0013-7227/97/$03.00/0
Endocrinology

Vol. 138, No. 9
Printed in U.S.A.

Activin Is a Local Regulator of Human Cytotrophoblast Cell Differentiation*

ISABELLA CANIGGIA, STEPHEN J. LYE†, AND JAMES C. CROSS‡

Program in Development and Fetal Health, Samuel Lunenfeld Research Institute, Mount Sinai Hospital (I.C., S.J.L., J.C.C.), and Departments of Obstetrics and Gynaecology (S.J.L., J.C.C.), Pediatrics (I.C.), Physiology (S.J.L.), and Molecular and Medical Genetics (J.C.C.), University of Toronto, Toronto, Ontario, M5G 1X5, Canada

ABSTRACT

Cytotrophoblast cells of the first trimester placenta are highly invasive when removed from villi and cultured *in vitro*. *In vivo*, however, only selected cytotrophoblast cells break through the overlying syncytiotrophoblast to form cytotrophoblast columns and ultimately invade the endometrium. To explore the role of paracrine growth factors in regulating cytotrophoblast development, we cultured explants of first trimester chorionic villi *in vitro*. Both activin and inhibin, as well as the activin binding protein follistatin, are produced by various trophoblast cells throughout pregnancy. We found that addition of activin-A, but not inhibin-A, stimulated the outgrowth of cytotrophoblast cells into the surrounding matrix. This outgrowth was characteristic of that observed in extravillous cytotrophoblast cells *in vivo*; it was accompanied by cell division within the proximal region of the cytotrophoblast outgrowth, synthesis of fibronectin, as well as the expression of markers characteristic of invasive cytotrophoblast cells, human leukocyte antigen-G and matrix metalloproteinase (MMP)-9. Activin also specifically induced the early expression of MMP-2 within villous cytotrophoblast cells. Addition of the activin binding protein, follistatin, blocked all of the effects of exogenous activin. The morphological and biochemical effects of activin were similar to those observed when signaling of endogenous transforming growth factor-β was blocked. Interestingly, the latter effects were also reversed by the addition of follistatin. These data suggest that activin plays a local role in promoting cytotrophoblast column formation, likely by regulating the differentiation of villous cytotrophoblast into extravillous cytotrophoblast cells. (*Endocrinology* **138:** 3976–3986, 1997)

THE BULK OF the human placenta consists of branching chorionic villi with an outer syncytiotrophoblast, underlying cytotrophoblasts, and a stromal core (1). These villi float within the intervillous space that is filled with maternal blood, providing the primary surface area for nutrient and gas transport to the developing fetus. Other structures, called anchoring villi, are attached to the uterine wall by virtue of cytotrophoblast cell columns that contain several cell types that represent the stepwise transition from basement membrane-associated, villous cytotrophoblast cells to invasive extravillous cytotrophoblast cells that migrate into the decidua and invade the maternal arterioles of the myometrium. Normal development of the placenta depends on the orchestrated balance of cytotrophoblast cell proliferation and differentiation into either one of the differentiated cell types, syncytiotrophoblast or invasive cytotrophoblasts (2). Abnormalities in these processes may lead to gestational abnormalities such as miscarriage, fetal growth restriction, and preeclampsia (3, 4). Preeclampsia is an interesting disease of pregnancy in which there is an excess of cytotrophoblast cell proliferation (5, 6) as well as an interruption in the progression from villous to invasive extravillous cytotrophoblast cells (7, 8).

The stepwise progression starting from villous cytotrophoblasts, to transitional extravillous cytotrophoblasts in the column, finally to invasive extravillous cytotrophoblasts is characterized by dramatic changes in the expression of cell adhesion molecules and proteinases that degrade the extracellular matrix (9, 10). Understanding the importance of these changes has been assisted by the development of systems for culturing isolated cytotrophoblast cells. Invasion *in vitro* is dependent on the expression of MMPs, particularly MMP-9 (gelatinase B) (11). While *in vitro* differentiation of villous cytotrophoblast cells into either syncytiotrophoblast or invasive cytotrophoblasts can occur under relatively simple culture conditions, several factors can influence cytotrophoblast cell differentiation. For example, culturing first trimester cytotrophoblasts in lowered oxygen tension arrests their acquisition of invasiveness and sustains their proliferation (12). Cytokines and growth factors produced locally also affect cytotrophoblasts *in vitro*. For example, hepatocyte growth factor (13) and vascular endothelial growth factor (14) can stimulate trophoblast DNA replication. Transforming growth factor (TGF)-β suppresses cytotrophoblast invasion (15–17) and endocrine differentiation (18), whereas IL-1 increases cell invasiveness (19). Epidermal growth factor (EGF) has multiple effects, promoting either cell proliferation (20) or invasion (21) depending on the gestational age. The influence of growth factors and cytokines on interactions between cell types in the intact villus, such as can be studied using villus explant cultures, have been less well documented.

Received April 14, 1997.
Address all correspondence and requests for reprints to: Dr. James Cross, Program in Development and Fetal Health, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 1X5 Canada. E-mail: cross@mshri.on.ca.
* This work was supported by the Department of Obstetrics and Gynaecology and by Medical Research Council of Canada Grant MT-12894 (to J.C.C.) and Medical Research Council of Canada Group Grant in Development and Fetal Health (to S.J.L. and J.C.C.).
† Career Scientist of the Ontario Ministry of Health.
‡ Medical Research Council of Canada Scholar.

Activin is also produced in the human placenta (22–26). The presence of activin receptors on cytotrophoblast cells suggests that activin may have autocrine or paracrine effects (27). In support of this idea, addition of activin to cultured cytotrophoblast cells stimulates their production of human CG (hCG) and progesterone (22, 28, 29). Because activin is produced by cytotrophoblast cells themselves, the response to exogenous hormone may at first seem somewhat paradoxical. This is reconciled by the fact that trophoblast cells are also a source of inhibin, a hormone related to activin that can antagonize the effects of activin on hCG secretion (28). The activin binding protein, follistatin, is also produced in the placenta (30). Like inhibin, follistatin antagonizes the effects of activin in a variety of systems, including the endocrine activity of cultured cytotrophoblasts, in which it has been shown to affect GnRH-stimulated hCG secretion from choriocarcinoma cells (30). This suggests that the activity of the activin system depends significantly on the degree to which inhibin and follistatin are also expressed.

The objective of the current study was to explore the possibility that activin and follistatin play general roles in cytotrophoblast development, apart from their endocrine effects. To do this, we cultured explants of first trimester chorionic villi which under normal circumstances retain the normal architecture of floating villi over several days. Addition of activin to these cultures, however, induced a massive outgrowth of cytotrophoblast cells that differentiated along the normal pathway including invasion into the surrounding matrigel.

