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TROPHO-UTERONECTIN (TUN): A UNIQUE ONCOFETAL FIBRONECTIN DEPOSITED IN THE EXTRACELLULAR MATRIX OF THE TROPHO-UTERINE JUNCTION AND REGULATED *IN VITRO* BY CULTURED HUMAN TROPHOBLAST CELLS

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INTRODUCTION

The precise biochemical mediators of human implantation are unknown, although trophoblast-specific cell adhesion proteins are thought to promote this process. On a cellular level, the trophoblast shell of the early embryo establishes initial contact with the endometrial lining (Hertig and Rock, 1956). Subsequently, an expanding zone of trophoblast adherence to the extracellular matrix (ECM) establishes the uteroplacental junction. Previous studies examining trophoblast behavior *in vitro* (Kao et al., 1988; Kliman and Feinberg, 1990) have suggested a significant role for fibronectin in modulating trophoblast contact, spreading, and syncytial formation. However, more specific information about the function of fibronectin in trophoblast-uterine interactions *in vivo* is not known.

Fibronectin molecules bearing an extra glycopeptide domain within the type III connecting segment (IIICS), defined as the oncofetal fibronectin class, have recently been described in human tumors, pregnancy tissues, and amniotic fluid (Matsuura and Hakomori, 1985; Matsuura et al., 1988; Matsuura et al., 1989; Loridon-Rosa et al., 1990). In order to understand the biological significance of this class of fibronectins in human gestation, we utilized the monoclonal antibody FDC-6 (Matsuura and Hakomori, 1985) as a specific probe for immunohistochemical localization and immunoblot analyses. The unusual features of FDC-6 are the antibody's high specificity to an O-linked glycosylated hexapeptide within IIICS, its purported low reactivity with normal adult plasma and cellular fibronectins, and its requirement for both carbohydrate and peptide moieties within the epitope binding site.

Whereas normal adult plasma fibronectins have a more generalized distribution *in vivo*, we found that FDC-6 reactive fibronectin is specifically present in

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the placental-uterine junction and chorion wherever trophoblast cells make contact with the ECM (Feinberg et al., 1991). As such, we have proposed that a specific isoform of oncofetal fibronectin—*tropho-uteronectin* (TUN)—may function as a trophoblast-uterine connecting protein for implantation of the early conceptus and anchorage of the developing placenta to the uterine wall.

In this study, we further hypothesize that the production of a fibronectin protein with such unique localization characteristics could be sensitive to regulation signals in the environment of implanting trophoblast cells. In order to approach these issues, we demonstrate that 1) TUN has a unique molecular weight compared to plasma or amniotic fluid fibronectin; 2) FDC-6 is highly specific for oncofetal fibronectins, but does have a low and significant binding capability for plasma fibronectin; and 3) TUN production by human trophoblast cells in culture is under profound stimulatory and inhibitory signals in the culture environment.

MATERIALS AND METHODS

Immunohistochemical Studies

Five micron sections from Bouin's-fixed and paraffin-embedded tissue were placed on glass slides previously coated with a film of 1% poly-d-lysine, 30-70,000 molecular weight (Sigma), dried at temperatures no greater than 60°C and stored at room temperature until used. Immunoperoxidase staining was carried out as described previously (Kliman et al., 1986). Hybridoma supernatant containing monoclonal antibody FDC-6 (American Type Culture Collection, Bethesda, MD), was utilized at dilutions of 50 to 100-fold. Control slides were incubated with undiluted P3X63Ag8 mouse myeloma cell line supernatant (American Type Culture Collection).

Cell Culture and Serum Regulation Studies

Human cytotrophoblast cells were isolated and cultured from term placentae as previously described (Kliman et al., 1986). The cytotrophoblast cells were cultured in Dulbecco's Modified Eagles' Medium (DMEM) containing 25 mM glucose and 25 mM HEPES (DMEM-HG) supplemented with gentamicin (50 μg/ml) and glutamine (4 mM). For serum regulation studies, cytotrophoblast cells were grown in different concentrations (1 to 10%) of heat-inactivated fetal calf serum or human cord serum. Human cord serum was obtained from discarded umbilical vein blood immediately following uncomplicated vaginal deliveries. For studies involving cyclic AMP regulation, 8-bromo-cAMP (Sigma) was added directly to the culture media at concentrations of 0.05 to 1.5 mM. Suspensions of cytotrophoblast cells were cultured with or without Matrigel (Collaborative Research, Bedford, MA) as previously described (Kliman et al., 1990; Feinberg et al., 1991).

