Molecular Human Reproduction vol.2 no.9 pp. 679–684, 1996

Purification and characterization of placental heparanase and its expression by cultured cytotrophoblasts

Ran Goshen[1,2,4], Abraham A.Hochberg[1], Gil Korner[3], Ehud Levy[3], Rivka Ishai-Michaeli[3], Michael Elkin[1], Nathan de Groot[1] and Israel Vlodavsky[3]

[1]Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University, Jerusalem, [2]Department of OB/Gyn, Hadassah University Hospital, Mount Scopus, Jerusalem, and [3]Department of Oncology, Hadassah University Hospital, Ein-Kerem, Jerusalem, Israel

[4]To whom correspondence should be addressed at: Department of Biological Chemistry, The Silberman Institute of Life Sciences, The Hebrew University, Givat-Ram, Jerusalem, 91904 Israel

The role of different extracellular matrix (ECM)-degrading enzymes in the normal functioning of the placenta is well documented. Heparan sulphate proteoglycan (HSPG) is an integral constituent of the placental and decidual ECM. Because this proteoglycan specifically interacts with various macromolecules in the ECM, its degradation may disassemble the matrix. Hence, in the case of the placenta, this may facilitate normal placentation and trophoblast invasion. Crude placental specimens were collected from first and third trimester placentas. Heparanase (endo-β-glucuronidase) was isolated and purified by ammonium sulphate precipitation followed by sequential chromatographies on carboxymethyl-, heparin- and ConA-Sepharose columns. The placental enzyme was further characterized for its molecular weight and specific inhibition by heparin, and was shown to resemble heparanase expressed by highly metastatic tumour cells and activated cells of the immune system. In order to locate the source of heparanase activity in the placenta, primary cytotrophoblast cultures were established. Intact cells, as well as conditioned medium and cell lysates, were analysed for heparanase activity using metabolically sulphate-labelled ECM as a natural substrate. Heparanase was highly active in lysates of cytotrophoblasts. This activity was also expressed by intact cytotrophoblasts seeded on ECM, but no activity could be detected in the culture medium. Incubation of the cytotrophoblasts in contact with ECM resulted in release of ECM-bound basic fibroblast growth factor (bFGF). We propose that the cytotrophoblastic heparanase facilitates placentation, through cytotrophoblast extravasation and localized neovascularization.

Key words: cytotrophoblast/extracellular matrix/heparanase/heparan sulphate proteoglycan/placenta

Introduction

Extravasation of blood-borne cells, whether normal or malignant, is initiated by specific adhesive interactions between circulating cells and the vascular endothelium. Once enveloped between endothelial cells (EC) and the basal lamina, the invading cells must degrade the subendothelial glycoproteins and proteoglycans in order to migrate out of the vascular compartment (Nicolson, 1988; Vlodavsky *et al.*, 1992).

Our studies on the extravasation of normal and malignant blood-borne cells focused on the degradation of heparan sulphate proteoglycans (HSPG) in a basement membrane-like extracellular matrix (ECM) produced by cultured corneal and vascular endothelial cells (Vlodavsky *et al.*, 1980, 1983). This ECM closely resembles the subendothelium *in vivo* in its morphological appearance and molecular composition (i.e. collagen type IV, HSPGs, laminin, fibronectin, and etactin) (Vlodavsky *et al.*, 1980). HSPG have been isolated from a variety of basement membranes and cell surfaces of normal and malignant cells (Hook *et al.*, 1984; Hassell *et al.*, 1986; Kjellen and Lindhal, 1991). The ability of HSPG to interact with ECM macromolecules such as collagen, laminin and fibronectin and with different attachment sites on plasma membranes suggests a key role for this proteoglycan in the self-assembly and insolubility of ECM components as well as in cell adhesion and locomotion (Wight *et al.*, 1992). Cleavage of heparan sulphate (HS) may therefore result in disassembly of the ECM and hence may play a decisive role in cell invasion associated with metastasis, inflammation and placentation (Vlodavsky *et al.*, 1992, 1995). The ability of cells to degrade HS in the ECM was studied by allowing cells to interact with a metabolically sulphate-labelled ECM, followed by gel filtration (Sepharose 6B) analysis of degradation products released into the culture medium (Vlodavsky *et al.*, 1983; Nakajima *et al.*, 1988). Expression of a HS-degrading endo-glucuronidase (heparanase) was found to correlate with the metastatic potential of various tumour cells (Vlodavsky *et al.*, 1983; Parish *et al.*, 1987; Nakajima *et al.*, 1988) and with the ability of activated cells of the immune system to leave the circulation and elicit both inflammatory and autoimmune responses (Lider *et al.*, 1989; Vlodavsky *et al.*, 1992).