Materials and Methods

Reagents

The murine monoclonal antibody specific for human endoglin (Mab 44G4; 31) was kindly provided by Dr. Michelle Letarte (Toronto, Ontario, Canada) and the rat monoclonal antibody against cytokeratin (MAb 7D3; 9, 32) was a generous gift from Drs. Susan Fisher and Caroline Damsky (San Francisco, CA). The mouse monoclonal antibodies to human MMP-9 (MAb 56–2A4) and MMP-2 (MAb 75–7F7) were from Oncogene Science (Cambridge, MA), and to human MHC class I (Mab W6/32) was from Dako Corporation (Carpinteria, CA). Recombinant human activin A and inhibin A were generously provided by Genentech (South San Francisco, CA), and recombinant human follistatin (rhFS-288; lot no. B3904) was from the National Hormone and Pituitary Program (National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Child Health and Human Development and U.S. Department of Agriculture, Bethesda, MD). TGF-β3 was from R&D Systems (Minneapolis, MN).

Chorionic villus explant cultures

Chorionic villous explant cultures were established from first trimester human placentae as described in detail previously (17, 33, 34). Briefly, placentae from 5–7 week elective pregnancy terminations, derived by dilatation and curettage, were dissected to remove endometrial tissue and fetal membranes. Small fragments of villus tips (15–20 mg wet weight) were placed on Millicell-CM culture dish inserts (Millipore Corp, Bedford, MA) that were precoated with 0.2 ml of undiluted matrigel (Collaborative Research, Inc., Bedford, MA) and placed in 24-well plates. Explants were cultured in DMEM/F12 (GIBCO, Grand Island, NY) supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.25 mg/ml ascorbic acid, pH 7.4. In all experiments, a single placenta was used and triplicate explants were set up for each treatment. Explants were incubated overnight in regular medium to allow attachment to the matrigel before the addition of treatments.

The next day, media were replaced with either regular medium (control), or medium supplemented with activin A (0.1–10 ng/ml), inhibin A (10 ng/ml), follistatin (100 ng/ml), TGF-β3 (10 ng/ml), a monoclonal antibody reactive with human endoglin (MAb 44G4 IgG, 10 μg/ml), or antisense phosphorothioate oligonucleotides (10 μM) used to block endoglin or TGF-β3 synthesis. The antisense oligonucleotides were directed against sequences around the translation start site (Endoglin: 5′-GCGTGCCGCGGTCCAT-3′; TGF-β3: 5′-CCTTTGCAAGTGCATC-3′), whereas the sense sequences were used as negative controls. Culture media including treatments were routinely changed every 48 h thereafter. hCG and progesterone concentrations in conditioned media were measured by RIA (Coat-A-Count, DPC, Los Angeles, CA).

Immunostaining

Villous explants were fixed for 1 h at 4°C in 4% (wt/vol) paraformaldehyde and then embedded in OCT compound (Tissue Tek, Miles, IN) for cryosectioning. Ten micron sections were subjected to immunostaining using a rat monoclonal antibody directed against cytokeratin (used at 1:100) followed by horseradish peroxidase conjugated antirat IgG (Sigma, St. Louis, MO; used at 1:50), and mouse monoclonal antibodies against MHC class I (used at 1:1000), MMP-2 (used at 1:100) and MMP-9 (used at 1:100) followed by horseradish peroxidase conjugated antimouse IgG (Amersham, Buckinghamshire, UK; used at 1:50). Reactions were developed using diaminobenzidine substrate (Sigma, St. Louis, MO).

BrdU labeling

Villous explants exposed to activin (10 ng/ml) for 2 days, were then incubated for 6 h in the presence of 1 μM 5-bromo-2′-deoxyuridine (BrdU) (Sigma, St. Louis, MO). Tissues were fixed in Carnoy's fixative for 1 h at 4 C, then embedded in OCT. BrdU-positive cells were detected using biotin conjugated, anti-BrdU (Zymed, South San Francisco, CA; used at 1:1000) and streptavidin-TRITC (Sigma; used at 1:150). Cell nuclei were stained with bisbenzimide (Sigma). For some experiments, streptavidin-horseradish peroxidase was used as the secondary antibody (Sigma; used at 1:150).

Fibronectin synthesis and release

On day 5 of culture, media were replaced by methionine- and cysteine-free, low glucose DMEM containing treatments and 25 μCi/ml of [^{35}S]-methionine/cysteine. After 18 h, media were collected and diluted with an equal volume of 25 mM Tris-HCl buffer, pH 7.4, 0.15 M NaCl and 0.5% (vol/vol) Triton X-100, and fibronectin was isolated by binding to gelatin-Sepharose as previously described (35). Briefly, 50 μl of a gelatin-Sepharose suspension (Pharmacia Biotech, Uppsala, Sweden) was added to 500 μl of diluted medium and the samples were incubated overnight at 4 C. The gelatin-Sepharose beads were centrifuged, washed three times in Tris/Triton X-100 buffer. Proteins were eluted by boiling for 5 min in 1% (vol/vol) SDS and separated on a 4–12% (wt/vol) polyacrylamide gradient gels (NOVEX, San Diego, CA). Radioactive bands were revealed by autoradiography. Gels typically showed a single band around 200 kDa, characteristic of fibronectin. The radiolabeled band at 200 kDa was then quantitated using a PhosphoImager (410A and Image Quant software, Molecular Dynamics, Sunnyvale, CA).

Detection of metalloproteinases by zymography and Western blotting

Conditioned media were harvested from explants at day 1, 2 and 5 of treatment. Two microliters of media were mixed with 10% (vol/vol) glycerol, 2% (wt/vol) SDS, 0.0025% (wt/vol) bromophenol blue, 0.5 M Tris, pH 6.8 and subjected to substrate-gel electrophoresis on a 10% polyacrylamide gel (wt/vol) impregnated with 0.1% gelatin (NOVEX, San Diego, CA). After electrophoresis, gels were washed in 2.5% (vol/vol) Triton X-100 washes (2 $\times$ 30 min at room temperature) to remove the SDS, then equilibrated with developing buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, Brij 35, pH 7.2) for 30 min and incubated overnight in the same buffer at 37 C. They were stained with 0.1% (wt/vol) Coomassie brilliant blue G-250 then destained to visualize zones of gelatinase activity.

For Western blot analysis of MMP expression, 5 μl of conditioned

media were electrophoresed through 10% SDS-polyacrylamide gels. Proteins were then blotted to Westran (Schleicher and Schuell, Keene, NH) PVDF membrane. Primary antibodies were used at 1:100 and detected using horse radish peroxidase conjugated antimouse IgG (Amersham; used at 1:10,000) and enhanced chemiluminescence (ECL; Amersham).

Statistical analysis

Differences among treatment means were assessed by *t* test, or ANOVA and Student-Newman-Keul's test.

Results

Activin induces cytotrophoblast outgrowth from villous explants

Villous explants from first trimester placentae that are cultured on matrigel remain viable for at least 7 days. Remarkably their structure remains intact, and few cells emerge from the villous tips under control conditions (Fig. 1). However, addition of recombinant activin-A to the culture media stimulated substantial outgrowth of cells from a region that was restricted to the villous tip (Fig. 1B). The effect of activin was very rapid, being apparent as early as 12 h after addition, though outgrowth continued over the course of several days (Fig. 1A). Significant outgrowth was observed at doses of 1 and 10 ng/ml, but not 0.1 ng/ml (data not shown). Recombinant inhibin in the same dose range was without effect (data not shown). Staining with anticytokeratin confirmed that all of the cells in the villous outgrowths from activin-treated explants were cytotrophoblasts (Fig. 2B). Because activin was previously shown to stimulate the production of progesterone and hCG from isolated cytotrophoblast cells in culture, we also measured the release of these hormones by cultured explants. Cumulative production of both progesterone and hCG increased throughout the course of the 5-day culture period and was significantly elevated by activin treatment (see Fig. 8; $P < 0.05$).

