Gelatinase and oncofetal fibronectin secretion is dependent on integrin expression on human cytotrophoblasts*

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Collagenolytic activity of cytotrophoblasts is stimulated by glycoproteins of the extracellular matrix and since this stimulation can possibly occur through integrins, we measured the gelatinolytic activity of villous and extravillous cytotrophoblasts according to the type of integrins expressed on these cells. Cytotrophoblasts were isolated from legal abortions, immunopurified with anti-CD45, separated according to their expression of histocompatibility-linked antigen (HLA)-G, α_6 or α_5 integrin subunits and cultured for 5 days on plastic or agarose. Fetal fibronectin, human chorionic gonadotrophin (HCG) and the gelatinolytic activity were measured in the culture supernatants. Following immunopurification with anti-CD45, the gelatinolytic activity of cytotrophoblasts was significantly higher than before, indicating that contaminating lymphomyeloid cells secreted gelatinolytic inhibitors. HLA-G positive cells secreted significantly more gelatinases than HLA-G negative cells but their HCG secretion was similar. Compared to α_5 positive cells, α_6 positive cytotrophoblasts secreted significantly more gelatinases, significantly less fibronectin but similar amounts of HCG. We conclude that during trophoblast invasion, extravillous cytotrophoblasts (HLA-G positive) expressing the α_6 integrin subunit represent the invasive population of cells (high gelatinase and low fibronectin secretion). When expression of the α_5 integrin subunit is turned on, their invasive behaviour ceases and they secrete low amounts of gelatinases and high concentrations of fibronectin.
Key words: fibronectin/gelatinase/integrins/trophoblast invasion

Introduction

Implantation and placentation in humans are dependent upon a tightly controlled invasion of the maternal endometrium by fetal trophoblast. Deregulation of this process in either direction leads to severe pathological conditions: insufficient invasion is associated with pre-eclampsia and inadequate fetal growth (Khong et al., 1986; Zhou et al., 1993), whereas unrestricted invasion leads to pre-malignant conditions such as hydatidiform mole.

After initial attachment of the blastocyst to the uterine lining, mononuclear cytotrophoblasts, which surround the embryonic disc, fuse to form syncytia (Kao et al., 1988). These multinucleated giant cells invade the pseudodecidualized endometrium (Weitlauf, 1988). Once the definitive placental villi are formed, some cytotrophoblasts of anchoring villi (which contact the uterine wall) acquire a transiently invasive phenotype and invade the decidualized endometrium, while the cytotrophoblasts of floating villi (in the extravillous space) remain attached to the villous basement membrane. Thus, cytotrophoblasts follow one of two existing differentiation pathways: villous cytotrophoblasts form a monolayer of polarized epithe-

lial stem cells which eventually differentiate by fusion to form a syncytial layer (Kao et al., 1988) which covers the entire surface of the villous. Cytotrophoblasts, in anchoring villi, either follow the differentiation pathway to form syncytia or break through the syncytium at selected sites to form multi-layered columns of non-polarized cytotrophoblasts. These motile and highly invasive cells, also referred to as intermediate trophoblasts (Enders, 1968), are found as cytokeratin positive cells in the decidua, the intima of uterine blood vessels and the proximal third of the myometrium. The molecular mechanism which directs cytotrophoblasts into one or the other differentiation pathway is the subject of intensive research in many centres.

Villous and extravillous cytotrophoblasts are morphologically and functionally distinct (Enders, 1968; Damsky et al., 1992; Genbacev et al., 1993). They are both cytokeratin positive, indicating their common epithelial nature, but only intermediate cytotrophoblasts express a non-classical human leukocyte class I antigen (HLA-G) (Chumbley et al., 1993; Shorter et al., 1993) whereas only villous cytotrophoblasts have epidermal growth factor receptors (Mühlhauser et al., 1993). Furthermore, villous and intermediate cytotrophoblasts are in a different microenvironment. Villous cytotrophoblasts

are bound to a basement membrane composed of collagen type IV, fibronectin and laminin whereas intermediate cyto-trophoblasts, depending on their location (proximal or distal), are surrounded by a matrix composed either of modified laminin or fibronectin and collagen type IV respectively (Damsky et al., 1992).

Extracellular matrix components are known to influence adhesion, spreading, migration and differentiation of cells. The cells recognize their microenvironment through specific membrane receptors. Among the different adhesion molecules, integrins mediate essentially but not exclusively the interactions between cells and the glycoproteins of the extracellular matrix (Ruoslahti, 1991; Heino, 1993). Integrins are heterodimeric transmembrane glycoproteins composed of an α and a β subunit. So far eight β and 14 α subunits have been identified, forming a family of about 20 receptors. Depending on the type of α/β combination the integrins will bind to one or another matrix glycoprotein, i.e. $\alpha_5\beta_1$ to fibronectin, $\alpha_6\beta_1$ to laminin etc. The specificity of integrins, however, is still a matter of speculation since several integrins can bind the same glycoproteins and since several glycoproteins can bind to the same integrin (Ruoslahti, 1991; Heino, 1993).

In the particular case of trophoblast, several studies (Damsky et al., 1992; Korhonen et al., 1991; Aplin, 1993; Burrows et al., 1993) including our own (Bischof et al., 1993) have shown that villous and intermediate cytotrophoblasts express different integrins. While villous cytotrophoblasts express predominantly the $\alpha_6\beta_4$ integrin (a probable laminin receptor, Lee et al. 1992) in a polarized manner (towards the basement membrane), intermediate cytotrophoblasts modulate their integrins: in the proximal region (cytotrophoblasts columns) they express $\alpha_6\beta_4$ in a non-polarized way whereas in the most distal part (the placental bed) they express the $\alpha_5\beta_1$ integrin, a fibronectin receptor. Thus, while cytotrophoblasts migrate from the villous into the decidua they modulate their integrin repertoire from being $\alpha_6\beta_4$ positive and $\alpha_5\beta_1$ negative to becoming $\alpha_6\beta_4$ negative and $\alpha_5\beta_1$ positive. This change in integrin expression goes along with the acquisition of an invasive phenotype.