Trophoblast cells form the fetal portion of the human placenta and exhibit certain tumour-like properties (Yagel *et al.*, 1988). A number of regulatory processes were shown to be involved in the interaction of cytotrophoblasts and ECM

during implantation and placentation (Cross *et al.*, 1994). During the process of implantation the trophectoderm acquires adhesive properties, where carbohydrate–lectin interactions play a crucial role. The possible role of HSPG in the pre-implantation period of mouse embryos was demonstrated by the ability of heparin or bacterial heparanase to block embryonic outgrowth on laminin, fibronectin, or isolated mouse uterine epithelial cells (Farach *et al.*, 1987). A strong correlation between HS expression and acquisition of attachment competence was further suggested by immunofluorescent studies during the attachment and outgrowth phases of mouse embryo development *in vitro* and *in utero* (Carson *et al.*, 1993). Moreover, specific HS binding proteins were identified in both mouse and human uterine epithelial cells during implantation (Raboudi *et al.*, 1992).

In view of the involvement of HS in placental development, we investigated heparanase (HS degrading enzyme) activity. For this purpose we purified the heparanase enzyme from human placenta, and characterized its properties using crude placental tissue as well as primary cultures of first and third trimester cytotrophoblasts.

Materials and methods

Materials

Recombinant human basic fibroblast growth factor (bFGF) was kindly provided by Takeda Chemical Industries (Osaka, Japan). Percoll, Sepharose 6B, heparin-Sepharose, CM-Sepharose and Superdex 75 were purchased from Kabi-Pharmacia (Uppsala, Sweden). Sodium heparin from porcine intestinal mucosa (PM-heparin, Mr 14000, Anti Fxa 165 IU/mg) was obtained from Hepar Industries (Franklin, OH, USA). Dulbecco's modified Eagle's medium (DMEM), nutrient mixture F-12, fetal calf serum (FCS), newborn calf serum (NBCS), penicillin, streptomycin, L-glutamine and saline containing 0.05% trypsin, 0.01 M sodium phosphate (pH 7.4) and 0.02% EDTA (STV) were obtained from Biological Industries (Beit-Haemek, Israel). Tissue culture dishes were obtained from Falcon Labware Division, Becton Dickinson (Oxnard, CA, USA). Tissue culture plates (4-well) were obtained from Nunc (Roskilde, Denmark). Na[^{125}I] and $Na_2[^{35}S]O_4$ were obtained from Amersham International (Amersham, UK). Trypsin, DNase-I, ConA-Sepharose, α-methyl mannoside and all other chemicals were of reagent grade, purchased from Sigma (St. Louis, MO, USA).