Cytotrophoblasts at the periphery of the outgrowth do not proliferate

To determine if the cytotrophoblast cell outgrowth was primarily due to cell division or to migration of cells away from the villous tips, we determined whether or not cells in the outgrowth were cycling. To do this, activin-treated explants were labeled with BrdU to identify cells undergoing DNA replication. BrdU-positive cytotrophoblast cells were present but were clustered close to the core of the villus with few present within the outgrowth itself (Fig. 2D). Counting cells on several histological sections showed that the labeling index of cytotrophoblast cells was highest in the villous cytotrophoblast population and dropped progressively as cells moved distally within the extravillous region (Table 1). Although we have not addressed this point, we cannot rule out the possibility that activin may first induce higher levels of cell division in the villous-associated cytotrophoblast population, an effect secondarily leading to more extravillous cells. Nonetheless, these data indicate that cells in the cytotrophoblast outgrowth are primarily not cycling, and therefore their appearance in the outgrowth must have been due to the migration of cells away from the villous core. Within the outgrowths, epithelial cell morphology changed dramatically. Cytotrophoblast cells close to the stroma were smaller and tightly clustered, whereas more distal cells became progressively larger and at the outgrowth edge were often isolated in the surrounding matrigel (Fig. 2B). These morphological features were reminiscent of the cytotrophoblast cell phenotypes within anchoring type chorionic villi *in vivo*.

Induction of migration- and invasion-associated markers

To confirm that the cytotrophoblast population that appeared in activin-treated cultures underwent a differentiation program typical of extravillous cytotrophoblast cells *in vivo*, we tested explants for the expression of markers characteristic of both transitional cells of the column and invading cytotrophoblast cell populations. After cytotrophoblast cells leave the basement membrane to form a column they express the $\alpha5\beta1$ integrin (9) as well as express its ligand, fibronectin (36). The synthesis of fibronectin was 3- to 8-fold higher in activin-treated explants compared with controls (Fig. 3), consistent with the presence of significantly more transitional cytotrophoblast cells. Human leukocyte antigen (HLA)-G is expressed on the surface of extravillous cytotrophoblast cells in the distal portion of the column and in invasive cells that enter the decidua (37–39). Immunostaining of activin-treated explants with monoclonal antibody W6/32, which recognizes HLA-G, demonstrated expression on cells in the distal portions of the outgrowth and in cells that were invading the surrounding matrigel (Fig. 2F).

Cytotrophoblast invasion into matrigel *in vitro* is depen-

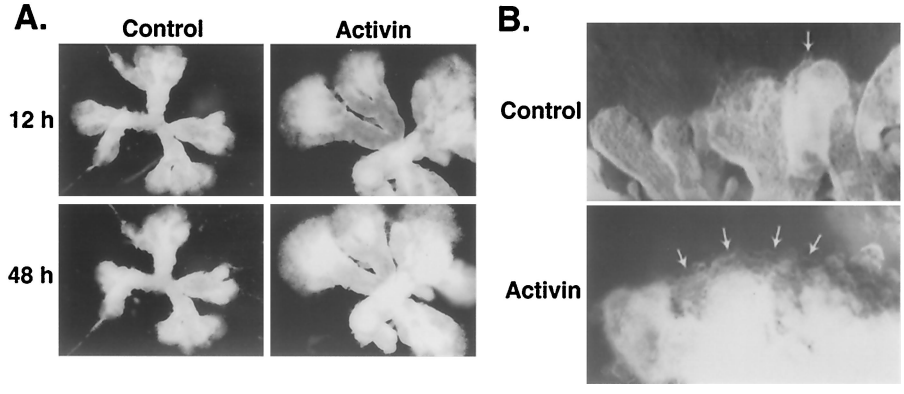

FIG. 1. Activin induces cytotrophoblast migration and invasion. A, Morphology of control and activin-treated explants within 12 and 48 h of treatment (40× magnification). B, View of explant edges showing the collar of the cytotrophoblast cells that emerge (200× magnification).

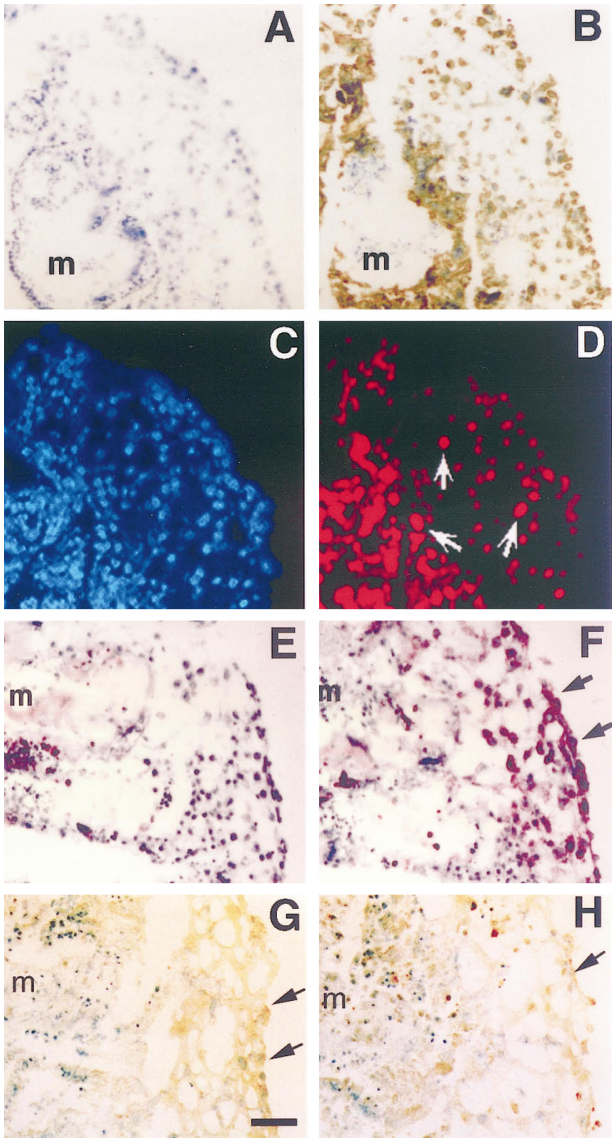

FIG. 2. Cytotrophoblast marker expression and DNA synthesis in activin-treated villous explants. A–D, Explants were treated with activin for 48 h, then pulse labeled with BrdU for 6 h before fixation. Serial sections show negative control (no primary antibody) (A) and anticytokeratin visualized by *brown* staining following immunoperoxidase histochemistry (B), or, on the same section, Hoescht DNA stain (C) and anti-BrdU visualized by immunofluorescence (D). Note that all cells in B show *brown* staining with the exception of the mesenchymal core (m). In D, note that the majority of cells at the outgrowth periphery are not labeled with BrdU, whereas cells proximal to the villus stromal core are more frequently labeled (*white arrows*). E–H, Sections show immunoperoxidase staining for E) negative control (no primary antibody); F, anti-MHC class I; G, anti-MMP-9; and H, anti-MMP-2. Note that MHC class I immunostaining was restricted to extravillous cells at the edge of the outgrowth (*arrows*), similar to the pattern of MMP-9 immunostaining (*arrows*), whereas MMP-2 staining was detected in the stroma and weakly in extravillous cytotrophoblasts. *Bar* represents 50 μm.