Invasion is not due to passive growth pressure but to an active biochemical process. A cell is recognized to be invasive by virtue of its ability to secrete proteases and cytotrophoblasts are no exception (Bischof and Martelli, 1992). Cytotrophoblasts have been found to secrete metalloproteinases (Fisher et al., 1985, 1989; Bischof et al., 1991). These enzymes which are secreted as inactive proenzymes are grouped into three subfamilies according to their substrate specificities: colla-genases, gelatinases and stromelysins (for review, see Matri-sian, 1990). The collagenases digest collagen types I, II, III, VII and X and are thus suited for digesting the collagens of the interstitium. The gelatinases are represented by two enzymes: the 72 kDa gelatinase (gelatinase A) and the 92 kDa gelatinase (gelatinase B). These matrix metalloproteinases digest collagen type IV (the major constituent of basement membranes) and denatured collagen (gelatin). The stromelysins have a much broader digestion spectrum and degrade fibro-nectin, laminin, collagens IV, V, VII and proteoglycans. Cyto-trophoblasts essentially secrete gelatinases, and their invasive

potential seems to be mediated by the 92 kDa gelatinase (Librach et al., 1991).

Since it has been shown that the collagenolytic activity of cytotrophoblasts is specifically regulated by the glycoproteins of the extracellular matrix (Bischof et al., 1991; Emonard et al., 1990) and since this regulation could possibly occur through integrins (Werb et al., 1989, at least in rabbit skin fibroblasts), we decided to compare the gelatinolytic activity of villous and intermediate cytotrophoblasts and to see to what extent this proteolytic capacity was dependent on the type of integrins expressed on cytotrophoblasts.

Materials and methods

Cell preparation and separation

Cytotrophoblasts were prepared and purified according to a method already reported by us (Bischof et al., 1991). Briefly, trophoblastic tissue obtained from induced abortions (6–12 weeks of pregnancy) was digested with trypsin, separated from blood cells and syncytia on a discontinuous Percoll gradient and immunopurified by an antibody (anti-CD45) coupled to magnetic particles (Dyna beads; Dynal, Milian, Geneva, Switzerland) in order to eliminate all contaminating lympho-myeloid cells. These cytotrophoblasts were counted in a Neubauer haemocytometer in the presence of Trypan Blue (Sigma) and either put into culture as such (10^6 cells/ml) or separated further. In order to separate the purified cytotropho-blasts into HLA-G positive and negative cells, the purified cytotrophoblasts were resuspended (5×10^6 cells in 2 ml) in Dulbecco's modified Eagle's medium (DMEM; Gibco, Basel, Switzerland) containing 10% fetal bovine serum. The suspen-sion was then incubated for 30 min at 4°C with 30 μl of a mouse monoclonal antibody which detects a common frame-work epitope of histocompatibility-linked antigen (HLA)-A, B, C (Dako-HLA-ABC, W6/32; Dako, IG Geneva, Switzerland). Since extravillous cytotrophoblasts do not express HLA-A, B or C (Hunt et al., 1990) but express HLA-G which is also recognized by the antibody W6/32, the cytotrophoblasts, positive for W6/32 will be called HLA-G positive hereafter.

After incubation the cells were washed with phosphate-buffered saline solution containing 0.1% bovine serum albumin (BSA, radioimmunoassay grade; Sigma). Prewashed (with phosphate-buffered saline solution and BSA) magnetic particles coated with a second antibody (30 μl Dyna beads) were then incubated with the cell suspension for 20 min at 4°C. A magnet was then applied along the test tube to retain the particles bound to the cells expressing HLA-G on their surface. The supernatant (HLA-G negative cells) was poured into a clean test tube and cells counted (as mentioned above). The cells immobilized by the magnet (HLA-G positive) were washed and resuspended in culture medium.

The same separation technique was also used with primary antibodies to the α_5 integrin subunit (CDw49e, clone SAM 1) and with antibodies to the α_6 integrin subunit (CDw49f, clone GoH3; both antibodies from Immunotech, Marseille, France). This allowed us to obtain α_6 positive and α_6 negative as well as α_5 positive and α_5 negative cytotrophoblast suspensions.

735

Culture conditions

Purified cytotrophoblasts (200 μl), HLA-G positive and HLA-G negative cytotrophoblasts, α_5 positive and α_5 negative cytotrophoblasts and α_6 positive and α_6 negative cytotrophoblasts (10^6 cells/ml) were incubated in duplicates in 12-well tissue culture plates (Costar, Cambridge, MA, USA) under a 5% CO_2 and 95% air atmosphere in an incubator at 37°C. Certain wells were precoated with 300 μl of 0.5% agarose in phosphate-buffered saline (Agar Noble Difco). The culture medium was DMEM containing 2 mmol/l L-glutamine (Gibco), 4.2 mmol/l magnesium sulphate, 25 mmol/l HEPES, 1% gentamycin, 1% amphotericin B, 100 μg/ml streptomycin and 100 IU/ml penicillin in the absence of serum. Medium was harvested on day 2, and the culture stopped on day 5. The supernatants were divided into aliquots and stored at −20°C until assayed. The cells were lysed with 200 μl of Triton X-100 (2.5% in water) and stored at −20°C.