Immunoblotting

For preparation of cell extracts, cells were washed with phosphate buffered saline (PBS), scraped from the culture dish, and total cellular protein was extracted with a cell lysis buffer containing PBS, 1.0% sodium cholate, 0.1% sodium dodecyl sulfate (SDS), and 0.02% sodium azide. Unconcentrated conditioned trophoblast media or cell protein was collected and electrophoresed on a 6% SDS-PAGE gel under non-reducing or reducing conditions. The gels were electrotransferred to nitrocellulose

(Schleicher and Schull) overnight, incubated with diluted FDC-6 supernatant as primary antibody, with immunodetection using a biotinylated anti-mouse secondary antibody (ABC Vectastain, Vector Labs, Burlingame, CA). This immunoblot method detects as little as 50 ng of intact TUN or amniotic fluid fibronectin. Both amniotic fluid fibronectin and TUN were purified in parallel by gelatin-Sepharose (Pharmacia) chromatography (Engvall and Ruoslahti, 1977). Purifed plasma fibronectin was obtained from Boehringer-Mannheim.

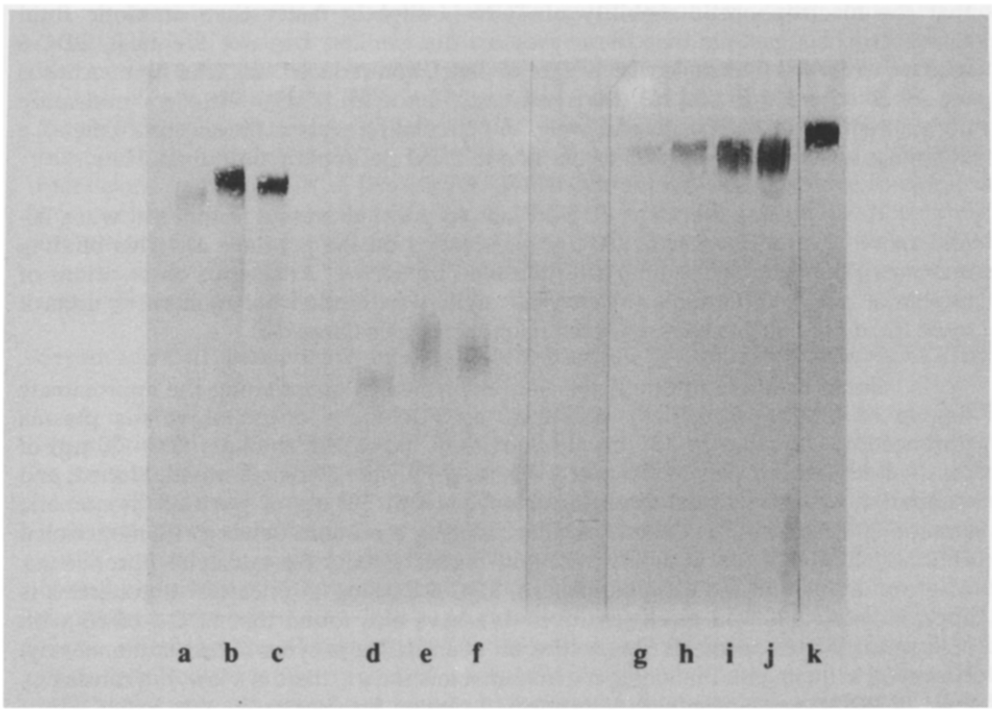


Figure 1. FDC-6 immunoblots of purified plasma fibronectin, amniotic fluid fibronectin, and tropho-uteronectin (TUN).
Electrophoretic mobility and FDC-6 binding specificity were analyzed on high resolution 4% polyacrylamide gel immunoblots under non-reducing (lanes a-c) or reducing (lanes d-f) conditions. Ten micrograms of purified plasma fibronectin (lanes a, d) were compared to 500 ng of purified amniotic fluid oncofetal fibronectin (lanes b, e) and 500 ng of purified TUN (lanes c, f). In order to further analyze the binding specificity of FDC-6, 2.0 μg (lane g), 5.0 μg (lane h), 10 μg (lane i), and 20 μg (lane j) of reduced monomeric plasma fibronectin was compared to the signal intensity achieved with 500 ng of amniotic fluid oncofetal fibronectin (lane k). FDC-6 binds 40 to 50-fold more specifically to oncofetal fibronectins under reducing conditions, and approximately 100-fold more specifically under non-reducing conditions, when compared to purified plasma fibronectin.