dent on the production and activation of MMPs, particularly MMP-9 (11, 19). Therefore, to test if the cytotrophoblast cells in the villous outgrowths produced MMPs we performed gelatin zymography. Conditioned media collected from villous explants contained gelatinases with different molecular

TABLE 1. BrdU labeling index of individual cell types in activin-treated villous explants

Cell type	Labeling index (%)[a]
Villous stroma	11.7 ± 1.2
Villous cytotrophoblast	16.6 ± 2.4
Extravillous cytotrophoblast	
- Proximal	12.7 ± 3.0
- Distal	3.3 ± 0.6

[a] BrdU was detected as described in *Materials and Methods*. Labeling indices were estimated as the percentage of BrdU-positive cells in each region of the villus within a section. Numbers represent the mean ± SEM calculated by examining five serial sections each separated by 20 μM. The proximal extravillous cytotrophoblast region was defined as the half of the cytotrophoblast outgrowth closest the stromal core, whereas the distal region represented the region outside this zone.

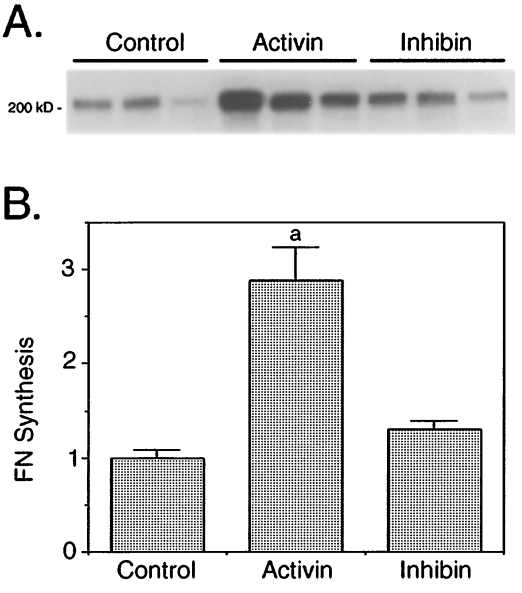

FIG. 3. Fibronectin synthesis is elevated in activin-treated villous explant cultures. Explants incubated in medium alone (Control), or in the presence of activin A or inhibin A (10 ng/ml) were pulsed with [^{35}S]-methionine/cysteine on day 5. Fibronectin in the medium was collected by binding to gelatin and assessed by PAGE and fluorography. A representative analysis of triplicate samples from a single experiment is shown (A). Samples from three separate experiments were quantified by Phosphorimager analysis (B). Data represent mean ± SEM (a, $P < 0.05$)

weights including weak bands at 92 and 84 kDa, and intense bands at 68 and 60 kDa (Fig. 4A). MMP-9 is produced as a 92-kDa proenzyme, which becomes activated by cleavage to form an 84-kDa species. It is likely that the gelatinase activity at 84 kDa was due to activated MMP-9 because Western blotting with an MMP-9-specific monoclonal antibody identified an immunoreactive band at 84 kDa (Fig. 4B). The relatively weak 84-kDa immunoreactive band observed was consistent with the observation on the zymogram that the comigrating gelatinolytic activity at 84 kDa appeared to be of relatively lower abundance. Because MMP-9 is known to be produced by activated macrophages, which might have been present in the stroma of the cultured villi, we performed immunostaining to determine if it was expressed by cytotrophoblast cells. The MMP-9 monoclonal antibody detected

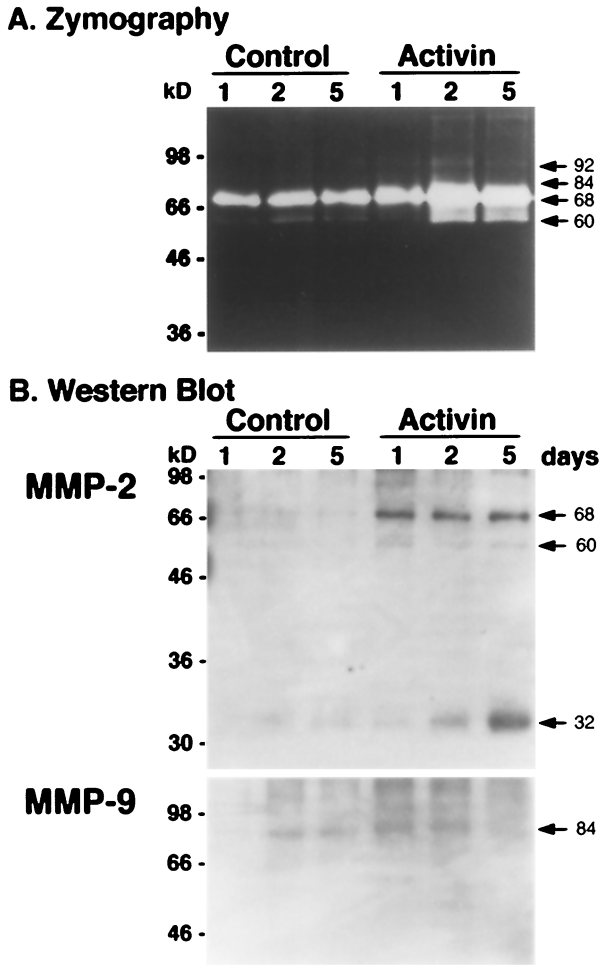

FIG. 4. Production of matrix metalloproteinases by control and activin-treated (10 ng/ml) villous explants. Samples of conditioned medium were collected from individual explants on days 1, 2, and 5 of culture and subjected to analysis by gelatin zymography (A), or Western blotting with MMP-2 and MMP-9 antisera (B). *Arrows* indicate positions of gelatinase activity (92, 84, 68, and 60 kDa) and immunoreactive bands.

scattered, weakly positive cells in the villous stroma (Fig. 2G). MMP-9-positive (albeit weakly) cytotrophoblast cells were also present but were largely restricted to the distal region of the outgrowth and in cells invading the matrigel in control explants (Fig. 2G). This restricted site of expression was similar to the pattern of HLA-G staining (Fig. 2F).

Neither the amount nor site of MMP-9 expression was significantly affected by activin treatment. By Western blot analysis, MMP-9 was released into the medium to a roughly similar level by control and activin-treated cultures. The only difference that was apparent was that the 84 kDa, MMP-9 immunoreactive band was detectable earlier during the culture period following activin treatment, compared with controls (compare bands at day 1 in Fig. 4B). There was also no difference in the extent to which MMP-9 was activated. Immunostaining of activin treated explants showed that activin treatment also did not apparently affect the site of MMP-9 expression (data not shown).