Hormone and protein assays

Total human chorionic gonadotrophin (HCG + βHCG) was measured in the supernatants by a microparticle enzyme immunoassay with a sensitivity of 1 mIU/ml and a coefficient of variation of 3.6% (Abbott, Abbott Park, IL, USA). Fetal fibronectin was measured by a commercially available enzyme immunoassay with a sensitivity of 50 ng/ml and a coefficient of variation of 7.5% (Adeza Biochemical, Sunnyvale, CA, USA). Total cell proteins were measured by the Bio-Rad protein assay according to the manufacturer's instructions and using BSA as the standard (Bio-Rad, Munich, Germany).

Gelatinase assays

Qualitative assessment of the secreted gelatinases was performed by zymography as previously reported by us (Bischof *et al.*, 1991). Quantitative estimation of gelatinolytic activity in the culture supernatants was performed by measuring the degradation of heat-denatured radiolabelled collagen type IV, using a method modified from Emonard *et al.* (1990). Briefly, tritiated human type IV collagen (N-[2,3-^{3}H]propionate), sp. act. 0.0067 GBq/mg; du Pont de Nemours, Geneva, Switzerland) was neutralized at pH 7.4 with Tris-HCl buffer (50 mM) containing 0.15 M NaCl, 4 mM $CaCl_2$ and incubated in a water bath at 60°C for 30 min to form gelatin. After cooling, N-ethylmaleimide (NEM, 0.5 mM; Sigma) and phenylmethylsulphonyl fluoride (PMSF, 0.1 M; Sigma) were added to inhibit thiol and serine proteases respectively. This solution contained 10 000 cpm of radiolabelled gelatin per 100 μl. Collagenase (EC 3.4.24.3) from *Clostridium histolyticum* (330 U/mg; Sigma) was used as a standard. The standard curve ranged from 0.8 to 50 ng/ml (0.26–16.5 mU/ml).

Aliquots of 100 μl of sample or standards were pipetted in duplicates into assay tubes in the presence or absence of 15 μl of 0.4 M ethylenediaminotetraacetate (EDTA, final concentration 25 mM). In order to activate the gelatinases to be measured, all tubes received 15 μl of 2.4 mM 4-aminophenylmercuric acetate (APMA; Sigma) in Tris buffer (0.05 M Tris, 0.05% Triton X-100, 5 mM $CaCl_2$, 0.02% NaN_2, pH 7.0) and the tubes were incubated at 25°C for 15 min. Substrate (100 μl, 10 000 cpm) was added and the tubes

incubated at 37°C overnight. After incubation, the tubes were put on ice and undigested gelatin was precipitated by addition of 100 μl of 36% trichloroacetic acid (TCA; Sigma) and incubated for 1 h at 4°C. The incubation mixture was finally centrifuged at 6000 g for 25 min at 4°C and an aliquot of the supernatants (100 μl) was added to 3 ml of scintillation cocktail (Luma Gel; Medipro, Teufen, Switzerland) and the samples counted in a liquid scintillation counter (Packard) at an efficiency of 35%. The gelatinolytic activity of the cell supernatants was calculated by comparison to the standard curve run in parallel and expressed (as mean of duplicates in the absence of EDTA minus mean of duplicates in the presence of EDTA) in ng per mg total cell proteins or ng per 10^6 cells.

Adhesion assay

Cytotrophoblasts and the different cytotrophoblast subsets were cultured for 2 days (as described above) in 12-well tissue culture plates (Costar, Cambridge, MA, USA) precoated (50 μg/ml for 4 h at room temperature) with fibronectin (Boehringer, Mannheim, Germany), laminin (Sigma), BSA (Sigma), agarose (as described above) and Matrigel (200 μl, diluted 1/5; Collaborative Research, Inotech, Switzerland). After culture, the supernatants were aspirated and the unattached cells pelleted by centrifugation. The attached and unattached cells were lysed by water and by two freezing/thawing cycles. Total DNA was measured by fluorimetry and compared to a plasmid DNA of known concentration. Results were expressed as percentage of DNA of attached cells versus DNA of total cells (attached and non-attached).

Immunohistochemistry

Aliquots of cytotrophoblasts, HLA-G negative, α_5 negative and α_6 negative cytotrophoblasts were taken before the cells were put into culture. The aliquots were centrifuged at 700 g onto glass slides with a cytospin centrifuge (Shandon, IG, Geneva, Switzerland). They were fixed in acetone for 5 min at −20°C and stained as described previously (Bischof *et al.*, 1991) using anti-CD45, anti HLA-G, anti-CDw49e and anti-CDw49f as primary antibodies (dilution 1:20). The slides were evaluated under the microscope and the results expressed as percentage of positive cells per 300 total cells counted.

Results

Immunohistochemistry

As shown in Table I, purified cytotrophoblasts (after immunoadsorption of CD45 positive cells) were still contaminated by

Table I. Immunohistochemical study on cell separation (percentage of positive cells, mean ± SEM, n = 3)

Antibodies	Cytotrophoblasts		
	Purified	α_6 negative	α_5 negative
Anti-CD45	3.0 ± 0.5	−	−
Anti-HLA-G	29.6 ± 8.6	−	−
Anti-α_6	30.5 ± 8.1	6.4 ± 0.8	27.9 ± 7.1
Anti-α_5	19.2 ± 1.8	30.5 ± 8.1	4.4 ± 0.3

~3% of lymphomyeloid cells. About one-third of the cells expressed HLA-G and one-third expressed the integrin subunit α_6, whereas 19.2% of the purified cytotrophoblasts expressed the α_5 subunit. After separation of the cells with anti-integrin antibodies, the α_6 negative cytotrophoblasts still contained ~6% of cells expressing the α_6 integrin subunit, whereas only 30.5% of them expressed the α_5 subunit. Similarly, the α_5 negative cytotrophoblasts were still contaminated by 4.4% of cells expressing the α_5 subunit, and 27.9% of the α_5 negative cells expressed the α_6 subunit.

