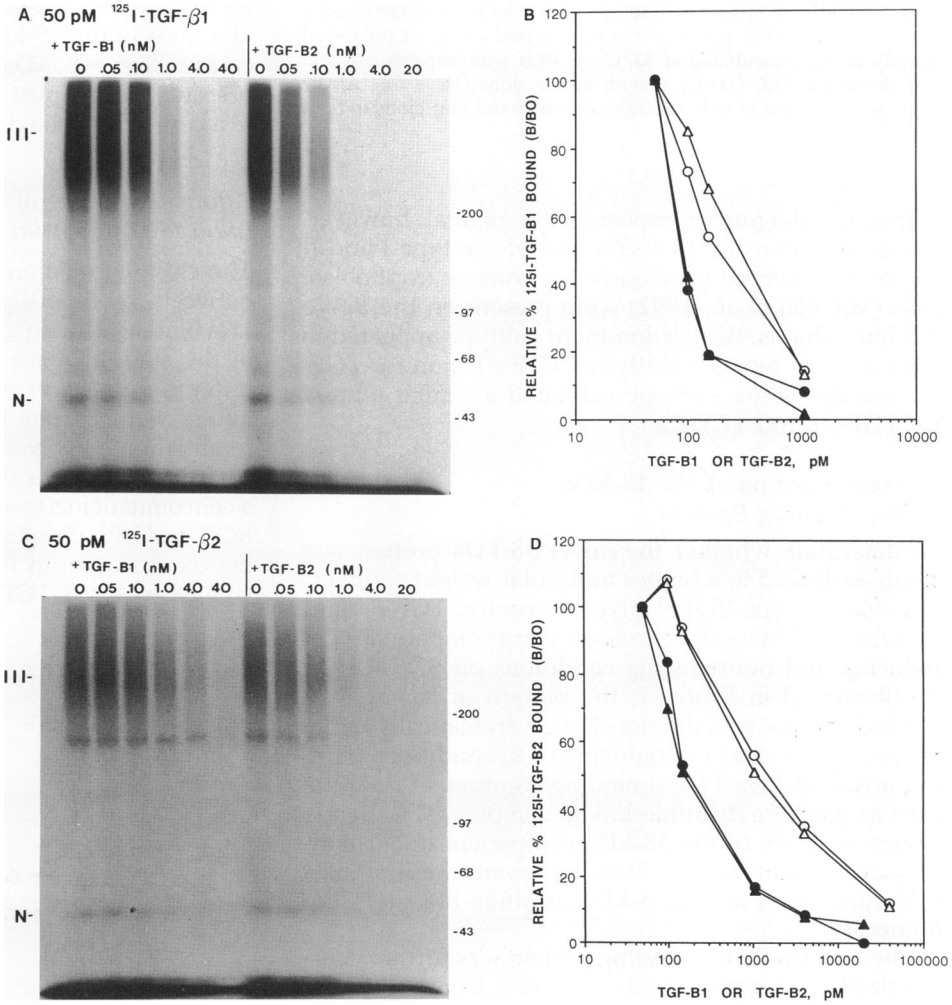

Figure 5. Affinity-labeled receptor competition patterns for TGF-β1 and TGF-β2 on BeWo cells. Cells were affinity labeled with 50 pM ^{125}I-TGF-β1 (A and B) or 50 pM ^{125}I-TGF-β2 (C and D) in the presence of the indicated concentrations (nM) of TGF-β1 or TGF-β2. Autoradiograms (A and C) of polyacrylamide gels of Triton X-100 extracts of affinity-labeled cells are shown after a 1- (A) or 4- (C) wk exposure time. Specific activities for ^{125}I-TGF-β1 and ^{125}I-TGF-β2 in this experiment were 2.4 and 3.5 μCi/pmol, respectively. Type III/betaglycan and novel 'N' 38-kDa TGF-β receptors, and molecular mass markers in kilodaltons are indicated. Competition curves (B and D) were derived by densitometric scanning of the autoradiographic exposures shown in A and C and are indicated as (△) for the type III/betaglycan receptor and (○) for the 38-kDa receptor. Competition with TGF-β1 (○, △) or TGF-β2 (●, ▲) is shown. Note that the x-axis in D is 10-fold larger than in B.