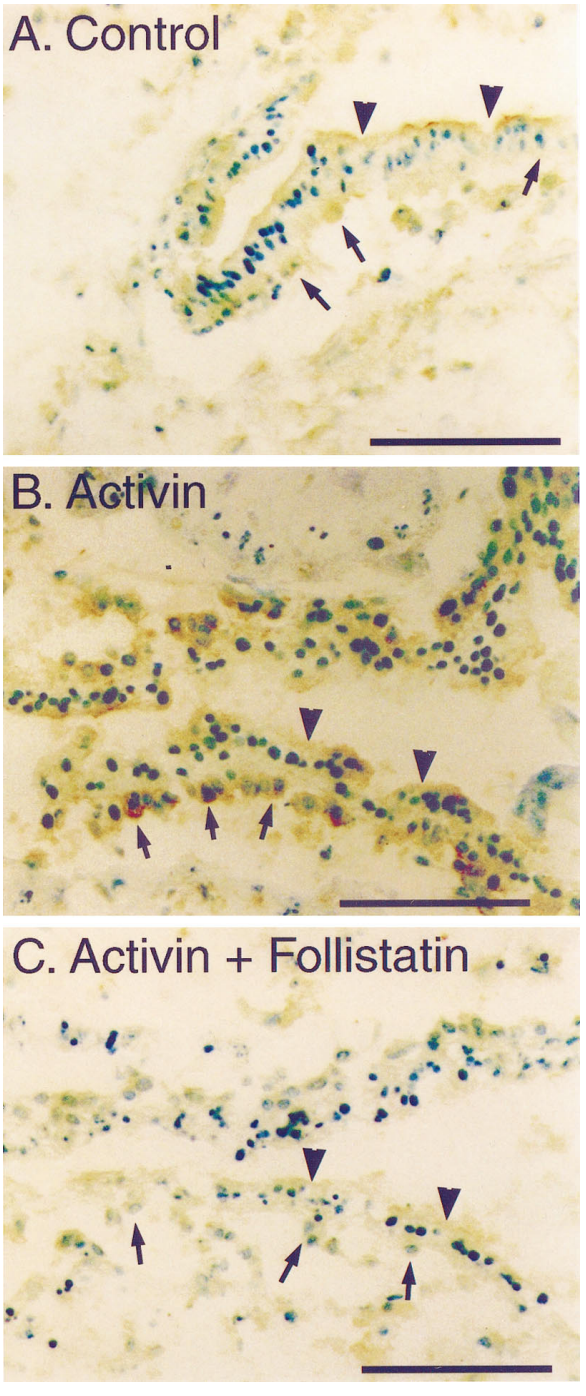

FIG. 5. Immunolocalization of MMP-2 in villous cytotrophoblast cells of activin-treated explants. Explants were cultured for 5 days in control medium (A), 10 ng/ml activin (B), or 10 ng/ml activin + 100 ng/ml follistatin-treated (C). Note that MMP-2 immunoperoxidase staining in control and activin + follistatin explants was weak in all cells (A) but is strongly expressed in villous cytotrophoblasts of activin-treated explants (B). *Bar* represents 50 μm. *Small arrows*, villous cytotrophoblast cells; *arrowheads*, syncytiotrophoblast.

Activin increases MMP-2 production by villous cytotrophoblast cells

Besides MMP-9, zymography showed that villous explants also released large amounts of gelatinase activity at 68

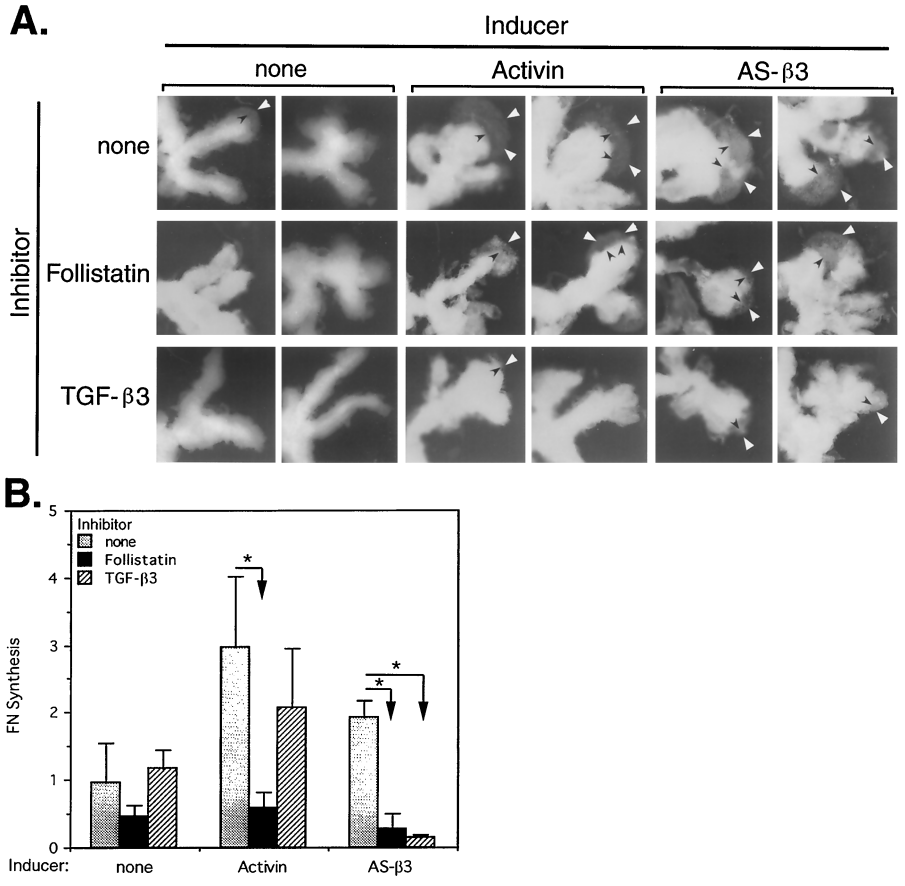

FIG. 6. Mutually antagonistic effects of activin and TGF-β3 on cytotrophoblast outgrowth and fibronectin production. Explants were treated with an inducer of outgrowth, either activin (10 ng/ml) or antisense oligonucleotides to TGF-β3 (AS-β3; 10 μM), for 24 h, before the addition of follistatin (100 ng/ml) or TGF-β3 (10 ng/ml). Explants were photographed 3 days later and pulse labeled with ^{35}S-methionine for assessment of fibronectin synthesis. A, Morphology of two examples of each treatment group are shown; the *black arrows* indicate the edge of the villous tip, whereas the *white arrows* indicate the edge of the outgrowth. B, Fibronectin synthesis measured by Phosphorimager analysis. Data represent mean ± SEM. Statistically significant effects (pairwise t test) of the inhibitors are indicated ($P < 0.05$).

and 60 kDa, which are the sizes of the proenzyme and activated forms of MMP-2, respectively (Fig. 4A). The lower molecular weight bands around 60 kDa may also represent interstitial collagenase, which is known to be produced by isolated cytotrophoblasts in culture (40). The release of both these species was significantly increased by activin treatment. Western blotting with an MMP-2-specific monoclonal antibody showed immunoreactive proteins at 68, 60, and 32 that were abundant in the conditioned media from activin-treated cultures, but were weak or undetectable in control media (Fig. 4B). The identity of the 32 kDa immunoreactive band is unknown, though it likely represents a proteolytic fragment of MMP-2. Activin induction of MMP-2 release was apparent even after only 1 day of treatment. Therefore, the induction of MMP-2 was a specific and early effect of activin treatment.