RESULTS

Electrophoretic Properties and Reactivity of FDC-6 with TUN, Amniotic Fluid Fibronectin, and Plasma Fibronectin

In order to examine the molecular weight profiles of two pregnancy-related oncofetal fibronectins, as well as the specificity of binding for monoclonal antibody FDC-6, we performed immunoblot analyses of three different fibronectin forms. As can be seen in Figure 1, FDC-6 reacted with 500 ng of purified intact amniotic fluid fibronectin (lane b), as well as 500 ng of purified intact TUN (lane c). It can also be seen that the electrophoretic mobility of TUN is slightly faster than amniotic fluid fibronectin, suggesting that these proteins are similar, but not identical, FDC-6 reactive isoforms. The molecular weight of intact, non-reduced oncofetal fibronectins is reported to be 500 to 550 kD (Matsuura and Hakomori, 1985). This is significantly higher than the 450 kD molecular weight of circulating plasma fibronectins (lane a), a difference which can be detected on our non-reduced electrophoretic immunoblots.

It can also be seen that FDC-6 binds to purified plasma fibronectin when 20-fold more plasma fibronectin (10 μg) was loaded on the gel (lane a). This binding indicates FDC-6 cross-reactivity with plasma fibronectin. Analogous observations of molecular weight differences and cross-reactivity were made when monomeric units of these three fibronectins were analyzed on reducing gels (lanes d-f).

Based on these findings, we were interested in quantitating the approximate degree of binding specificity exhibited by FDC-6 for oncofetal versus plasma fibronectins. In order to address this question, increasing amounts (2.0 - 20 μg) of purified monomeric plasma fibronectin (lanes g - j) were electrophoresed, blotted, and compared with the signal intensity achieved with 500 ng of purified monomeric amniotic fluid fibronectin (lane k). Under reducing conditions (lanes g - k), monoclonal antibody FDC-6 binds with 40 to 50 fold higher affinity for oncofetal fibronectins, whereas under non-reducing conditions, FDC-6 binding to oncofetal fibronectins is approximately 100-fold more sensitive. We have also found that FDC-6 binds with high sensitivity to oncofetal fibronectins on quantitative enzyme-linked immunoassay. However, in analogous manner to the immunoblots shown, there is a low, but consistent, level of FDC-6 cross-reactivity with purified plasma fibronectin.

TUN Immunohistochemistry *In Vivo* and *In Vitro*

The high specificity of FDC-6 for amniotic fluid and trophoblast forms of fibronectin gave us the impetus to examine the immunolocalization patterns of FDC-6 reactive fibronectins in pregnancy tissue. Our results with such tissues from previously fixed specimens revealed that there was specific and consistent deposition of FDC-6 reactive material at the tropho-uterine junction. As shown in a histologic section from a 6 week implantation site, specific and intense staining for TUN was noted within the attachment zone of the placental-uterine junction (Figure 2a). TUN staining was localized primarily to the ECM connecting extravillous anchoring trophoblast cells and trophoblast cell columns to the uterus. Both placental villi and uterine tissue remote from the implantation site were negative for TUN. We observed this specificity of staining in different placental attachment sites across multiple gestational ages, ranging from 20 days post-conception to term. An analogous zone of TUN was present within the ECM of anchoring trophoblast cells from pregnancies which implanted