Thus, the separation technique with the anti-α_5 or anti-α_6 antibodies and the magnetic particles allowed a relatively good separation of cells expressing the integrin α_5 or α_6, but left ~4–6% of positive cells in the fractions which were supposed to be negative for these integrins. Immunohistochemistry for the α_5 and α_6 integrin subunits could not be performed on the positive cells since these cells were already saturated with the antibodies and the magnetic particles used during the separation procedure.

Proteolytic activity of cytotrophoblasts

In the absence of APMA, which activates the progelatinases into active gelatinases even when bound to tissue inhibitor of metalloproteinases (TIMP), the assay measures the gelatinases secreted as active enzymes. These were below the sensitivity of the assay (0.4 ng/ml) for cytotrophoblasts grown for 2 days on agarose or plastic (Figure 1). Cytotrophoblasts grown on Matrigel released low but measurable amounts of active gelatinases (0.5 + 0.02 ng/ml, SD).

In the absence of EDTA but in the presence of APMA, the test measures the total gelatinolytic activity due to metalloproteinases but also to other enzymes. The secreted activity was high for all cytotrophoblasts irrespective of the substrate on which the cells were grown.

The gelatinase activity measured in the presence of both APMA and EDTA (APMA is inhibited by EDTA) represents the secreted activity of in-vitro activated prometalloproteinases (called gelatinolytic activity hereafter). This activity was significantly higher in supernatants of cytotrophoblasts grown on Matrigel or plastic ($P < 0.01$, $P < 0.05$ respectively) as compared to cells grown on agarose (Figure 1).

As shown in Figure 2, after immunopurification of the cytotrophoblasts with antibodies to CD45 (to remove lymphomyeloid cells), their secreted gelatinolytic activity was significantly higher ($P < 0.05$) than before immunopurification. This difference could be observed irrespective of the substrate on which the cells were grown.

Adhesion assay

Irrespective of the cytotrophoblasts subset, attachment of the cells to agarose or to BSA was very weak (6–20%, Figure 3). Attachment of cytotrophoblasts to fibronectin or to laminin (48.3 and 45.9% respectively) was significantly weaker ($P < 0.05$) than to plastic (88.0%). As compared to purified cytotrophoblasts, the number of α_5 positive cytotrophoblasts and α_5 negative cytotrophoblasts attaching to plastic or Matrigel was significantly reduced ($P < 0.05$ to $P < 0.001$, Figure 3).
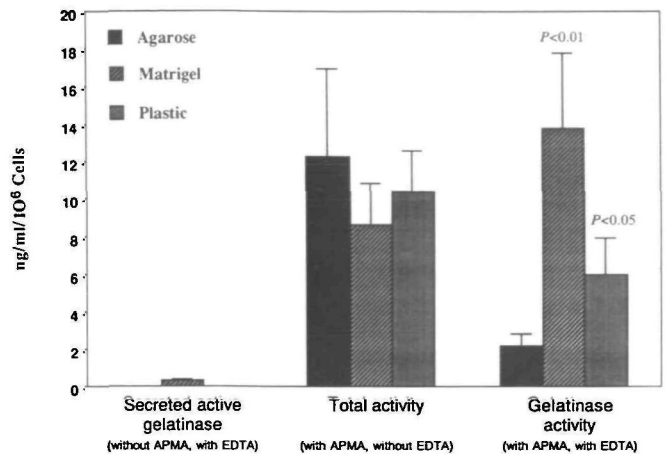
Figure 1. Gelatinolytic activities secreted by purified cytotrophoblasts grown for 2 days on agarose, Matrigel or plastic ($n = 4$; mean ± SD). APMA = 4-aminophenylmercuric acetate; EDTA = ethylenediaminotetraacetate.
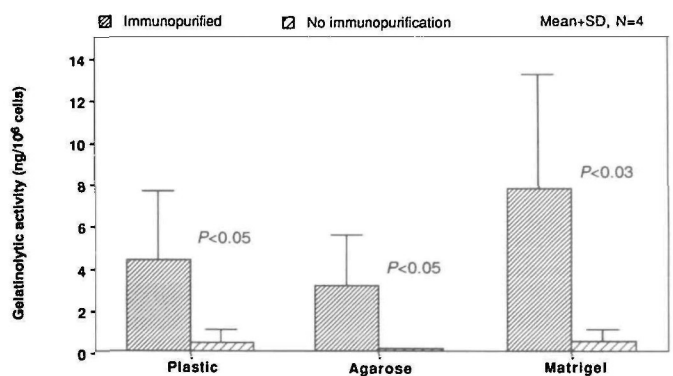

Figure 2. Gelatinolytic activity secreted by cytotrophoblasts grown for 2 days on agarose, Matrigel or plastic. Comparison between purified (with CD45) and non-purified cells.

Attachment of α_5 negative cells to fibronectin and to laminin (29.6 and 23.7% respectively) was significantly reduced when compared to α_5 positive cytotrophoblasts (67.5 and 48.2%, $P < 0.05$ and $P < 0.001$ respectively). The number of α_6 positive cytotrophoblasts which attached to plastic (50.4%) was significantly lower ($P < 0.03$) than purified cytotrophoblasts (88.0%). Attachment of α_6 negative cytotrophoblasts to laminin was significantly ($P < 0.05$) reduced as compared to purified cytotrophoblasts (16.9 versus 45.9%, Figure 3).

