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Transforming Growth Factor-β Stimulates Trophoblast Oncofetal Fibronectin Synthesis *in Vitro*: Implications for Trophoblast Implantation *in Vivo**

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ABSTRACT

In pregnancy tissues, oncofetal fibronectin (onfFN) has been localized specifically to the extracellular matrix (ECM) surrounding extravillous anchoring trophoblasts of the placental-uterine junction and chorion. When isolated from first or third trimester placentas, human cytotrophoblasts in culture secrete and deposit onfFN in the ECM. In addition, onfFN synthesis is significantly up-regulated in response to serum stimulatory factor(s). The goal of this study was to examine the role of transforming growth factor-β (TGFβ), a cytokine present in uterine decidua, as a stimulator of trophoblast onfFN production. Our initial insight into the significance of TGFβ resulted from the serendipitous use of cord serum from a neonate with severe alloimmune thrombocytopenia. Trophoblasts cultured in medium containing this serum underwent normal morphological differentiation, but produced markedly less onfFN. In an analogous fashion, trophoblasts cultured in normal serum preincubated with anti-TGFβ neutralizing antibodies also produced significantly less onfFN. Exogenously added TGFβ1 restored the ability of trophoblasts to produce onfFN by a factor of 4- to 5-fold in medium containing thrombocytopenic serum. In platelet-poor serum derived from human or bovine plasma, TGFβ1 also induced onfFN synthesis, as assayed both in the conditioned medium and by immunocytochemical localization of onfFN in cell-associated ECM fibrils. Dose-response analysis demonstrated that the onfFN stimulatory response is sensitive to TGFβ, with an ED_{50} of 0.1–0.2 ng/ml. In a reciprocal fashion, TGFβ inhibited βhCG secretion 3- to 4-fold. Our results demonstrate that TGFβ is a significant stimulator of trophoblast onfFN production. Furthermore, TGFβ appears to modulate trophoblast differentiation by up-regulating the expression of an anchoring trophoblast marker (onfFN) and down-regulating a phenotypic marker of villous syncytiotrophoblast (hCGβ). We speculate that trophoblast responsiveness to TGFβ in the implantation milieu contributes to trophoblast adhesion by stimulating the production of a trophoblast-derived implantation site fibronectin. (*J Clin Endocrinol Metab* **78:** 1241–1248, 1994)

ONCOFETAL fibronectin (onfFN) is closely associated with extravillous trophoblasts in the placental-uterine junction throughout pregnancy (1–3). In normal human implantation sites, onfFN is localized to a highly specific region, the extracellular matrix (ECM), connecting extravillous trophoblasts and trophoblastic cell columns to the uterine decidua. In extrauterine pregnancies, onfFN is absent from the uterus, but it is found at ectopic trophoblastic implantation sites, further suggesting that the protein is trophoblast derived. Trophoblast-associated onfFN has been identified as early as 20 days postconception, and at this gestation it is specifically localized to the early cytotrophoblastic shell. Based on these immunolocalization studies, it has been hypothesized that onfFN functions in the placental-uterine junction as a tropho-uteronectin or trophoblast-uterine connecting protein (1–3). Similar, if not identical, oncofetal fibronectins are also found within the ECM of the chorion-decidual junction (2, 4), and these chorion-derived forms may be the source of cervico-vaginal "fetal" fibronectin detected clinically in patients at risk for preterm labor and delivery (4, 5). The novel feature of these fibronectins, as originally described by Matsuura and Hakomori (6–8), is the presence of a specific *O*-glycosylated hexapeptide epitope within the type III connecting segment (IIICS) region, reactive with monoclonal antibody FDC-6.

Human cytotrophoblasts isolated from first trimester or term placentas synthesize and secrete abundant quantities of FDC-6-reactive fibronectin, and we have found that this fibronectin is specifically deposited at sites of trophoblast-ECM attachment *in vitro* (1, 2). As such, we have hypothesized that trophoblasts *in vitro* are recapitulating an important *in vivo* trophoblast function by producing and depositing an endogenous fibronectin within newly formed ECM. More recently, we have found that onfFN production *in vitro* is regulated by a specific stimulatory factor(s) in serum. Trophoblasts cultured in medium containing increasing percentages of serum produce increasing quantities of onfFN (2). Interestingly, human newborn serum derived from cord blood is 5–10 times more stimulatory for onfFN production than equivalent amounts of fetal calf serum (2).

We have further hypothesized that identification of an onfFN stimulatory factor(s) in serum might give us insight into potential uterine-derived promoters of trophoblast implantation. While working with numerous cord sera, we

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observed that trophoblasts cultured in one serum derived from a neonate with severe alloimmune thrombocytopenia exhibited normal morphology, yet produced significantly less onfFN. As transforming growth factor-β (TGFβ), a potent growth factor released into serum by activated platelets, has also been localized to the placental-uterine junction (9), we initiated studies to determine whether TGFβ affects trophoblast synthesis of onfFN. Using cultured cytotrophoblasts, we assayed the production of two phenotypic markers of trophoblast differentiation, onfFN and hCGβ, and found a reciprocal response to TGFβ.