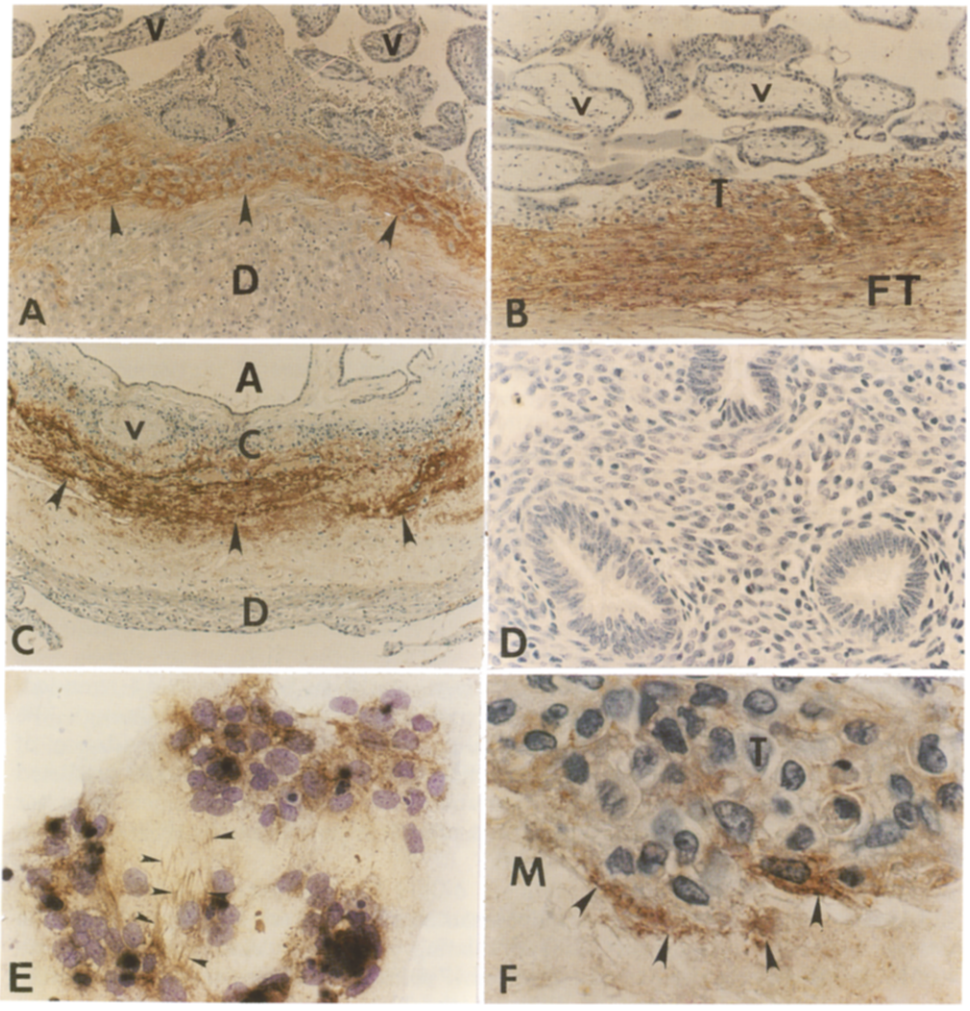
outside of the uterus, including gestations within the fallopian tube (Figure 2b), ovary, and cervix. These results clearly linked TUN deposition in the ECM with implanting trophoblast cells, whether intra- or extra-uterine in location. TUN was also prominent within the ECM of the chorionic membrane at the chorion-decidual junction (Figure 2c). This finding fits the pattern of staining seen in the implantation site, since the chorion is actually the remnant of the trophoblastic shell which did not form a placenta, and only contains extravillous trophoblast cells attached to maternal decidual stroma. Normal human endometrium was uniformly negative for TUN staining (Figure 2d), whether the endometrium was obtained from non-pregnant patients or from patients with extra-uterine pregnancies.

Freshly purified placental villous cytotrophoblast cells contained no detectable TUN in agreement with the negative villous staining for FDC-6 reactive fibronectin in the placenta. A small quantity of TUN was present intracellularly after 24 hours, suggesting that TUN synthesis had been initiated by the cultured cells. Trophoblast cells cultured for 48 hours produced significant amounts of TUN, as seen by immunocytochemical staining of 48 hour differentiated trophoblast cells with monoclonal antibody FDC-6 (Figure 2e). When cytotrophoblast cells were co-cultured in suspension with fragments of Matrigel, multiple aggregates attached and burrowed into the matrix. Immunohistochemical analysis of these sections with FDC-6 revealed a specific band of TUN which had been deposited by the trophoblast cells in the extracellular space at the trophoblast-Matrigel junction (Figure 2f).

Serum and Cell Attachment Stimulate TUN Production by Cultured Trophoblast Cells

On glass or plastic substrates, trophoblast cells synthesize and secrete TUN when cultured in the presence of FCS-containing media. We found that when cultured in serum-free media (SFM), no TUN production was detectable on immunoblots, either in the cellular extract or in the media. This finding is contrary to the notion that serum does not affect other markers of trophoblast biochemical differentiation (Kao et al., 1988). In order to explore this issue further, we cultured trophoblast cells in FCS-containing media at different concentrations and found that TUN stimulation is dose dependent, from 1 to 10% FCS concentrations. Cells in SFM appeared rounded, mononuclear, and not firmly attached to the underlying substrate. In 2% through 10% FCS, trophoblast cells appeared morphologically identical, with evidence of attachment, spreading, and aggregate and syncytial formation. As would be predicted from the work of Kao et al. (1988), immunoblot levels of plasminogen activator inhibitor type 1 (PAI-1), another trophoblast marker (Feinberg et al., 1989), were not significantly affected by serum (not shown).