Separation of cytotrophoblasts by HLA-G

When grown on plastic, HLA-G positive cells secreted significantly ($P < 0.05$) higher amounts of gelatinolytic activity than HLA-G negative cells (209 and 19.5 ng/mg protein respectively, Figure 4). HLA-G negative cells grown on agarose secreted significantly more ($P < 0.05$) gelatinases than the same cells grown on plastic (31.8 and 19.5 ng/mg protein respectively). HLA-G positive or negative cells, irrespective of the substrate on which they grew, secreted similar concentrations of HCG.
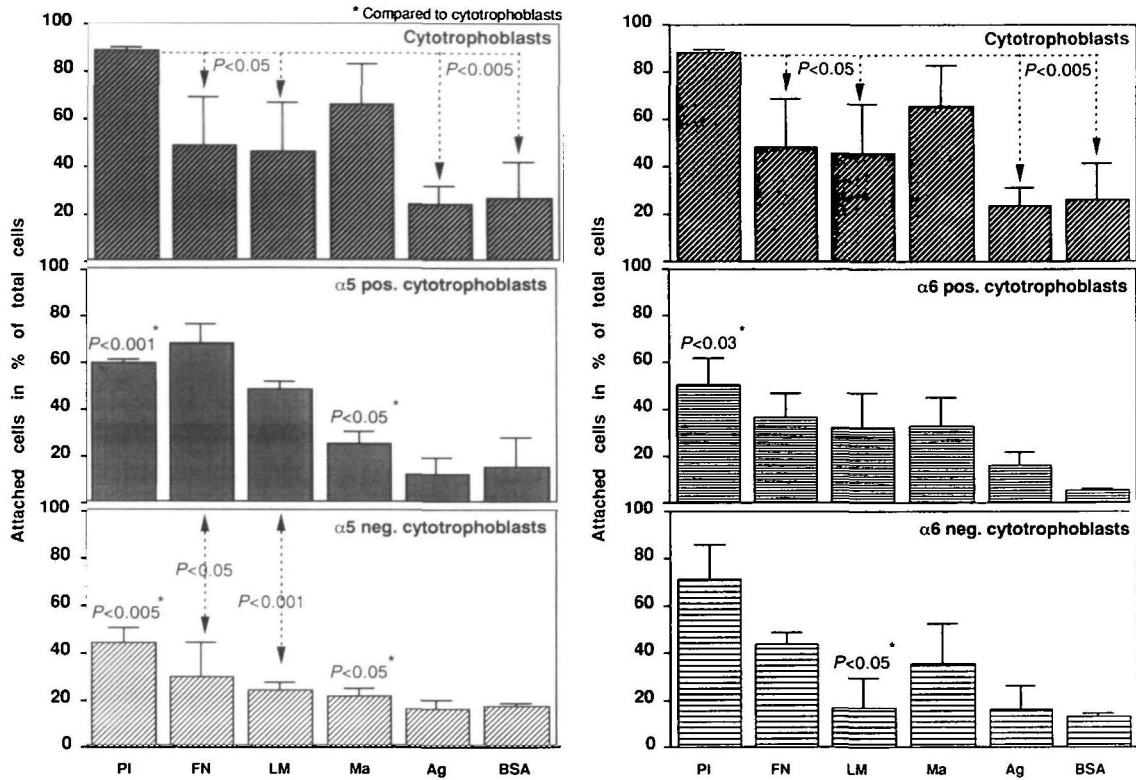
Figure 3. Attachment of different cytotrophoblast subsets after 48 h of culture on plastic (Pl), fibronectin (FN), laminin (LM), Matrigel (Ma), agarose (Ag) and bovine serum albumin (BSA). Values are mean ± SD of three different cultures. Pos. = positive; neg. = negative.

Separation of cytotrophoblasts by integrins

As can be seen on the zymogram (Figure 5), α_6 positive cytotrophoblasts expressed a much higher proteolytic activity than α_6 negative cytotrophoblasts, irrespective of the substrate on which they grew. The major digestion band had a molecular size of 92 kDa. A minor faint digestion band with a molecular size >200 kDa was also visible. Similar results were observed when supernatants of α_5 positive and negative cells were compared (results not shown).
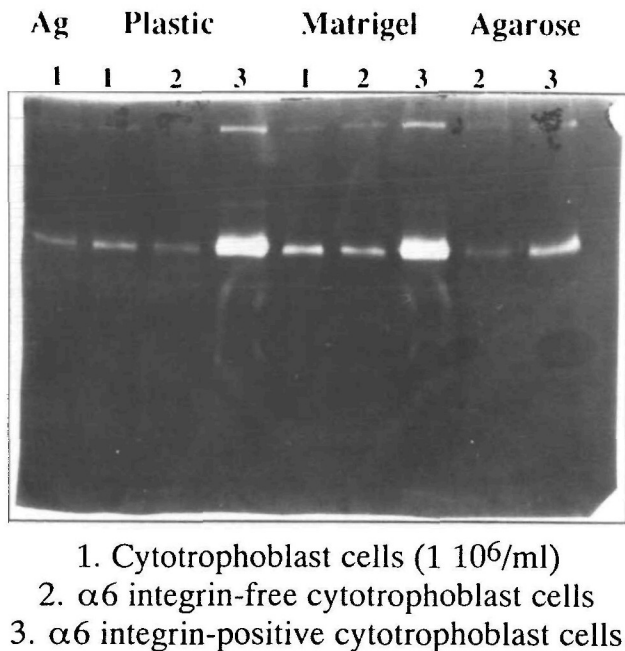
The gelatinolytic activity secreted by α_6 positive cytotrophoblasts grown on agarose or plastic was significantly higher ($P < 0.03$ to $P < 0.001$) than the activity secreted by purified cytotrophoblasts or by the other cytotrophoblast subsets (α_6 negative, α_5 positive, α_5 negative). Compared to agarose, α_6 positive cytotrophoblasts grown on plastic secreted a significantly ($P < 0.05$) higher gelatinolytic activity. In addition, α_5 positive cells secrete a significantly ($P < 0.05$ on plastic, $P < 0.03$ on agarose) higher gelatinolytic activity than α_5 negative cells (Figure 6).