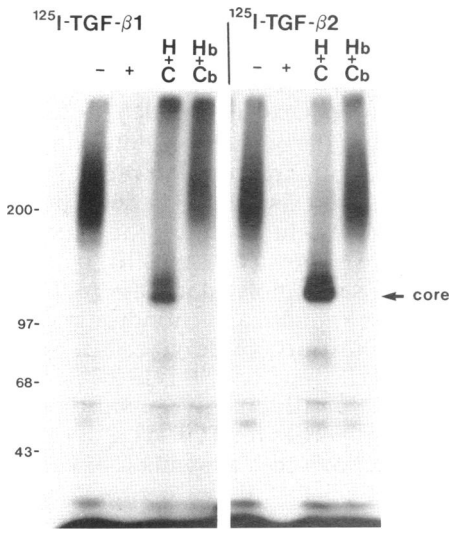

Figure 6. Enzymatic deglycosylation by Heparitinase and Chondroitinase ABC of the BeWo type III/betaglycan receptor affinity labeled with ^{125}I-TGF-β1 or ^{125}I-TGF-β2. BeWo cells were affinity labeled with 100 pM ^{125}I-TGF-β1 or ^{125}I-TGF-β2. Triton X-100 extracts were either immediately treated with sample buffer for SDS-PAGE (control lanes, absence (−) or presence (+) of 100-fold excess of unlabeled ligand); or were incubated at 37°C for 18 h with heparitinase and chondroitinase ABC (H+C) or with buffer alone (H_b + C_b). Arrow indicates the major type III/betaglycan core band that binds to both TGF-β isoforms.

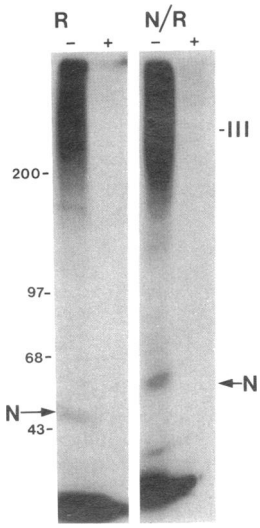

Figure 7. The effect of reducing agent on the affinity-labeled TGF-β binding components on BeWo cells. Shown are the BeWo cell binding components that were affinity labeled with 100 pM ^{125}I-TGF-β2 and electrophoresed under reducing (R) vs. (N/R) conditions. The band migrating at ~25 kDa on the nonreducing gel represents ^{125}I-TGF-β2 dimers that are bound but not cross-linked.

long autoradiographic exposures. In general, however, it appeared that the two subtypes of the type I and II receptors observed previously on primary trophoblast cells (Mitchell *et al.*, 1992) were present on the BeWo cell line. That is, the predominant subtype appeared to have a much higher affinity for TGF-β1 than for TGF-β2 and the minor subtype exhibited a similar affinity for TGF-β1 and TGF-β2.

Characterization of the 38-kDa TGF-β Binding Protein

To determine whether the novel 38-kDa protein was disulfide-linked to a higher molecular weight complex, possibly the type III/betaglycan receptor, TGF-β affinity-labeled BeWo cell complexes were compared under reducing and nonreducing conditions on SDS-PAGE. As illustrated in Figure 7, the pattern of ^{125}I-TGF-β2 labeled components did not change dramatically under reducing (R) versus nonreducing (NR) conditions. Thus the novel 38-kDa TGF-β binding component does not exist as part of a disulfide-linked complex. The slightly slower mobility of the 38-kDa component under nonreducing conditions most likely represents the additional molecular mass of a 12.5-kDa disulfide-linked TGF-β monomer.

The 38-kDa TGF-β binding protein was further characterized by enzymatic deglycosylation. BeWo cells were affinity labeled with ^{125}I-TGF-β2, and the Triton-soluble extract was incubated with endoglycosidase F and analyzed on SDS-PAGE (Figure 8). The apparent molecular mass of the 38-kDa TGF-β binding component decreased by ~8 kDa after digestion, indicating the presence of complex N-linked carbohydrates attached to a core protein having a molecular mass of ~30 kDa.

Affinity-Labeling of BeWo Cells after Methotrexate-Induced Differentiation

The close correlation in affinities for TGF-β1 and -β2 between the type III/betaglycan receptor and the 38-kDa binding protein led us to examine whether a change in the state of these cells might be correlated with a difference in the expression of either of these components. Methotrexate has been shown to induce a differentiation response in BeWo cells from a cytotrophoblast-like state to a syncitiotrophoblast-like state with a concomitant increase in hormone production including hCG and hPL (Friedman and Skehan, 1979). In the experiment shown in Figure 9, BeWo cells were affinity labeled with ^{125}I-TGF-β2 on day 0 and on day 4 after