Since early implanting trophoblast cells *in vivo* are exposed directly to maternal serum, we wondered if TUN stimulating factors were also present in human serum. To date, we have collected and tested 17 different cord blood sera from healthy term uncomplicated deliveries. In all cases, the TUN stimulating capability of cord blood sera was 5 to 10-fold higher than fetal calf serum (Figure 3). In addition, TUN production was stimulated by these human sera in a dose-dependent manner, when comparing serum-free conditions with different concentrations of human cord sera. By quantitative immunoblots, we have measured the TUN in conditioned media from trophoblast cells grown in 10% human cord sera, and found concentrations of 50 to 100 μg/ml after 72 hours of culture. The dose response of human cord sera is parallel to that seen with fetal calf sera, with an accentuated response at each percent of serum tested.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In order to determine that immunoblots of trophoblast media truly reflected cellular synthesis of TUN, we assayed identical quantities of total cell protein from trophoblast cells grown in SFM or in different percent serum-containing medias. We found that cellular content of TUN completely mirrored the results we had seen with conditioned media containing secreted TUN (immunoblots not shown).

The TUN stimulating factor(s) present in serum were not diminished by dialysis with molecular weight cut-offs of 10,000 or 50,000 daltons. Ten percent human cord serum in DMEM-HG was dialyzed against serum-free DMEM-HG for up to 72 hours and assayed for TUN stimulating activity. No significant difference was noted in TUN stimulation with dialyzed media when compared to control non-dialyzed 10% human cord serum. This finding suggests that TUN stimulating factor(s) may either be larger molecular weight species or exist in serum bound to larger molecular weight proteins.

In addition to the importance of serum factors in stimulating TUN production, we wondered about the role of trophoblast-substrate attachment in regulating TUN. Since attachment to the ECM material Matrigel appeared to induce a specific zone of TUN production and deposition, we assayed the capability of trophoblast cells cultured in suspension to produce TUN. We found that in suspension culture with 10% fetal calf serum, aggregates of cytotrophoblast cells produced 20 to 40-fold less TUN than control trophoblast cells in standard two-dimensional cultures. This finding suggests that trophoblast recognition of either a plastic or ECM substrate may trigger intracellular TUN production and subsequent TUN secretion and deposition in the ECM.

Figure 2. TUN immunohistochemistry.
Bouin's fixed, paraffin embedded tissue sections or cultured trophoblast cells were immunohistochemically stained with mouse monoclonal anti-human oncofetal fibronectin antibody FDC-6. A) Utero-placental junction from a 6-week gestation exhibits distinct band of TUN staining (arrow heads) at zone of contact between extravillous trophoblast cells and decidualized endometrium (D). Note the negatively stained chorionic villi (V). B) A distinct band of TUN staining can be seen at the junction of the extravillous trophoblast cells (T) and fallopian tube (FT) in an tubal pregnancy. The extravillous trophoblast cells nearest the junction have a heavier ECM deposit of TUN, while the trophoblast cells farther away from the tropho-tubal junction appear to have a more delicate membrane staining pattern. Note the negatively stained cytotrophoblast cells and syncytiotrophoblast cells of the chorionic villi (V). C) External amniochorionic membrane from an uncomplicated term pregnancy reveals distinct band of TUN staining (arrow heads) at junction between chorionic trophoblast cells (C) and adherent decidual tissue (D). Amniotic cavity (A). Note the ghost villous (V) within the chorion layer. D) TUN staining of proliferative endometrium reveals no reactive oncofetal type fibronectin within either the glands or stroma of normal endometrium. E) Human trophoblast cells cultured for 48 hours express significant quantities of immunoreactive TUN. The staining is present as fine linear streaks (arrow heads), possibly following stress filaments within the cell. F) Immunohistochemistry of trophoblast-Matrigel suspension co-culture. Cross-section of a tongue of trophoblast cells (T) which have penetrated into the Matrigel (M) fragment. The majority of the TUN immunoreactivity is restricted to the periphery of the trophoblastic aggregate, at the junction with the surrounding Matrigel (arrow heads). Magnifications: X250 (A-D), X500 (E,F).