Despite the fact that adhesion of the cells to plastic increased significantly ($P < 0.05$) their fetal fibronectin secretion, α_6 positive cytotrophoblasts secreted significantly less fetal fibronectin than α_5 positive cytotrophoblasts ($P < 0.03$ on agarose, $P < 0.05$ on plastic). Furthermore, the fetal fibronectin secretion of α_5 positive cytotrophoblasts was significantly higher that of α_5 negative cytotrophoblasts or of purified cytotrophoblasts ($P < 0.03$, $P < 0.05$ respectively, Figure 6).

In contrast, α_6 positive and α_5 positive cells secreted similar
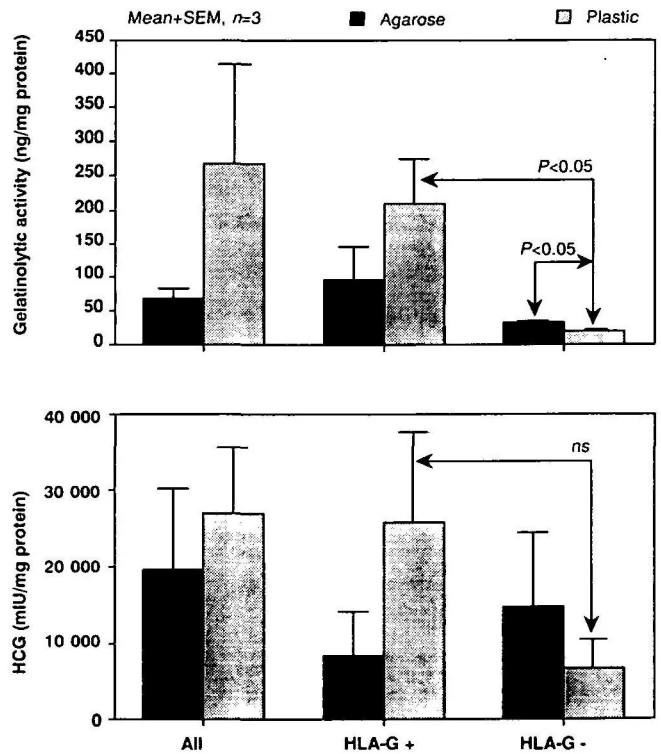
Figure 4. Gelatinolytic activity and human chorionic gonadotrophin (HCG) secretion of purified cytotrophoblasts (all), HLA-G positive cytotrophoblasts (HLA-G +) and HLA-G negative cytotrophoblasts (HLA-G−) grown in culture for 48 h.

Ag   Plastic   Matrigel   Agarose

1   1   2   3   1   2   3   2   3

1. Cytotrophoblast cells (1 10^6/ml)
2. $\alpha 6$ integrin-free cytotrophoblast cells
3. $\alpha 6$ integrin-positive cytotrophoblast cells

Figure 5. Zymogram of 2 day culture supernatants from cytotrophoblast subsets grown on agarose (Ag), plastic or Matrigel.

Figure 6. Gelatinolytic activity, fibronectin and human chorionic gonadotrophin (HCG) secretion of different cytotrophoblast subsets grown for 48 h on agarose or plastic. Values are mean ± SD of three different cultures.

amounts of HCG. Adhesion of these cells to plastic, however, increased significantly ($P < 0.05$) their HCG release as compared to cells grown on agarose. Finally, α_5 positive cytotrophoblasts grown on plastic secreted significantly ($P < 0.05$, Figure 6) more HCG than α_5 negative cytotrophoblasts or purified cytotrophoblasts grown on plastic. In summary, α_6 positive cytotrophoblasts secreted large amounts of gelatinolytic activity and low concentrations of fetal fibronectin, whereas α_5 positive cytotrophoblasts secreted small amounts of gelatinolytic activity and high concentrations of fetal fibronectin; their HCG secretion was not significantly different.

Comparison of gelatinolytic activity secretion rates

Figure 7 compares, for all cytotrophoblast subsets, their localization in the anchoring villous (from Korhonen *et al.*, 1991; Damsky *et al.*, 1992; Aplin 1993; Burrows *et al.*, 1993; Bischof *et al.*, 1993) together with their gelatinolytic activity secretion rate expressed as the activity secreted per mg protein and per 24 h over a period of 5 days in culture. Cytotrophoblasts, HLA-G positive and α_5 positive cytotrophoblasts secreted gelatinases at a similar rate to purified cytotrophoblasts. In contrast, α_6 positive cytotrophoblasts secreted gelatinases at a significantly higher rate ($P < 0.03$) compared with α_5 positive cytotrophoblasts. As compared to their positive counterparts or to purified cytotrophoblasts (data not shown), HLA-G negative, α_5 negative and α_6 negative cytotrophoblasts secreted gelatinases at a significantly lower rate ($P < 0.05$, Figure 7).