Methods

Isolation and purification of placental heparanase

Human third trimester placentas were homogenized and suspended in 10 mM phosphate citrate buffer (PCB; pH 6.0), sonicated and centrifuged at 900 *g* in 4°C for 15 min. Ammonium sulphate (35%) was added to the collected supernatant on ice for 1 h and centrifuged at 10 000 *g* in 4°C for another 30 min. The supernatant fraction was again collected and the ammonium sulphate concentration was increased to 55% and processed in the same manner. The pellet was resuspended in a minimum volume of 10 mM PCB pH 6.0 containing 100 mM NaCl, collected and frozen until all the placentas had been processed identically. The final pellet was dialysed against the same buffer (10 mM PCB, pH 6.0) for 3 days; the buffer being changed daily. The dialysed material was then subjected to cation exchange chromatography on CM-Sepharose (8×85 cm) equilibrated with PCB (pH 6.0) containing 0.1% 3-([3-cholamido-propyl]-dimethyl-ammonio)-1-propane-sulphonate (CHAPS). Following a wash of 400 ml with the same buffer, the column was subjected to a linear 0.25–1.4 M NaCl gradient, at a flow rate of 1 ml/min and 5 ml fractions were collected (Figure 1A). The fractions were analysed for heparanase activity by incubation with sulphate-labelled ECM. Enzyme activity was found to elute between 0.6–0.7 M NaCl. These fractions were pooled, dialysed and applied to heparin-Sepharose (1.5×15 cm column) in 10 mM PCB containing 0.2 M NaCl and 0.1% CHAPS. Elution was with a NaCl gradient (0.35–1.1 M); again fractions were analysed for heparanase activity by incubation with sulphate-labelled ECM. Enzyme activity was found to elute at 0.7–

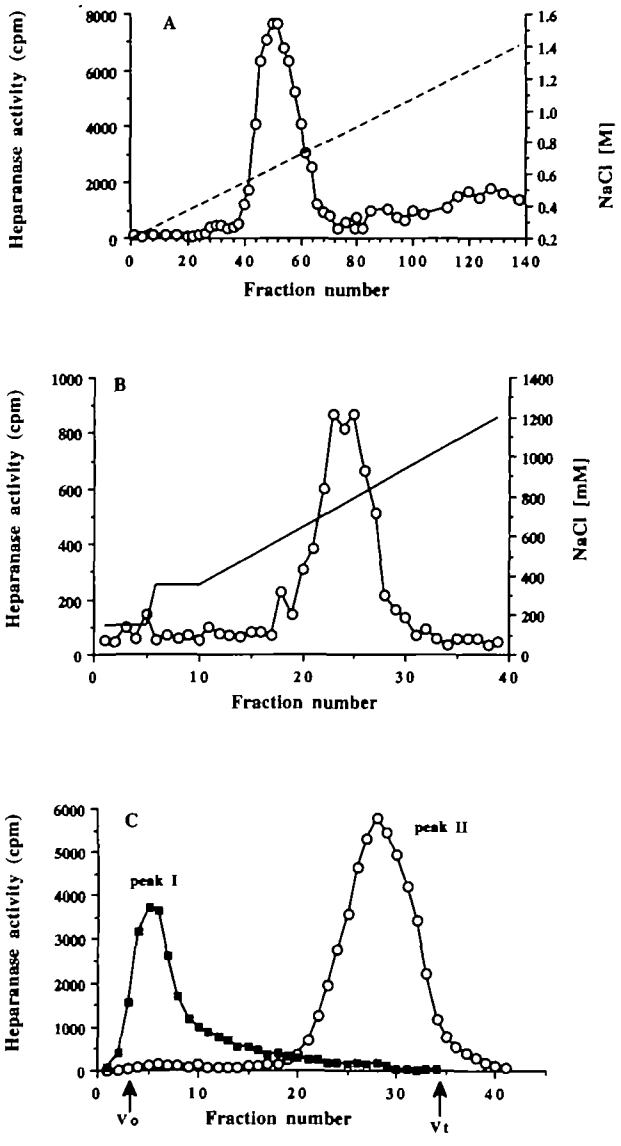

Figure 1. Purification of placental heparanase by cation exchange, heparin-Sepharose and ConA-Sepharose chromatographies. Human third trimester placentas were homogenized, precipitated with ammonium sulphate (35–55%) and subjected to chromatography on: (**A**) CM-Sephadex, and (**B**) heparin-Sepharose. Heparanse activity was determined on aliquots of each fraction as described in Materials and methods (-O-) (**C**) Con A-Sepharose chromatography. An aliquot (2.5 μl) of the material eluted from ConA-Sepharose was incubated (18 h, 37°C, pH 6.2) with sulphate-labelled ECM in the absence (○) and presence (■) of heparin, and degradation products released into the incubation medium were analysed by gel filtration over Sepharose 6B, as described in Materials and methods.