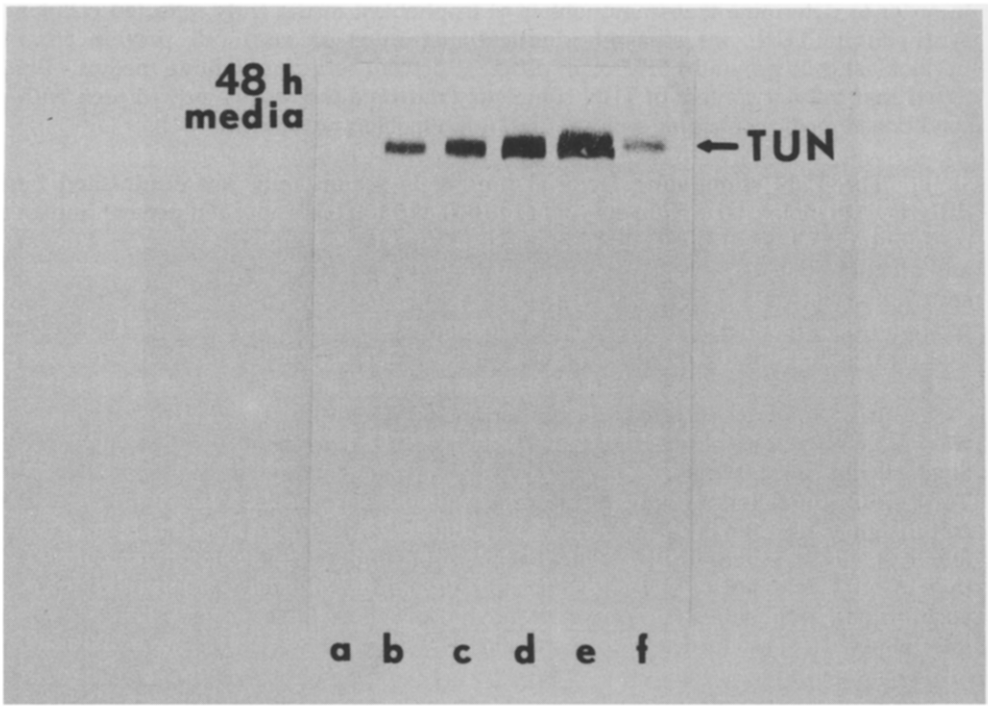

Figure 3. Serum dose-response stimulation of TUN production.
Trophoblast cells grown in serum free media (lane a) secrete no detectable fibronectin, whereas trophoblast cells grown in the presence of 1% human cord serum (HCS) (lane b), 2% HCS (lane c), 5% HCS (lane d), and 10% HCS (lane e) secrete increasing amounts of TUN, in a dose-dependent fashion. Trophoblast cells grown in 10% fetal calf serum (FCS) (lane f) secrete 5-10-fold less TUN than cells grown in 10% HCS. Fifty µl of unconcentrated conditioned media was loaded per lane. An identical dose response to serum was seen when 25 µg of trophoblast cell protein was analyzed. Trophoblast cells grown in 1% through 10% HCS or in 10% FCS appear to be indistinguishable morphologically.

Cyclic AMP Agonists Inhibit TUN Production

Cyclic AMP agonists are major stimulators of trophoblast hCG synthesis and secretion *in vitro* (Feinman et al., 1986). The addition of 1.5 mM 8-bromo-cAMP to trophoblast cultures, a concentration which maximally stimulates hCG synthesis, resulted in almost complete inhibition of cellular and secreted TUN at all time points examined. Treatment of freshly isolated cytotrophoblast cells for only 6 hours with 1.5 mM 8-bromo-cAMP resulted in an equivalent inhibition of TUN synthesis and secretion. Even a 30-fold lower concentration of 8-bromo-cAMP (0.05 mM) resulted in 20-fold inhibition of TUN after 48 hours and 30-fold reduction after 72 hours (Figure 4). Forskolin (100 µM) also inhibited TUN production. The cyclic AMP agonist effect appears specific, since no modulation of TUN synthesis was noted with 1.5 mM 8-bromo-cyclic GMP or 155 nM of the phorbol ester 12-O-tetradecanoyl-phorbol 13-