Materials and Methods

Cytotrophoblast preparation and culture

Human cytotrophoblasts were purified from the placentas of uncomplicated term pregnancies immediately after delivery by serial trypsin-DNase digestions followed by Percoll gradient centrifugation, as previously described by Kliman *et al.* (10). Yields of viable trophoblast cells ranged from 60–100 × 10^6 cells/30 g starting placental tissue. The purified cytotrophoblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 25 mmol/L glucose and 25 mmol/L HEPES supplemented with 4 mmol/L glutamine and 50 μg/mL gentamicin. The DMEM contained different sera, depending on the experimental conditions. Trophoblasts were typically cultured for 48 or 72 h, and the media were collected at these time points for immunoblot and/or enzyme-linked immunoassay (ELISA) analyses.

Human cord blood samples (80–100 mL) were collected from the umbilical vein immediately after normal vaginal deliveries of healthy neonates, but before delivery of the placenta. For normal serum collection, the blood was transferred to red-top Vacutainer tubes (Becton Dickinson, Rutherford, NJ) containing no additives and allowed to clot. The clotted blood was centrifuged for 20 min in a clinical centrifuge, and the resultant serum supernatant was collected and aliquoted. Human and bovine plasma were either purchased commercially (Sigma Chemical Co., St. Louis, MO) or prepared from whole blood by collection in blue-top Vacutainer tubes containing buffered sodium citrate as an anticoagulant. These tubes were centrifuged to remove all cells and platelets, and the resultant plasma supernatant was collected. Platelet-poor serum was then prepared from plasma by generating a clot with 0.1 mL thrombin (Thromboquik, Organon Teknika, Durham, NC)/1.0 mL plasma, which was removed from the serum.

TGFβ immunoneutralization and stimulation

For immunoneutralization of endogenous TGFβ activity in DMEM-containing normal serum, turkey (Collaborative-Becton Dickinson, Bedford, MA) or chicken (R & D Systems, Minneapolis, MN) antihuman TGFβ panneutralizing antibodies were employed. The neutralization results we obtained were identical for each antibody. DMEM containing either 2% normal newborn human serum or 5% fetal calf serum were incubated at antibody concentrations of 10–100 μg/mL for 6 h before and throughout the duration of the cell culture.

To examine the effects of exogenous TGFβ on onfFN production, purified human TGFβ1 was obtained from two different commercial sources (Collaborative-Becton Dickinson and R & D Systems). As recommended by the manufacturers, the lyophilized samples were reconstituted with sterile 4 mmol/L hydrogen chloride containing 1 mg/mL BSA to a final concentration of 1 ng/mL TGFβ1. Appropriate dilutions were made from frozen aliquots of TGFβ1 stock solutions, as needed for stimulation experiments.

Quantitative onfFN immunoassays

For Western immunoblots, trophoblast-conditioned medium samples were electrophoresed in 6% sodium dodecyl sulfate-polyacrylamide gels under reducing conditions. Known standards of 50–1000 ng amniotic fluid fibronectin, previously purified by gelatin-Sepharose 4B chromatography (Pharmacia, Piscataway, NJ) (11), were electrophoresed in parallel. The gels were then electrotransferred to nitrocellulose (Schleicher and Schuell, Keene, NH) overnight. To detect onfFN, the nitrocellulose blots were incubated with a 1:50 dilution of hybridoma supernatant containing murine monoclonal antibody FDC-6 (American Type Culture Collection, Rockville, MD). Immunodetection of FDC-6 was performed with a biotinylated antimouse secondary antibody, avidin, and a biotinylated horseradish peroxidase, according to the manufacturer's instructions (ABC Vectastain, Vector Laboratories, Burlingame, CA). The chromagen reaction was carried out with 3,3′-diaminobenzidine and hydrogen peroxide (Sigma). For more exact quantitation of medium samples, an ELISA was used. Known purified standards (0–400 ng) of amniotic fluid fibronectin were plated on 96-well plates (Corning, Corning, NY) in parallel with medium samples diluted 1:20 to 1:100. Immunodetection was identical to that described for the Western blots (ABC Vectastain), except that *o*-phenylenediamine was used as the soluble chromagen. Samples were read at 492 nm. A detailed standard curve for each ELISA, based on quadruplicate samples, was created with CricketGraph 1.3.2 for the MacIntosh (Computer Associates, Garden City, NY), with r^2 values typically greater than 0.98.