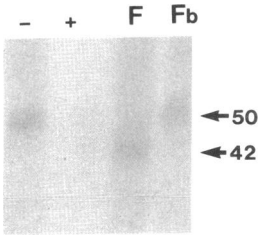

Figure 8. Enzymatic deglycosylation of the 38-kDa TGF-β binding component by endoglycosidase F. BeWo cells were affinity labeled with 100 pM ^{125}I-TGF-β2, and the Triton X-100 soluble extracts were treated directly with sample buffer (control lanes, absence (−) and presence (+) of 100-fold excess of TGF-β2) or were incubated at 37°C for 18 h with (F) or without (Fb) endoglycosidase F. The apparent molecular mass of the 38-kDa component was reduced by ~8 kDa as determined on this 10% polyacrylamide gel.

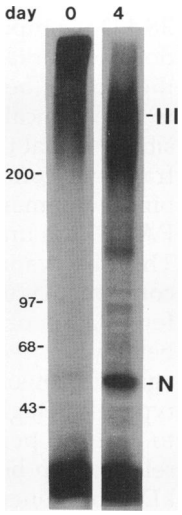

Figure 9. Affinity labeling of BeWo cells after methotrexate induced differentiation. Shown are autoradiographs of BeWo cell TGF-β receptors affinity labeled with 200 pM ^{125}I-TGF-β2 before treatment (day 0; ~60% confluency), or 96 h after treatment (day 4) with 1 μM methotrexate.

treatment with 1 μM methotrexate. At this time the cells have stopped growing and the process of differentiation into the syncitiotrophoblast-like state appears complete. This experiment indicated that differentiation was accompanied by a consistent increase (~10-fold as determined by densitometry) in the level of the 38-kDa component with relatively little change in the level of the type III/betaglycan component. In some experiments, as in Figure 9, there was a difference in the appearance of the type III/betaglycan band in that it migrated faster after differentiation. This is probably due to changes in carbohydrate modification. There also appeared to be a slight increase in the level of the type I and II receptor bands as well as the appearance of another band of ~120 kDa that may be the type III/betaglycan core.

DISCUSSION

Identification and Characterization of TGF-β Receptors on BeWo Cells

Our earlier affinity-labeling studies suggested that subtypes of all three types of TGF-β receptors (I, II, and III/betaglycan) existed on human trophoblast cells in primary culture (Mitchell *et al.*, 1992). Unfortunately, it was difficult to perform more quantitative studies due to inherent variations in primary cell culture including cell density and differentiation. In the present study, both affinity-labeling and equilibrium binding assays were used to characterize TGF-β1 and TGF-β2 binding to an established trophoblastic cell line derived from a human choriocarcinoma (BeWo). The BeWo cell line is a more uniform model than primary trophoblast cell culture for quantitative receptor studies. Scatchard analyses indicated that the average affinity and total number of binding sites on BeWo cells for TGF-β1 and TGF-β2 are similar and that these cells are among the richest in terms of numbers of TGF-β binding sites. Competitive equilibrium binding experiments indicated that TGF-β1 and TGF-β2 are equipotent and that all the BeWo binding sites are capable of recognizing both isoforms. The affinity-labeling studies demonstrated the presence of a predominant type III/betaglycan receptor that exhibits an apparently higher capacity for TGF-β1 than for -β2 yet a higher affinity for TGF-β2 than for TGF-β1. A low number of type I and II receptors were detected that appeared very heterogeneous in nature. Finally, we have also identified a novel 38-kDa TGF-β binding glycoprotein, not previously described, that shows a similar affinity profile as the type III/betaglycan component and that appears to be upregulated upon methotrexate-induced differentiation of BeWo cells.