To identify the cellular source of MMP-2, control explants as well as those treated with activin for 48 h were fixed and sectioned for immunostaining. In control explants, MMP-2 immunoreactivity was only weakly detectable in villous stroma and extravillous cytotrophoblasts (Fig. 2H). Activin treatment had no effect on these sites of expression (data not shown). However, in villous cytotrophoblast cells that underlie the syncytiotrophoblast layer, whereas MMP-2-immunoreactivity was undetectable in control explants (Fig. 5A), it was significantly elevated in activin-treated explants (Fig. 5B). MMP-2 immunoreactivity was also slightly elevated in the syncytiotrophoblast of activin-treated explants compared with controls (Fig. 5, B *vs.* A, data not shown), though this effect was much less dramatic than the change in villous cytotrophoblast expression. Together these data demonstrate that the increase in MMP-2 expression following activin treatment is primarily due to MMP-2 production from villous cytotrophoblast cells.

Follistatin blocks cytotrophoblast outgrowth

Because activin is expressed by cytotrophoblast cells *in vivo*, we wished to determine if it is an essential regulator of differentiation during the transition from villous to extravillous cytotrophoblasts. To do this, we tested if follistatin, an activin-binding protein, could block cytotrophoblast outgrowth. Addition of follistatin (100 ng/ml) alone to cultures reduced both cytotrophoblast outgrowth (Figs. 6A) and fibronectin synthesis (Fig. 6B) compared with controls, though the latter effect was not statistically significant in all experiments. As noted earlier, though, the outgrowth of cytotrophoblasts from intact villi is relatively small under control conditions, and therefore our ability to observe a reduction was likely limited. To get around this problem, we tested whether follistatin could block cytotrophoblast outgrowth after it had been initiated by treatment for a short period with activin alone. Follistatin (100 ng/ml) was therefore added after 24 h exposure to activin (10 ng/ml). The high concentration of follistatin added was such that we were trying to block the effects of both exogenous as well as any endog-

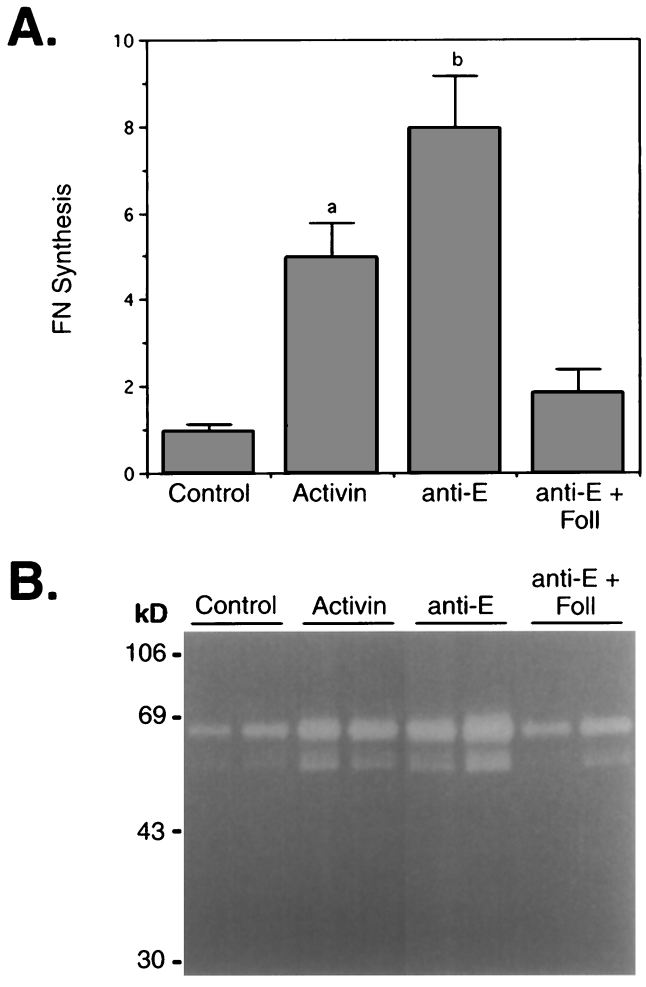

FIG. 7. Follistatin blocks the increase in cytotrophoblast production of fibronectin and gelatinase activity in response to inhibition of TGF-β signaling. Explants were treated with medium alone (control), activin (10 ng/ml), or an antibody against the TGF-β binding protein endoglin (anti-E) with and without follistatin (100 ng/ml) for 5 days. A, Fibronectin synthesis (mean ± SEM; significant differences are indicated by different superscripts, $P < 0.05$); B, gelatinase activity present in the medium on day 5 of culture. The prominent gelatinolytic bands in B comigrated with the 68- and 60-kDa bands shown in Fig. 4.

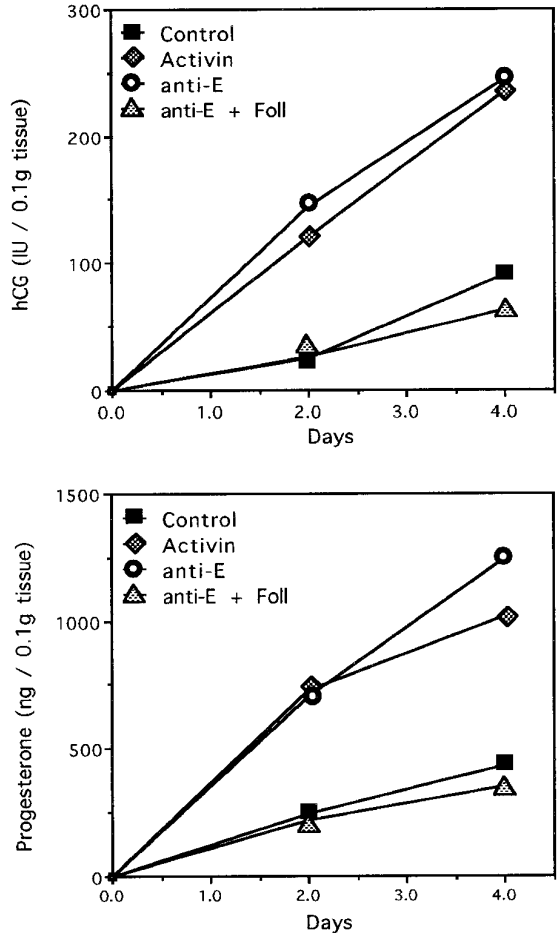

FIG. 8. Cumulative production of hCG (*top*) and progesterone (*bottom*) by villous explants. Hormone concentrations in the conditioned media of explants from the experiment shown in Fig. 7 were measured by RIA. Points represent mean of triplicate samples. The *error bars* are not shown to simplify the presentation; hCG and progesterone production were significantly higher in activin and antiendoglin (anti-E) treated cultures compared with control and anti-E + follistatin cultures at both days 2 and 4 ($P < 0.05$; ANOVA).

enously produced activin. Follistatin effectively reversed the effects of exogenous activin including morphological outgrowth (Fig. 6A) and fibronectin synthesis (Fig. 6B). These data suggest that sustained activin function, including via endogenously produced hormone, is required to elicit full outgrowth.