Trophoblast immunocytochemistry

Cytotrophoblasts were cultured on sterile glass coverslips (Corning) for 48 or 72 h in defined DMEM containing 2% platelet-poor human or bovine serum, with or without 2.0 ng/mL TGFβ1. For fixation, coverslips were washed with phosphate-buffered saline, fixed for 15 min with formalin, and stored in 0.1% sodium azide until use. For immunostaining, FDC-6 supernatant was diluted 1:50 and used as the primary antibody. Immunodetection was performed as described above for immunoblots with ABC Vectastain. Coverslips were counterstained with hematoxylin. Undiluted P3X63Ag8 mouse myeloma cell line supernatant (American Type Culture Collection) was used as a control for negative staining. The specificity of the FDC-6 antibody has previously been demonstrated by immunoabsorption (1, 4) and the inability of the antibody to bind to *O*-deglycosylated oncofetal fibronectin (7, 8).

Results

Trophoblasts cultured in thrombocytopenic serum produce significantly less onfFN than trophoblasts cultured in normal serum

We reported previously that isolated human cytotrophoblasts demonstrate normal morphological differentiation (*i.e.* attachment and spreading, formation of aggregates, and syncytia) when cultured in medium containing as little as 1% human cord blood serum (2). Although the cord sera we collected postdelivery were generally derived from normal pregnancies, we obtained one cord serum sample from a neonate who was later found to have an extremely low platelet count of 11,000/mm^3 (normal range, 150–400 × 10^3/mm^3). This neonate was subsequently diagnosed with severe alloimmune thrombocytopenia and did well with platelet transfusions. As with other cord sera we had collected, this particular sample was unremarkable in its clotting and serum separation characteristics.

When trophoblasts were cultured in medium containing the thrombocytopenic cord serum, they appeared indistinguishable morphologically from cells cultured with normal cord sera. Figure 1 demonstrates the results of an immunoblot assay comparing the quantity of onfFN secreted into the media when trophoblasts from the same placental isolation prep were cultured in medium containing 10% cord serum from a normal neonate thrombocytopenic cord serum (lanes

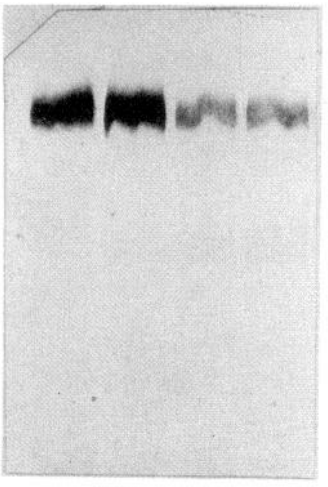

FIG. 1. Normal *vs.* thrombocytopenic serum as a stimulator of onfFN synthesis. Immunoblot comparison of secreted onfFN in parallel trophoblast cultures containing 10% cord serum from a normal neonate (lanes a and b) *vs.* 10% cord serum from a thrombocytopenic neonate (lanes c and d) after 48 h (lanes a and c) and 72 h (lanes b and d). Unconcentrated conditioned medium (50 μL) was loaded in each lane.

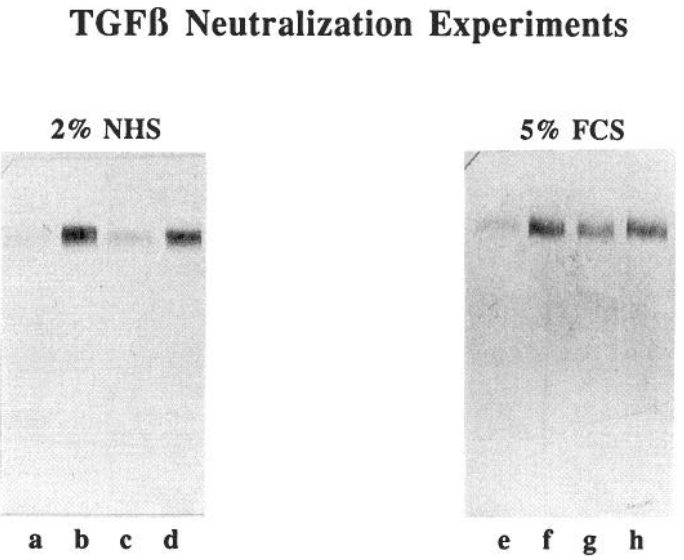

FIG. 2. Effect of TGFβ neutralization on onfFN production. In two different TGFβ neutralization experiments, 100 μg/mL pan-neutralizing anti-TGFβ antibody (lanes a and e) were effective in reducing onfFN secretion to almost undetectable levels compared to control values (lanes b and f) in medium containing either 2% normal newborn human serum or 5% fetal calf serum. Using the same TGFβ neutralizing antibody at 25 μg/mL (lanes c and g) resulted in partial inhibition of onfFN production compared to control values (lanes d and h; ~75% inhibition of the 2% newborn human serum and 50% inhibition of the 5% fetal calf serum). Antibody inhibition of endogenous TGFβ activity, whether serum or trophoblast derived, abrogates the onfFN stimulatory response. NHS, Newborn human serum; FCS, fetal calf serum.

a and b) *vs.* 10% thrombocytopenic cord serum from a normal neonate (lanes c and d). After culture times of 48 h (lanes a and c) and 72 h (lanes b and d), onfFN levels in the medium were 4- to 5-fold lower in the trophoblast cultures containing the thrombocytopenic serum. We further confirmed this observation by comparing the onfFN-stimulating capacity of the thrombocytopenic serum with other normal cord sera (not shown). Depending on the serum sample used, medium containing normal cord sera induced 4- to 10-fold higher levels of onfFN secretion than the thrombocytopenic serum, based on quantitative onfFN immunoblot and ELISA assays. These findings suggested that a platelet-derived soluble serum factor, such as TGFβ, might be required for trophoblast production of onfFN *in vitro*.