We first observed the apparently higher capacity of the trophoblastic type III/betaglycan receptor for TGF-β1 as compared with TGF-β2 on trophoblast cells in primary culture (Mitchell *et al.*, 1992). This same effect is retained in the BeWo trophoblastic cell line. Three possible explanations for this observation include 1) there are two separate type III/betaglycan subtypes, one of which binds TGF-β1 exclusively; 2) the trophoblast type III/betaglycan receptor exhibits positive cooperative effects with TGF-β1 but not with TGF-β2; and 3) the cross-linking reagent causes disproportional labeling of the trophoblast type III/betaglycan receptor with ^{125}I-TGF-β1 as compared with ^{125}I-TGF-β2. In the present study, the TGF-β2 presaturation study essentially ruled out the existence of an exclusively TGF-β1-binding type III/betaglycan subtype on BeWo cells. Moreover, the equilibrium competition studies indicated that there were no distinct sites that exclusively recognized either TGF-β1 or -β2. Furthermore, the equilibrium saturation experiments do not support the concept of differential cooperative effects for TGF-β1 versus -β2, because there was no evidence of nonlinear Scatchard plots or a higher capacity for TGF-β1 binding. With respect to cross-linking effects, we have previously demonstrated that the apparently higher capacity of the primary trophoblast type III/betaglycan receptor for TGF-β1 than TGF-β2 was not due to an inherent difference in the cross-linking efficiency to ^{125}I-TGF-β1 versus ^{125}I-TGF-β2 (i.e., the type III/betaglycan receptor on control placental mesenchymal cell cultures was labeled to the same extent by ^{125}I-TGF-β1 and ^{125}I-TGF-β2 in affinity labeling saturation studies [Mitchell *et al.*, 1992]). We cannot, however, rule out the possibility that, in the context of the trophoblast cell, the presence of cross-linking reagent in the affinity-labeling experiment disproportionally affects the amount of ^{125}I-TGF-β1 as compared with ^{125}I-TGF-β2 that becomes covalently cross linked to the type III/betaglycan receptor at equilibrium. The main point is that the type III/betaglycan receptor on both BeWo and primary trophoblast cells behaves differently

with respect to both apparent capacity and preferential affinity than the type III/betaglycan from most other sources. Although we cannot rule out the presence of multiple core proteins that comigrate on SDS-PAGE, our detection of a single core band for the trophoblast type III/betaglycan receptor together with the identification of a single mRNA species in several rat tissues (Lopez-Casillas *et al.*, 1991; Wang *et al.*, 1991) suggests that the unique binding properties of the trophoblast type III/betaglycan receptor may be inherent in a single core protein. Differences in affinity-labeling binding characteristics of the type III/betaglycan in different cell types may be due to the presence of undetected post-translational modifications or interacting modifier protein subunits. Eventually, studies using a recombinantly expressed type III/betaglycan receptor should clarify these issues. In addition, BeWo cells would be an interesting model in which to test chimeric TGF-β1-β2 molecules (Qian *et al.*, 1992) by affinity labeling.

The type I and II TGF-β receptors on BeWo cells are very heterogeneous in appearance and subtypes similar to those observed on primary trophoblast cells (Mitchell *et al.*, 1992) and some other cells types (Cheifetz *et al.*, 1990, 1991) were detected i.e., a predominant subtype with a higher affinity for TGF-β1 and a second minor subtype with an equal/slightly higher affinity for TGF-β2. Preliminary deglycosylation experiments with larger numbers of affinity-labeled cells to compensate for the low level of type I and II receptors suggest that the heterogeneity is not due to different sizes of carbohydrate modifications (Mitchell, unpublished results). It has been recently shown that the activin type II receptor family includes a heterogeneous group of four alternatively spliced isoforms that differ in ^{125}I-activin A binding affinity (Attisano *et al.*, 1992). We are currently testing whether the heterogeneity of the type II TGF-β receptor on BeWo cells is due to alternative splicing.

The major difference between the BeWo TGF-β receptor profile (as well as that of two other human choriocarcinoma cell lines, JEG-3 and JAR) and that of the primary trophoblast cells was the presence of the novel 38-kDa TGF-β binding glycoprotein. Our observation that the 38-kDa TGF-β binding component and the type III/betaglycan receptor showed a strikingly similar higher affinity for TGF-β2 as compared with -β1, leads us to speculate that the 38-kDa glycoprotein may be 1) an independent binding protein that happens to have a similar affinity profile to that of the type III/betaglycan receptor, 2) an accessory protein that interacts with the type III/betaglycan receptor, or 3) a discrete proteolytic fragment of the type III/betaglycan receptor. The first possibility is straightforward and would add to the list of novel TGF-β binding proteins recently detected and reviewed by Massagué (1992). The second possibility is similar to that proposed recently by Segarini *et al.* (1992) for the type VI binding component. That is, the 38-kDa component may be an accessory protein that does not contain a binding site per se but interacts with the type III/betaglycan receptor such that it is accessible to be chemically cross-linked to TGF-β. The third possibility is that the 38-kDa protein is a discrete proteolytic fragment containing the type III/betaglycan receptor binding domain, which becomes separated upon SDS-PAGE both under reducing or nonreducing conditions. The observation that the expression of this 38-kDa component is upregulated on methotrexate-induced differentiation of these cells, whereas that of the type III/betaglycan receptor is relatively unaffected, argues against proteolysis, because a concomitant decrease in type III/betaglycan would be expected. We attempted to perform peptide mapping studies to investigate the relationship between the type III/betaglycan and 38-kDa components, however owing to the small size of this glycoprotein, the major affinity-labeled fragments comigrated with fragments of the bound TGF-β radioligand itself (Mitchell, unpublished observations). The availability of anti-type III/betaglycan antibodies, anticipated soon after the recent cloning of this receptor (Lopez-Casillas *et al.*, 1991; Wang *et al.*, 1991), should help to determine the relationship (if any) between the 38-kDa and type III/betaglycan binding components on BeWo cells.