TGF-β3 inhibits activin-induced cytotrophoblast outgrowth

During the course of these studies, it became apparent that the effects of activin were opposite to those of TGF-β; specifically, adding activin to the villous explant cultures had effects similar to those observed when TGF-β signaling is blocked by several means (17). For example, inhibition of the TGF-β binding protein, endoglin, by addition of the endoglin specific monoclonal antibody 44G4, and inhibition of endogenously produced TGF-β ligand by antibody or antisense oligonucleotides, elicits cytotrophoblast outgrowth and increased fibronectin synthesis (17; Figs. 6 and 7). TGF-β isoforms are expressed by various cells in villous explants, and the fact that inhibition of their expression or their signaling, by interference with receptor expression, indicates that TGF-β functions as a negative regulator of cytotrophoblast outgrowth. To determine if exogenous TGF-β could block activin-stimulated outgrowth, we added TGF-β3 (10 ng/ml) to explants treated initially for 24 h with activin (10 ng/ml). Under these conditions, the effects of TGF-β3 were similar to those of follistatin in suppression of cytotrophoblast outgrowth (Fig. 6A). Interestingly, however, TGF-β3 did not significantly reduce the activin-stimulated increase in fibronectin synthesis (Fig. 6B).

Outgrowth induced by inhibition of TGF-β signaling is arrested by follistatin

One possible explanation for the combined effects of activin and TGF-β is that activin is a direct positive regulator

of outgrowth, whereas the role of TGF-β is to simply block activin action. In this way, the outgrowth promoting effects of observed blocking TGF-β would be dependent on activin signaling. To explore this possibility, we perturbed endogenous TGF-β signaling and determined whether follistatin affected outgrowth development (Fig. 6). TGF-β signaling was blocked in two ways; ligand expression was reduced using antisense oligonucleotides designed against TGF-β3 (AS-β3) (Fig. 6) and receptor function was affected through inhibition of endoglin, a TGF-β1 and -β3 binding protein, with a monoclonal antibody (Fig. 7). While both methods elicited outgrowth, as has been observed before (17), these effects were inhibited by follistatin. This effect was most significant with simultaneous addition of follistatin and the outgrowth inducer. However, when addition of follistatin was delayed for 24 h, it was noteworthy that although outgrowth was significantly reduced by follistatin, morphological outgrowth was still significantly greater compared with control or follistatin alone treatment (Fig. 6A).

The effect of follistatin on biochemical markers of cytotrophoblast differentiation was also examined. Like the effect of activin, inhibition of TGF-β signaling resulted in increased production of fibronectin (Figs. 6B, 7A), gelatinase activity at 68 and 60 kDa (Fig. 7B), hCG (Fig. 8), and progesterone (Fig. 8). All of these effects were reversed to control levels by addition of follistatin to the cultures (Figs. 6–8).

Discussion

The expression of activin as well as activin receptors by cytotrophoblast cells suggested that these factors may be local regulators of placental development and/or functions. Several earlier studies showed that indeed the secretion of hCG and progesterone by isolated cytotrophoblast cells in culture could be stimulated by exogenous activin (25, 26, 28, 29). In the present studies, we have extended these actions to include effects on promoting the differentiation of cytotrophoblast cells. Specifically we found that addition of activin to villous explant cultures promoted the rapid transition from relatively static explants, which maintain the structure of a floating type chorionic villus, into a villus that sprouted migrating cytotrophoblast cells that ultimately differentiated into invasive extravillous cytotrophoblast cells expressing the markers HLA-G and MMP-9. In contrast to the effect of activin, inhibin had no effect on development of villous explants. Moreover, by adding recombinant follistatin to the cultures, as an inhibitor of activin function, we found importantly that endogenously produced activin may be a mediator of cytotrophoblast differentiation. These are the first studies to implicate activin as an essential local regulator of human placental development.

Activin induces cytotrophoblast outgrowth

The cytotrophoblast cell outgrowths induced by activin resemble by morphology, sites of cell proliferation and expression of specific markers, those cytotrophoblast populations present in anchoring chorionic villi. Therefore, we suppose that activin initiates an early event leading to subsequent differentiation steps that progress uninterrupted. That this full differentiation program could occur *in vitro* is supported by the fact that purified cytotrophoblast cells undergo the same process (40–43). Therefore, the villous explant system is unique in that unless an initiating event occurs to trigger the process, villous cytotrophoblast cells within the explant remain relatively static (33, 34).

The mechanism by which activin induces cytotrophoblast outgrowth is obscure at present. Though activin and follistatin have effects in a variety of systems (44), effects on trophoblast cell differentiation or migration, as described here, appear unique. The closest parallel may be the effect of activin on promoting mesoderm differentiation, a process best studied in Xenopus embryos. During gastrulation, a specialized region of embryonic ectoderm transforms into mesoderm involving extensive cell migration and presumed changes in cell-cell or cell-extracellular matrix interactions (45, 46). Indeed, activin induces changes in integrin expression on Xenopus ectodermal cells (45). In a similar manner, during the transition from villous to extravillous cytotrophoblast cells, cells must first detach from the villus basement membrane, then migrate through the cytotrophoblast column and ultimately invade the decidua. Each step in this process in characterized by the expression of distinct sets of integrins (9) and cell-cell adhesion molecules (*e.g.* cadherins) (40). Additionally, cytotrophoblast cells express extracellular matrix-degrading enzymes such as plasminogen activator (41, 42) and metalloproteinases (11, 47) that are thought to be required for migration and invasion. MMP-9 is required for at least the terminal step of cytotrophoblast differentiation, invasion into the laminin-rich decidua, a process that can be studied by invasion of matrigel *in vitro* (11). Our direct demonstration here by immunostaining that MMP-9 is expressed by cells only at the distal fringes of cytotrophoblast outgrowths in culture supports this hypothesis and is similar to the expression pattern of MMP-9 *in vivo* (48).