Neutralization of endogenous TGFβ in medium containing normal serum

Although a lack of various platelet-derived factors could have been responsible for the diminished onfFN production observed in the thrombocytopenic serum, we focused specifically on TGFβ for the following reasons: 1) TGFβ upregulates fibronectin production in other cell systems (12–15); 2) TGFβ has been reported to modulate trophoblast differentiation *in vitro* (9, 16, 17); and 3) TGFβ has recently been immunolocalized *in vivo* to the placental-uterine junction in human pregnancies (9). To assess the significance of TGFβ in stimulating trophoblast onfFN production, we used a strategy of incubating trophoblast medium before and during the culture with anti-TGFβ neutralizing antibodies. As an assay for onfFN production by the cultured trophoblasts, conditioned media were analyzed by immunoblot. Figure 2 demonstrates the results of two different TGFβ neutralization experiments, in which 100 μg/mL added antibody (lanes a and e) were effective in reducing onfFN secretion to almost undetectable levels compared to control values (lanes b and f) in medium containing either 2% normal newborn human serum or 5% fetal calf serum. Using the same TGFβ neutralizing antibody at 25 μg/mL (lanes c and g) resulted in partial inhibition of onfFN production compared to control values (lanes d and h; ~75% inhibition of the 2% newborn human serum and 50% inhibition of the 5% fetal calf serum). These results provide good evidence that antibody inhibition of endogenous TGFβ, whether serum or trophoblast derived, abrogates the onfFN stimulatory response.

Exogenously added TGFβ1 stimulates onfFN synthesis

To further determine the importance of TGFβ as an inducer of trophoblast onfFN synthesis, we used two additional experimental approaches: 1) TGFβ1 stimulation of trophoblasts cultured in medium containing serum from the thrombocytopenic neonate; and 2) TGFβ1 stimulation of trophoblasts cultured in plasma-derived (*i.e.* platelet-poor) serum. Figure 3 demonstrates the effect of exogenously added TGFβ1 on onfFN production when trophoblasts were cultured for 48 h in 2% thrombocytopenic serum. TGFβ1 concentrations of 0.5–5.0 ng/mL induced onfFN production approximately 5-fold to the onfFN levels typically found when trophoblasts were cultured with normal sera. Thus, TGFβ1 appears to be the major onfFN stimulatory factor lacking in the thrombocytopenic serum. As shown in Fig. 4, concentrations of TGFβ1 ranging from 0.625–5 ng/mL in-

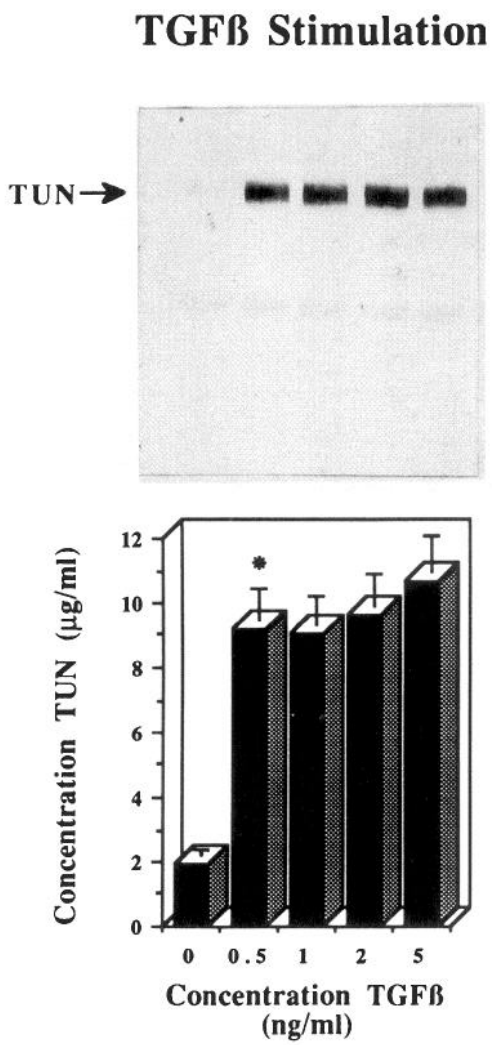

FIG. 3. TGFβ1 stimulates onfFN production by trophoblasts cultured in thrombocytopenic serum. Exogenously added TGFβ1 concentrations of 0.5–5.0 ng/mL induced onfFN production approximately 5-fold, to levels of onfFN typically found when trophoblasts are cultured with normal serum. onfFN concentrations in the medium were determined in quadruplicate by quantitative ELISA and are expressed on the bar graph as the mean ± SD. *, $P < 0.001$, based on Student's paired t test for each TGFβ concentration (0.5–2.0 ng/mL), compared to the unstimulated cells. TUN, Tropho-uteronectin.