Discussion

The population of cytotrophoblasts used here was purified by immunoadsorption with an antibody to CD45 and with a magnetic particle separation. This technique yielded a 97%
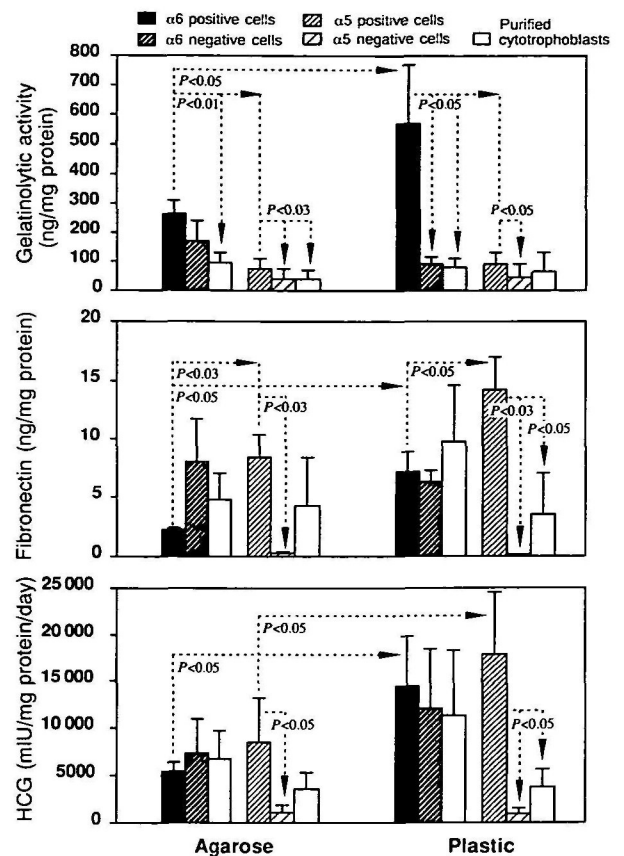
pure cytotrophoblast preparation with a 3% contamination by lymphomyeloid cells. Using the same technique to separate cytotrophoblasts according to the integrins they express, the same sort of results were observed: 4–6% of cells were left behind.

As reported by us here and previously (Bischof *et al.*, 1991), purified cytotrophoblasts grown on agarose do not attach to this substrate at all or do so very poorly, but remain viable and floating in the supernatant. Purified cytotrophoblasts grown on Matrigel and plastic do attach. These cytotrophoblasts did not secrete active gelatinases when grown on agarose or plastic, but released small amounts of active gelatinases when grown on Matrigel. Thus gelatinases are secreted by cytotrophoblasts mainly in their inactive proenzyme form. Binding of the cells to Matrigel or plastic activated their secretion of gelatinases, whereas when the cells remained in suspension (as on agarose), their secretion of gelatinases was low. This confirms previous reports where these same differences were also observed, either semi-quantitatively on zymograms (Bischof *et al.*, 1991) or quantitatively with a similar assay to ours (Emonard *et al.*, 1990). Discarding contaminating lymphomyeloid cells from the cytotrophoblast suspension had a marked effect on their gelatinolytic activity, irrespective of the substrate on which the cells grew. Since immunopurified cytotrophoblasts secreted significantly more gelatinolytic activity than non-purified cells,

739
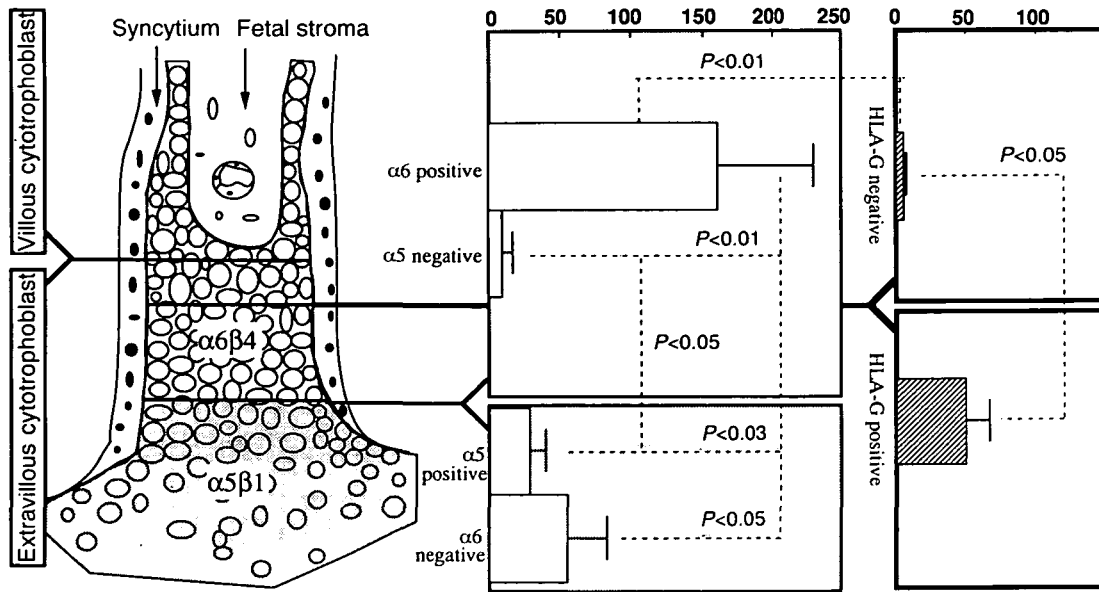
Figure 7. Comparison between localization of different cytotrophoblast subsets and their secretion rates of gelatinolytic activity as measured over 5 days in culture (mean ± SD, n = 3).

one must assume that contaminating lymphomyeloid cells did secrete factors which inhibited cytotrophoblast gelatinases. TIMP is a potent inhibitor of gelatinases which is produced by lymphomyeloid cells such as monocytes (Opdenakker *et al.*, 1991) and macrophages (Shapiro *et al.*, 1993). It could thus be that the lower gelatinolytic activity secreted by cytotrophoblasts in the presence of lymphomyeloid cells was possibly due to monocyte—macrophage-derived TIMP.