Activin stimulates MMP-2 production by villous cytotrophoblast cells

A significant early effect of activin on the villous explants was the induction of MMP-2 expression. The 60-kDa form of MMP-2 was stimulated by activin, as well as the 68-kDa proenzyme form, indicating that the enzyme becomes activated. The role of MMP-2 in cytotrophoblast differentiation has been somewhat controversial. By zymography analysis, MMP-2 is abundantly expressed by purified cytotrophoblast cells using some procedures (47), whereas in others it is barely detectable (11). The discrepancy has been attributed to the possibility of placental stromal cells contaminating the cultures. The fibroblasts could be a source of MMP-2 directly or may redirect cytotrophoblast differentiation toward syncytiotrophoblast (49), also a potential source of MMP-2. Our data support a third and more interesting possibility. The fact that MMP-2 expression by villous cytotrophoblast cells was significantly elevated in activin-treated explants at a time when extensive transition toward extravillous outgrowth was occurring, argues that activated villous cytotrophoblast cells are a significant source of MMP-2. They presumably would use this enzyme to migrate off the basement membrane. Thereafter, in the cytotrophoblast column, expression of MMP-2 is significantly reduced. Given this, the extent to

which MMP-2 is expressed by freshly isolated cytotrophoblast cells may depend significantly on the proportion of cells in the population that represent the earliest stages of differentiation. The fact that freshly isolated cytotrophoblasts often contain large fractions of $\alpha 5$ and even $\alpha 1$ integrin-positive cells (9, 32, 36), which represent extravillous populations, strongly argues that the numbers of these early cells may be low. This may be particularly true if older placentae are used for cell isolation because cytotrophoblast proliferation and invasiveness changes dramatically within the first trimester. An advantage of the explant system is, therefore, the ability to better study the behavior of villous cytotrophoblast cells in culture.

We conclude that activin plays an autocrine or paracrine role in modulating cytotrophoblast differentiation within chorionic villi. In both first and third trimester villi, activin expression has been detected in both syncytiotrophoblasts and the underlying cytotrophoblasts (24–26). In contrast, follistatin is expressed by syncytiotrophoblasts (30). Because receptors for activin are present on cytotrophoblasts (27), activin can have autocrine and paracrine effects in intact villi. Explants of floating chorionic villi placed into culture essentially consist only of a villous cytotrophoblast monolayer with covering syncytiotrophoblast, and an underlying basement membrane and stromal core. We suggest that a likely target for the effects of activin is the villous cytotrophoblast cell whose production of MMP-2 is acutely increased in response to activin. At the moment we cannot rule out the possibility that activin may play additional roles at subsequent steps along the progression ultimately toward invasive cytotrophoblasts. This would be best tested using isolated cytotrophoblast cells which, when put into culture, represent an intermediate cell population partially progressed along the differentiation pathway.

Activin and TGF-β have opposing effects on cytotrophoblast differentiation

Recently, TGF-β and activin were shown to have opposing effects on hCG production by human trophoblast cells (50), but the mechanisms of their effects were unexplored. In our studies, the discovery that activin has effects on villus development that are opposite to that of TGF-β, and moreover that cytotrophoblast outgrowth induced by inhibiting TGF-β signaling is reversed in turn by follistatin (an inhibitor of activin), indicates that the effects of these growth factors intersect to regulate cytotrophoblast differentiation. Though it is not surprising that growth factor networks affect complex biological events, this is the first study to systematically determine at what steps in cytotrophoblast differentiation different growth factors act and how their effects are integrated. The ability to induce the activin and TGF-β signaling pathways by the addition of ligands, as well as to block the effects with follistatin and antiendoglin, respectively, or with antisense oligonucleotides will allow the interactions between these cytokines to be described in further detail. Other growth factors and cytokines have been shown to affect cytotrophoblast growth and differentiation, including IL-1 (19), epidermal growth factor (20, 21), hepatocyte growth factor (13), and vascular endothelial growth factor (14). Because it is likely that these factors function as a network, it would be fruitful to use inducing and blocking strategies similar to the ones used here to unravel this complexity. Such strategies will likely be necessary to assign these different factors to regulating specific steps in cytotrophoblast differentiation. This critical understanding will be important to evaluate what specific processes are affected in a disease such as preeclampsia in which villous development and cytotrophoblast invasion are abnormal.

In considering how activin and TGF-β pathways may intersect to regulate cytotrophoblast development, we consider two general possibilities. In the first, activin and TGF-β independently regulate processes that control the ability of cytotrophoblast cells to migrate and differentiate along the invasive pathway. The sum of the inhibitory effects of TGF-β, the inducing effects of activin and blocking effects of its inhibitor, follistatin, would determine whether a floating-type villus tip develops sprouts of cytotrophoblasts to form an anchoring type villus. Alternatively, TGF-β and activin could act in series. TGF-β, which could be produced by syncytiotrophoblast (51), would act on the villous cytotrophoblasts and reduce activin expression or activity (perhaps through follistatin production or effects on activin receptor expression). Activin mediated processes necessary for cytotrophoblast migration and differentiation would in turn therefore be reduced. Based on our current data, we suggest that elements of both pathways may be correct and propose a general model summarized in Fig. 9. The basis of the model is the finding that, whereas exogenous TGF-β3 was a potent inhibitor of cytotrophoblast outgrowth, it was unable to block activin-induced increase in fibronectin synthesis. Conversely, follistatin ablated fibronectin synthesis, but only effectively blocked morphological outgrowth when added at the start of the experiment. Together these data suggest that while activin and TGF-β may each have direct effects on cytotrophoblast cells, activin may be the direct regulator of certain responses, such as fibronectin synthesis, and TGF-β effects may be indirect. Other experimental approaches that may be useful to clarify this pathway include determining if the effects of inhibition of TGF-β action and addition of

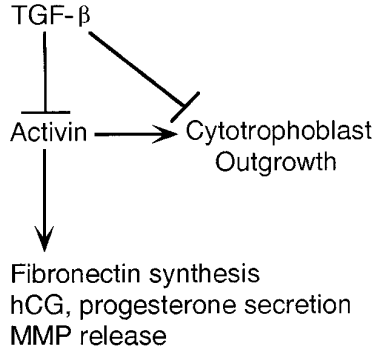

FIG. 9. Activin, its inhibitor follistatin, and TGF-β are local regulators of villous cytotrophoblast differentiation. Diagram depicts a model of how activin and TGF-β may interact to regulate cytotrophoblast outgrowth and the expression of specific cytotrophoblast responses (*arrows* indicate activation, whereas *bars* indicate inhibition). See text in *Discussion* for details.

activin have additive effects; if all effects of TGF-β occur upstream of activin one would predict that effects would not be additive. Such actions of TGF-β could be exerted through direct effects on activin production, or through induction of either follistatin, the activin inhibitor, or inhibin, a factor which has opposing effects in other cell systems, such as luteal cells (52). Conversely, TGF-β could affect activin receptor expression or downstream signaling events. Ongoing experiments will explore the nature of the interaction between activin and TGF-β in detail, by testing some of these hypotheses.

Acknowledgments

We thank Lindsay McWhirter for collection of placentae; Drs. Michelle Letarte, Susan Fisher, and Caroline Damsky for antibodies; Genentech for recombinant human activin-A and inhibin-A; the National Hormone and Pituitary Program for recombinant human follistatin; Dr. Martin Post for oligonucleotide synthesis and recombinant TGF-β3; and Dr. Knox Ritchie for enthusiastic support.

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