duced a 3- to 4-fold induction of onfFN when trophoblasts were cultured in plasma-derived serum from a normal neonate. Preparing serum in this manner prevents platelet activation and platelet incorporation into the clot, thus reducing the amount of platelet-derived factors released into the serum. TGFβ1 stimulation of trophoblasts cultured in two additional human and two bovine plasma-derived sera obtained commercially resulted in a similar 3- to 5-fold onfFN induction after 48 and 72 h of culture (not shown).

TGFβ1 also has a pronounced effect on enhancing the deposition of onfFN extracellularly. As shown in Fig. 5, trophoblasts cultured for 48 h in 2% bovine platelet-poor serum exhibited occasional immunocytochemical staining for onfFN in the absence of exogenously added TGFβ1 (Fig. 5A). However, in parallel cultures stimulated with 2 ng/mL

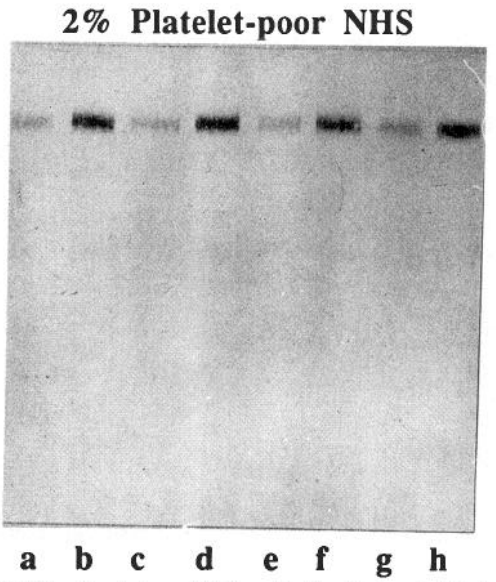

FIG. 4. TGFβ1 stimulates onfFN production by trophoblasts cultured in normal plasma-derived platelet-poor serum. Concentrations of TGFβ1 ranging from 0.625–5 ng/mL (lanes b, d, f, and h) induced a 3- to 4-fold induction of onfFN compared to controls (lanes a, c, e, and g) when trophoblasts were cultured in 2% plasma-derived serum from a normal neonate. Preparing serum in this manner prevents platelet activation and platelet incorporation into the clot, thus reducing the amount of platelet-derived factors released into the serum. NHS, Newborn human serum.

TGFβ1 (Fig. 5B), prominent fibrillar staining typical of fibronectin extracellular deposition (18) was observed. Figure 5C demonstrates the immunocytochemical appearance of onfFN fibrils associated with the periphery of a large syncytial cell in the presence of TGFβ1.

Dose response of TGFβ1-mediated onfFN stimulation

Because trophoblasts cultured in 2% neonatal thrombocytopenic serum appeared normal morphologically, yet produced low basal levels of onfFN, we used this culture system to determine a dose response for TGFβ1-mediated onfFN stimulation. The dose-response curve from four separate TGFβ1 stimulation experiments, including 48- and 72-h culture times, is shown in Fig. 6, with TGFβ1 concentrations ranging from 0.01–5 ng/mL. The lowest onfFN concentration (*i.e.* unstimulated control) was considered 0%, whereas the highest onfFN concentration for each experiment was considered a 100% response. Based on this graph, the 50% effective dose (ED_{50}) of TGFβ1 in this culture system was between 0.1–0.2 ng/mL. If the 48 h TGFβ-onfFN response was plotted separately from the 72 h results, the dose-response curves were essentially superimposable, with the same ED_{50} calculated (not shown).

As previously demonstrated by Morrish *et al.* (17), TGFβ1 down-regulates hCG *in vitro*. When trophoblast media from our TGFβ1-treated cultures were quantitatively assayed for hCGβ using the Abbott IMX automated microbead analyzer (courtesy of Dr. Marilyn Senior and Ms. Margaret Bulley, Clinical Endocrine Laboratory, Hospital of the University of Pennsylvania), we found, in agreement with Morrish *et al.*