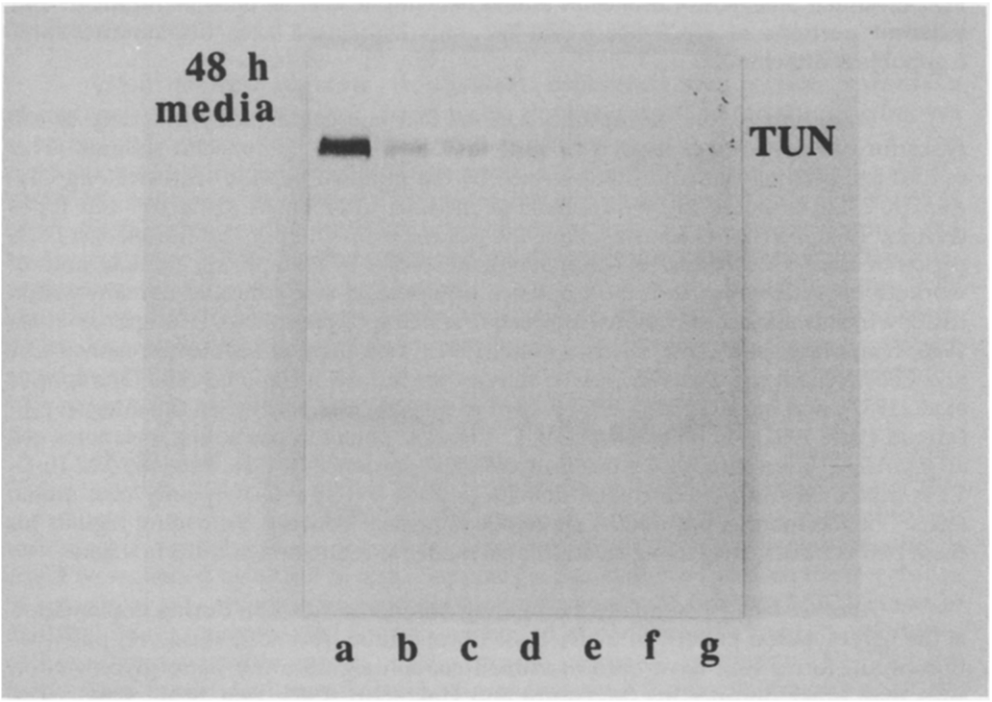

Figure 4. 8-bromo cyclic AMP inhibits TUN production.
Trophoblast cells were grown in 10% fetal calf serum in the absence (lane a) or presence (lanes b-g) of the cAMP agonist 8-bromo-cAMP. In a dose-response manner, significant inhibition of TUN production is seen at a low concentration of 0.05 mM 8-bromo-cAMP (lane b), with increasing inhibition at higher concentrations of 0.10 mM (lane c), 0.25 mM (lane d), 0.50 mM (lane e), 1.0 mM (lane f), and 1.5 mM (lane g) 8-bromo-cAMP.

acetate. Immunocytochemical staining of fixed cultured trophoblast cells treated with 1.5 mM 8-bromo-cAMP contained no detectable TUN. Inhibition of TUN by cAMP agonists may explain previous observations that 8-bromo-cAMP abolishes trophoblast spreading on and degradation of Matrigel™ (Kliman and Feinberg, 1990). Transcriptional inhibition of the fibronectin gene is likely to be one important regulatory mechanism for TUN modulation, since cAMP agonists prevent the accumulation of trophoblast fibronectin mRNA (Ulloa-Aguirre et al., 1987).

DISCUSSION

Previous immunohistochemical analyses have identified fibronectin staining in a variety of locations in pregnancy tissue, including ECM of the placental-uterine junction, uterine stroma, connective tissue core of placental villi, fetal membranes, and in walls of fetal blood vessels (Vartio et al., 1987; Yamada et al., 1987; Earl et al., 1990). These immunolocalization studies, while specific for fibronectin, were performed with antibodies which may have reacted with more ubiquitous, less well-characterized fibronectin epitopes. We conclude that the specificity of staining with

FDC-6, unlike other anti-fibronectin antibodies, implicates the oncofetal domain and adjacent portions of IIICS as critical moieties associated with implantation and trophoblast attachment.

Traditionally, one fibronectin domain that is recognized by a variety of cell types for cell adhesion is located close to the center of the fibronectin subunit. This central cell binding domain, characterized by the minimal peptide sequence Arg-Gly-Asp (R-G-D), appears to be sufficient to mediate attachment for many cell types (Hynes, 1990). What is known about the adhesive function of the fibronectin IIICS region in other cell systems? A series of elegant studies by Humphries, Yamada, and co-workers have demonstrated the existence of a second cell adhesive domain within IIICS, which is subject to complex alternative splicing of pre-mRNA (Humphries et al., 1986; Humphries et al., 1987; Mould et al., 1991) Two peptide backbones, named CS1 and CS5, contain significant adhesive activity for human melanoma cells (Humphries et al., 1987), and have recently been shown to mediate attachment via the integrin $a_4\beta_1$ (Mould et al., 1991). In melanoma cells, CS1 is most potent in promoting melanoma cell attachment. Interestingly, the minimal essential sequence in CS1, Leu-Asp-Val (L-D-V) is separated from the oncofetal domain peptide V-T-H-P-G-Y by only nine amino acids. Furthermore, a pre-mRNA splice site is present between the coding regions for these two peptides, suggesting an additional mode for regulation of IIICS function.