One-third of the purified cytotrophoblasts were HLA-G positive, one-third were positive for the α_6 integrin subunit and about one-fifth were positive for the α_5 subunit. An unknown proportion of the α_6 positive cytotrophoblasts must be villous cytotrophoblasts since these express also the α_6 integrin subunit (Korhonen *et al.*, 1991; Damsky *et al.*, 1992; Aplin, 1993; Burrows *et al.*, 1993; Bischof *et al.*, 1993). Extravillous cytotrophoblasts express both α_5 or α_6 integrin subunits, depending on their location within the placental bed (Korhonen *et al.*, 1991; Damsky *et al.*, 1992; Aplin, 1993; Burrows *et al.*, 1993; Bischof *et al.*, 1993). Thus the α_6 positive cytotrophoblasts used here represented a mixture of villous and extravillous cytotrophoblasts, whereas the α_5 positive cytotrophoblasts were purely extravillous cells. The extravillous trophoblast of first-trimester anchoring villi expresses both HLA-G protein (Shorter *et al.*, 1993) and mRNA (Chumbley *et al.*, 1993) together with the α_5 or α_6 integrin subunits. The cytotrophoblasts located in the proximal portion of the cell columns do not express HLA-G (Shorter *et al.*, 1993) but are α_6 positive (Korhonen *et al.*, 1991; Damsky *et al.*, 1992; Aplin, 1993; Burrows *et al.*, 1993; Bischof *et al.*, 1993). Thus, α_6 positive cytotrophoblasts and HLA-G positive cytotrophoblasts are not the same cell population but are overlapping populations. In our hands, the purely extravillous cytotrophoblast population (HLA-G positive) secreted a significantly higher gelatinolytic activity than villous cytotrophoblasts (HLA-G negative). Since the

invasive behaviour of a cell depends on its secretion of gelatinases (Mignatti and Rifkin, 1993), our observation could explain why extravillous cytotrophoblasts are invasive whereas villous cytotrophoblasts are not. It is interesting to note in this context that cytotrophoblasts positive for the α_5 integrin subunit are also exclusively extravillous cytotrophoblasts (Korhonen *et al.*, 1991; Damsky *et al.*, 1992; Aplin, 1993; Burrows *et al.*, 1993; Bischof *et al.*, 1993). These cells secrete a gelatinolytic activity which was not different from HLA-G positive cytotrophoblasts but which was significantly higher than HLA-G negative cytotrophoblasts.

The α_6 positive cytotrophoblasts expressed a gelatinolytic activity which was significantly higher than α_5 positive, α_5 negative or α_6 negative cytotrophoblasts. This cell population is a mixture of villous and extravillous cytotrophoblasts. Since villous cytotrophoblasts (HLA-G negative) expressed a very low gelatinolytic activity one must admit that the high activity secreted by the α_6 positive cells was mainly due to the extravillous α_6 positive cytotrophoblasts. The α_5 negative cytotrophoblast population is also a mixture of villous and extravillous cytotrophoblasts and should theoretically contain all α_6 positive cytotrophoblasts. In fact, these represent only 30% of the α_5 negative population. This might explain why the gelatinolytic activity of α_5 negative cytotrophoblasts was significantly smaller than that of the α_6 positive cytotrophoblasts. Obviously, cytotrophoblasts expressing neither α_6 nor α_5 were also present in the α_5 negative cytotrophoblasts suspension. An alternative explanation for the difference in the gelatinolytic activity between α_5 negative and α_6 positive cytotrophoblasts could relate to the use of α_6 antibodies used to isolate the cells. Function-perturbing anti-integrin antibodies have been shown to induce collagenases (Werb *et al.*, 1989). We cannot rule out the possibility that our α_6 antibody has induced the secretion of gelatinases. However, this is certainly not the case with the α_5 antibody (α_5 positive cells had a low

gelatinolytic activity). Furthermore, the anti-α_6 and anti-α_5 antibodies we used do not seem to be function-perturbing antibodies since the attachment of α_6 positive cytotrophoblasts or α_5 positive cytotrophoblasts to laminin or fibronectin was similar to that of purified cytotrophoblasts.

Compared to the α_5 positive cytotrophoblasts, the extravillous α_6 positive cytotrophoblasts are located more proximally to the villous stem, whereas the α_5 positive cytotrophoblasts are localized more distally in the placental bed (Korhonen et al., 1991; Damsky et al., 1992; Aplin, 1993; Burrows et al., 1993; Bischof et al., 1993). Our results thus suggest that once the cytotrophoblasts have reached the placental bed and express the α_5 integrin subunit, their gelatinolytic activity is lower than when they express the α_6 integrin subunit and migrate along the cell columns. Since the α_5 positive cytotrophoblasts secrete significantly higher concentrations of fibronectin than α_6 positive cytotrophoblasts, one could postulate that once cytotrophoblasts express the $\alpha_5\beta_1$ integrin, their invasive behaviour and gelatinase secretion have almost stopped and the cells become immobile and secrete fibronectin. In contrast, extravillous α_6 positive cytotrophoblasts are invasive and motile cells which secrete large amounts of gelatinases but little fibronectin.

As shown by immunohistochemistry (Korhonen et al., 1991; Damsky et al., 1992; Aplin, 1993; Burrows et al., 1993; Bischof et al., 1993) the $\alpha_6\beta_4$ integrin in villous cytotrophoblasts has a focal distribution along the villous basement membrane, whereas this integrin is not clustered on extravillous α_6 positive cytotrophoblasts. Since clustering of integrins occurs upon binding to its ligand (Kornberg et al., 1992), focally distributed integrins are thus observed on immobile, anchored cells. Therefore it seems reasonable to suppose that villous cytotrophoblasts become motile and invasive when they depolarize their $\alpha_6\beta_4$ integrins. If this is correct, then one must admit that depolarization of the $\alpha_6\beta_4$ integrin is accompanied by an up-regulation of the gelatinolytic activity and a down-regulation of the fibronectin secretion. How these events might be connected is unknown. Since Werb et al. (1989) elegantly demonstrated in rabbit synovial fibroblasts that collagenase induction was a result of transduction through the $\alpha_5\beta_1$ integrin, one might speculate that gelatinase expression in extravillous cytotrophoblasts might be turned on and fibronectin secretion turned off by a lack of transduction through the $\alpha_6\beta_4$ integrin due to the depolarization of this integrin.

Acknowledgements

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