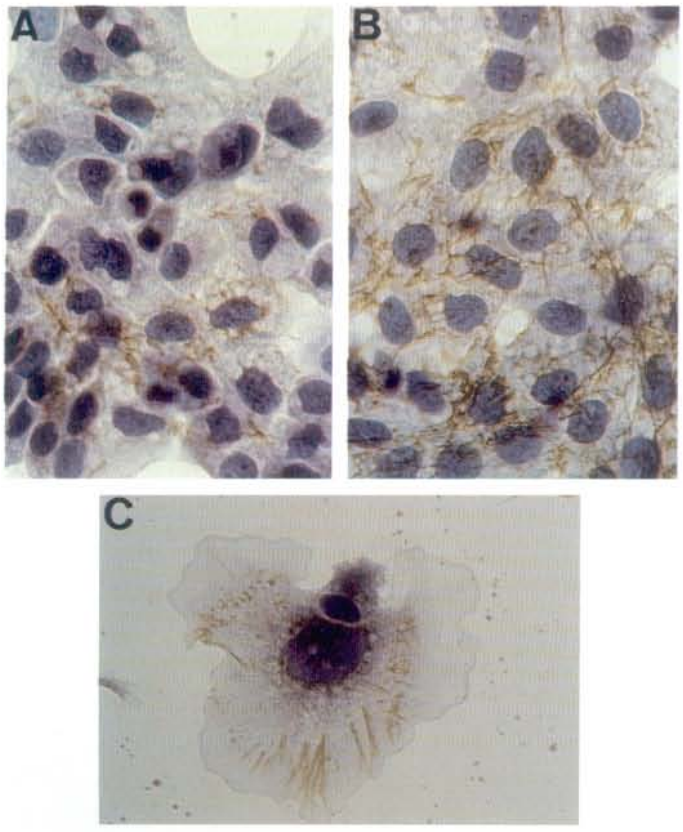

FIG. 5. TGFβ1 stimulates onfFN deposition *in vitro*. Immunocytochemical staining of 48-h cultured trophoblasts with monoclonal antibody FDC-6 demonstrates: A, occasional staining in the absence of TGFβ; B and C, more pronounced, organized fibrillar staining for onfFN in the presence of 2 ng/mL TGFβ, typical of subcellular fibronectin localization in the ECM of cultured cells (18); and C, a large syncytiotrophoblast in contact with a smaller cytotrophoblast exhibits fibrillar onfFN staining associated with the cell periphery.

(17), that TGFβ1 reduced secreted levels of hCGβ 3- to 4-fold (Fig. 7). Thus, TGFβ1 has a reciprocal regulatory effect on two specific trophoblast-derived proteins.

Discussion

Implanting trophoblasts make early contact with the uterine epithelium, decidual stroma, and maternal blood. Growth factors and other cytokines present in the uterine decidua and maternal circulation are likely to affect early trophoblast function by regulating the production of trophoblast-derived implantation site proteins. The specific immunolocalization *in vivo* of a trophoblast-derived oncofetal fibronectin to the placental-uterine junction (1–3) as well as previous studies demonstrating serum modulation of trophoblast onfFN (2) led us to wonder which specific factors are important for its regulation. Could such factors be found both in human serum and within the implantation milieu?

Our initial insight into which onfFN stimulatory factor(s) to investigate resulted from the serendipitous use of cord serum from a neonate subsequently diagnosed with severe alloimmune thrombocytopenia. Cell medium prepared with this serum resulted in normal trophoblast morphology in culture, but significantly less onfFN stimulation. TGFβ is one cytokine growth factor that is released in abundance from the α-granules of activated platelets upon whole blood collection and clot formation in the absence of anticoagulants (19, 20). Likewise, whole blood collected from a severely thrombocytopenic individual will typically clot, but the serum will contain significantly fewer platelet-derived factors, such as TGFβ. This consideration, coincident with a recent report localizing TGFβ to both decidual cells and trophoblasts of the placental-uterine junction (9), led us to speculate that TGFβ could be a significant regulator of onfFN production. As such, the major goal of this study was to determine the effects of endogenous TGFβ immunodepletion and exogenous TGFβ addition on onfFN production by trophoblasts *in vitro*. By using these experimental approaches, our results suggest that TGFβ is an important, if not requisite, factor for stimulating trophoblast onfFN synthesis and secretion *in vitro*.

Although decidual-derived TGFβ could act as a paracrine regulator of trophoblast adhesive activity during placentation, a contributory role for platelet-derived TGFβ should be considered. As reviewed by van der Weiden *et al.* (21), production of platelet-activating factor (PAF) by human and murine embryos appears to correlate with successful implantation. In mice, there is an increased consumption of platelets and an actual systemic thrombocytopenia measured as an early maternal response to pregnancy; the degree of thrombocytopenia is proportional to the number of implanted embryos. van der Weiden *et al.* point to studies by O'Neill *et al.* (22, 23), which also demonstrate a correlation between human embryo PAF production and the likelihood of successful implantation. This has led to human embryo therapy with PAF as a means for improving implantation rates in patients undergoing *in vitro* fertilization and embryo transfer (23). Based on these findings, we hypothesize that local platelet-derived TGFβ released at the implantation site in response to embryo-derived PAF could help stimulate early trophoblast onfFN production.