Another factor potentially modulating fibronectin function during implantation is the glycosylation pattern of trophoblast fibronectins. Amniotic fluid and placental fibronectin forms that have been examined contain significantly more glycosylation sites than other fibronectins (Matsuura and Hakomori, 1985; Zhu et al., 1984). The higher molecular weight of these pregnancy-related fibronectins, which we also have seen with trophoblast fibronectin, is probably due to additional carbohydrate residues. There is evidence demonstrating that unglycosylated fibronectin enhances fibroblast adhesion and spreading (Jones et al., 1986), suggesting a regulatory function for the glycosylation pattern seen in different forms of fibronectin. In addition, fibronectin containing certain carbohydrates has altered binding affinity to gelatin (Zhu and Laine, 1985), further suggesting a role in extracellular matrix assembly. The significance of glycosylation sites within the IIICS region is not known, although the FDC-6 binding epitope requires O-linked glycosylation of the hexapeptide backbone. The structure-function relationship of the oncofetal domain in mediating trophoblast adhesiveness has also not been determined.

Recently, the extravasation and release of FDC-6 reactive fibronectins into the cervix and vagina in late pregnancy has been proposed as a biochemical marker of chorionic membrane disruption and preterm delivery (Lockwood et al., 1991a, b). The clinical utility of such an assay depends on the high specificity of FDC-6 binding for oncofetal fibronectins, particularly since this material may be derived from inflamed or degraded chorionic trophoblast ECM. As shown in our immunoblot analyses, we have found that FDC-6 has at least a 40-fold lower binding capability for plasma fibronectin, when compared to either amniotic fluid fibronectin or TUN. This represents a low, but clinically significant cross-reactivity with plasma fibronectin. Because plasma proteins such as fibronectin could commonly be present in the cervix and vagina during late pregnancy, we urge caution regarding the use of FDC-6 based clinical assays for predicting the normal or abnormal state of a human gestation. However, based on the information that has been learned about the tissue localization of FDC-6

reactive fibronectins, we should be encouraged to pursue earlier and more specific markers for preterm chorionic membrane inflammatory processes.

What factors regulate trophoblast differentiation *in vivo* towards a phenotype of TUN expression? Previously, trophoblast differentiation *in vitro* was characterized morphologically by syncytial formation and biochemically by the production of placental hormones (Feinman et al., 1986; Kliman et al., 1986; Kao et al., 1988). We have now shown that TUN is another important marker both *in vivo* and *in vitro* which defines a differentiated form of trophoblast capable of penetrating and anchoring to the ECM. Serum factors and cellular attachment itself are significant up-regulators *in vitro* of TUN production. Although not yet identified, the TUN stimulating factor(s) in human serum are likely to be greater than 50,000 Dalton molecular weight species, based on our serum dialysis experiments. Since such factors could be similar to those found in a receptive endometrium, the aim of future studies will be to purify and characterize serum TUN stimulating factor(s).

Cyclic AMP agonists act as intracellular second messengers to increase hCG (Feinman et al., 1986) and inhibit TUN synthesis in cultured trophoblast cells. This coordinated response to cAMP *in vitro* suggests that trophoblast differentiation towards a syncytial villous phenotype versus an extravillous anchoring phenotype could be regulated by cAMP *in vivo*. Support for this model is based on the fact that in the placenta, only villous syncytiotrophoblast cells appear to produce hCG (Kliman et al., 1986). In the uteroplacental junction, anchoring trophoblast cells and cell columns — differentiated extravillous cells attached to the uterus — synthesize TUN, but not hCG. Our future goal is to identify the endogenous factors in the milieu of the implanting trophoblast cells which successfully modulate TUN synthesis and trophoblast-endometrial interactions. Since *in vitro* TUN production appears to recapitulate the *in vivo* situation, the human trophoblast system should provide a unique *in vitro* model for identifying those factors which regulate normal human implantation.

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