In a recent report, there was mention of a 50-kDa affinity-labeled complex on A549 human lung carcinoma cells, although it was largely insoluble in detergent (MacKay and Danielpour, 1991). In addition, of the four TGF-β binding components purified recently from porcine uterus (Ichijo *et al.*, 1991), the 40-kDa component could be related to our 38-kDa TGF-β binding glycoprotein. However, immunoblotting experiments with antibodies against this protein (kindly supplied by Dr. C.-H. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden) did not detect the BeWo 38-kDa component (Mitchell, unpublished observations). Lack of cross-reactivity between species cannot be ruled out in this case. Similarly, anti-decorin antibodies (kindly supplied by Dr. R. Spiro, Telios Pharmaceuticals, San Diego, CA) did not recognize our 38-kDa protein, in either immunoprecipitation or immunoblotting experiments (Mitchell, unpublished results), indicating that it was unrelated to decorin, which is a TGF-β binding proteoglycan that has a core protein of 40 kDa (Yamaguchi *et al.*, 1990). Thus further studies are needed to investigate the nature of this 38-kDa component.

Biological Significance of TGF-β Receptors on BeWo Cells

The activities of TGF-β in extracellular matrix remodeling, growth inhibition, and immunosuppression have led various investigators to examine the roles of TGF-β in trophoblast invasiveness (Lala and Graham, 1990;

Tamada *et al.*, 1990; Graham and Lala, 1991, 1992a,b), trophoblast differentiation (Morrish *et al.*, 1991; Graham *et al.*, 1992a), and in the maintenance of pregnancy (Altman *et al.*, 1990; Clark *et al.*, 1990, 1991; Lea *et al.*, 1992). A greater understanding of the control of trophoblast invasiveness is important for the treatment of certain conditions of pregnancy, such as the overinvasiveness of choriocarcinoma or the underinvasiveness of preeclampsia, as well as for cancer therapy in general. Metalloproteinases, which are locally activated by plasmin, a serine protease, appear to be the final mediators of human trophoblastic invasiveness (Yagel *et al.*, 1988; Fisher *et al.*, 1989; Graham and Lala, 1992b). In vitro, TGF-β1 appears to control human trophoblast invasiveness directly by regulating the expression of the mRNA for TIMP-1 (tissue inhibitor of metalloproteinase) (Graham and Lala, 1991) and indirectly by stimulating differentiation to syncitiotrophoblasts (Graham *et al.*, 1992a). However, in another study, TGF-β1 was found to be inhibitory to trophoblast differentiation and hCG and hPL secretion (Morrish *et al.*, 1991). Further studies are needed to differentiate the potencies of TGF-β1 and -β2 in controlling invasiveness and differentiation. TGF-β, TGF-β2 in particular, also appears to have an immunoregulatory role in mice, which is important for the maintenance of pregnancy. It has been shown that neutralizing antibodies against TGF-β2 appear to increase the spontaneous rate of abortion (Clark *et al.*, 1991). Furthermore, a TGF-β2-like immunosuppressive factor in murine amniotic fluid, which appears to be complexed with α-fetoprotein, has been identified (Altman *et al.*, 1990). Currently, there is some controversy over the levels and location of TGF-β2 mRNA in murine placenta and decidua (Miller *et al.*, 1989; Pelton *et al.*, 1990; Altman *et al.*, 1990; Schmid *et al.*, 1991; Lea *et al.*, 1992). However, together these studies illustrate that there are multiple autocrine and paracrine roles for TGF-β1 and TGF-β2 in placental function that are likely regulated both spatially and temporally during pregnancy. Recently a method for culturing BeWo cells as polarized monolayers has been reported (Cerneus and van der Ende, 1991). This model may permit a comparison of the apical and basolateral receptor patterns to help distinguish paracrine effects mediated through the decidua, on the one hand, or the mesenchymal layer underlying the villous trophoblast, on the other.

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