The effects of TGFβ on fibronectin expression have been examined in other cell systems. Consistent with the hypothesis that TGFβ promotes wound healing, fibroblasts have a coordinated and sustained stimulation of both fibronectin and collagen synthesis, with subsequent incorporation of these proteins into the extracellular matrix (11, 12). TGFβ induction of matrix proteins is regulated at the transcriptional level (13, 14). Both fibroblastic and nonfibroblastic cell lines, which do not typically produce fibronectin under basal conditions, can be stimulated to synthesize fibronectin in response to TGFβ (11). More recently, it has been shown that TGFβ also affects the pattern of fibronectin pre-mRNA splicing in rat and human cell lines (24). Specifically, the ratio of fibronectin isoforms containing integrin-binding domains within the IIICS region were found to be significantly modulated in response to TGFβ.

The trophoblast model system we have used for studying TGFβ-onfFN regulation *in vitro* displays remarkable phenotypic similarities to important trophoblast functions *in vivo*, *i.e.* peptide and steroid hormone production (10, 17), attach-

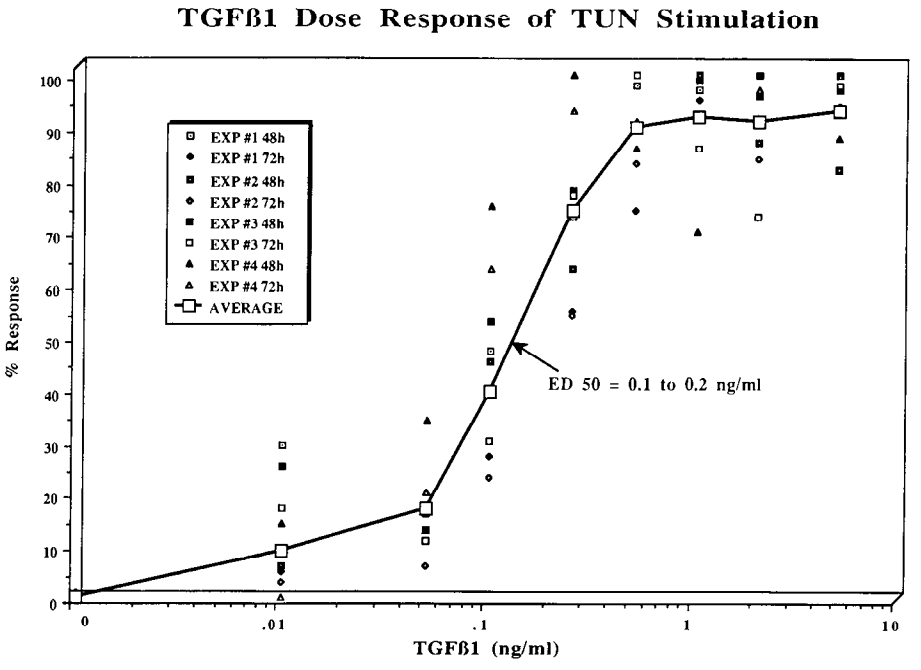

FIG. 6. TGFβ1 dose response of onfFN stimulation. Composite data from four separate TGFβ stimulation experiments were combined and normalized to the percent response from both 48- and 72-h cultures. A dose-response curve was generated on a semilogarithmic scale, with TGFβ1 concentrations ranging from 0.01–5 ng/mL. The lowest onfFN concentration (*i.e.* unstimulated control) was considered 0% for each experiment, whereas the highest onfFN concentration for each experiment was considered a 100% response. Based on this graph, the 50% effective dose (ED_{50}) of TGFβ1 in this culture system was 0.1–0.2 ng/mL. When the 48 and 72 h data were plotted separately, the ED_{50} was equivalent. TUN, Tropho-uteronectin.

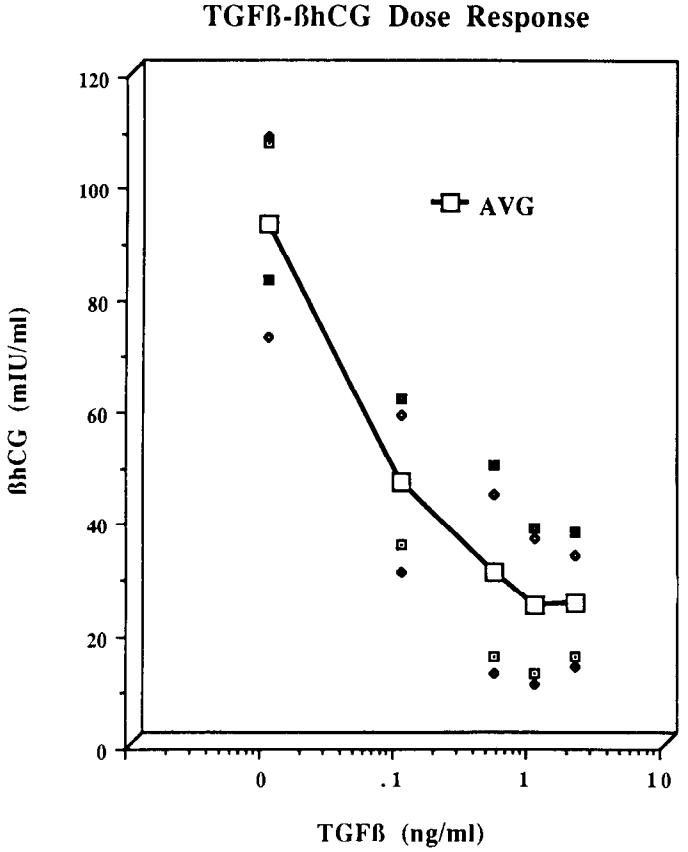

FIG. 7. TGFβ1 inhibits βhCG production. In a reciprocal fashion to onfFN stimulation, trophoblast production of βhCG is inhibited 3- to 4-fold in the presence of increasing concentrations of TGFβ1.

ment to and invasion/proteolysis of the extracellular matrix (25–29), and new ECM production (1, 2, 30). Interestingly, cultured trophoblasts can mimic implanting and anchoring trophoblasts *in vivo* by synthesizing and depositing onfFN at sites of cell-ECM attachment (1, 2). In addition, onfFN production by cultured trophoblasts is markedly sensitive to modulators in the culture environment; onfFN synthesis is stimulated by TGFβ, yet specifically inhibited at the mRNA level by cAMP agonists (30). Recently, Guller *et al.* (31) demonstrated 60–90% reduction of onfFN mRNA and secreted protein in response to medroxyprogesterone acetate and dexamethasone in primary cytotrophoblast cultures. Although specific immunoneutralization of functional TGFβ from serum markedly blunts the onfFN stimulatory response, other cytokine growth factors, such as TGFα, epidermal growth factor, platelet-derived growth factor, and leukemia inhibitory factor, could certainly be involved in regulating onfFN production and trophoblast differentiation pathways.

Based on findings that TGFβ inhibits hCG and human placental lactogen production *in vitro*, Morrish *et al.* (17) concluded that TGFβ inhibits trophoblast differentiation. Our studies indicate that TGFβ may actually have a more profound modulatory effect on determining which trophoblast phenotypes are expressed. Although we also found that hCG is down-regulated by TGFβ, the synthesis of onfFN, a different trophoblast marker, is significantly up-regulated. As anchoring trophoblasts at the placental-uterine junction are the only trophoblasts *in vivo* that are associated with onfFN, we hypothesize that TGFβ could be critical for normal trophoblast-ECM adhesive interactions. Support for this concept derives from recent observations we have made of enhanced trophoblast spreading on fibronectin substrates in response to TGFβ (unpublished results). Thus, the TGFβ domain of trophoblast adhesion-promoting proteins could include onfFN, integrin receptors for fibronectin, as well as other cell adhesion molecules. Graham and co-workers (9, 16) have

provided considerable evidence that TGFβ modulates trophoblast differentiation. In addition to their *in vivo* localization of TGFβ to decidual and trophoblast cells at the human placental-uterine interface (9), these investigators found that TGFβ promotes trophoblast syncytial formation and limits invasiveness *in vitro* (9, 16).

Although our work has generally focused on the adhesive aspects of trophoblast-uterine interaction, onfFN production and deposition within the placental-uterine junction could have additional roles involved with promoting and maintaining pregnancy. Fibronectin-lymphocyte and fibronectin-macrophage interactions have been well described, and fibronectin appears to have an important function in modulating leukocyte adhesiveness, chemotaxis, proliferation, and differentiation. Typically, these leukocyte-matrix interactions are mediated via cell surface integrins, such as the fibronectin receptors VLA-4 and VLA-5 (32–38). One particularly interesting feature of the VLA-4 integrin is its binding specificity for the CS-1 region of fibronectin IIICS, specifically the tripeptide Leu-Asp-Val (39). This VLA-4-binding site is separated from the FDC-6 epitope by only nine amino acids (18), raising the possibility that *O*-glycosylation at this IIICS site could have an effect on CS1 conformation and binding affinity. However, the precise functional consequences of the FDC-6 epitope on cellular VLA-4-fibronectin interactions are not known. TGFβ has also been found to have significant immunomodulatory properties, such as diminishing the production of inflammatory cytokines implicated in septic shock (40). In a model relevant to implantation, TGFβ can function as a potent immunosuppressant *in vivo* during organ transplant, and it has been suggested that one immunosuppressive effect of drugs such as cyclosporin-A may be mediated through induction of endogenous TGFβ (40). Thus, it is interesting to speculate that both TGFβ and onfFN, localized to the trophoblast-decidual junction, could act as immunomodulators to protect the implanting pregnancy from immunological rejection.

In summary, we have proposed one important role for TGFβ in normal human implantation and trophoblast development: the up-regulation of onfFN, an extravillous trophoblast-derived adhesive glycoprotein. Future studies will focus on other potential decidual and serum-derived modulators of trophoblast function as well as the implications of aberrant onfFN expression and regulation in the pathophysiology of implantation failure and pregnancy loss.

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