0.8 M NaCl (Figure 1B). The pooled fractions were adjusted to 1.0 M NaCl in 10 mM PCB (pH 6.0; 0.1% CHAPS) and applied to a ConA-Sepharose column. Elution was with the same buffer containing 0.25 M α-methyl mannoside.

Cells

Cultures of bovine corneal endothelial cells were established from steer eyes as previously described (Gospodarowicz *et al.*, 1977). Stock cultures were maintained in DMEM (1 g glucose/l) supplemented with 10% bovine calf serum, 5% FCS, penicillin (50 IU/ml), and streptomycin (50 μg/ml) at 37°C in 10% CO_2 humidified incubators; bFGF (1 ng/ml) was added every other day during the phase of active cell growth (Gospodarowicz *et al.*, 1977; Vlodavsky *et al.*, 1980, 1983).

Cytotrophoblasts were purified from human term placentas collected from elective Caesarean sections or first trimester placentas collected from elective termination of pregnancies during the first 8–12 weeks of pregnancy according to Gileadi *et al.* (1988). Cells were plated ($5.0–7.0\times10^5$ cells/cm^2) in 35 mm culture dishes and maintained in DMEM-F12 medium containing 10% FCS, 25 mM HEPES (pH 7.4), penicillin (180 IU/ml), streptomycin (100 mg/ml) and amphotericin B (0.2 mg/ml). The medium was replaced every 24 h.

Preparation of dishes coated with ECM

Bovine corneal endothelial cells were dissociated from stock cultures (second to fifth passage) with STV and plated into 4-well plates at an initial density of 5×10^4 cells/ml. Cells were maintained as described above, except that 5% dextran T-40 was included in the growth medium. Between 6 and 8 days after the cells reached confluence, the subendothelial ECM was exposed by lysing (3 min, 22°C) the cell layer with a solution containing 0.5% Triton-X 100 and 20 mM NH_4OH in phosphate-buffered saline (PBS) followed by four washes in PBS (Vlodavsky *et al.*, 1980, 1983, 1987). The ECM remained intact, free of cellular debris, and firmly attached to the entire area of the tissue culture dish.

For preparation of sulphate-labelled ECM, $Na_2[^{35}S]O_4$ (540–590 mCi/mmol) was added (40 μCi/ml) to cultured corneal endothelial cells, 2 and 5 days after seeding, and the cultures were incubated with the label without medium change (Vlodavsky *et al.*, 1983; Ishai-Michaeli *et al.*, 1990). The cell monolayer was dissolved and the ECM exposed 10–12 days after seeding, as described above.

Characterization of ECM degradation products

Sulphate-labelled ECM was incubated (12–48 h, 37°C, 10% CO_2 incubator) with freshly isolated cytotrophoblasts or precultured cytotrophoblasts, cell lysates or conditioned medium at pH 6.6. To evaluate the occurrence of proteoglycan degradation, the incubation medium was collected and applied for gel filtration on Sepharose 6B columns (0.9×30 cm). Fractions (0.2 ml) were eluted with PBS at a flow rate of 5 ml/h and counted for radioactivity using Bio-fluor scintillation fluid. The excluded volume (V_o) was marked by blue dextran and the total included volume (V_t) by phenol red. Peak I (Figure 1C) (Kav <0.2) was identified as virtually intact HSPG (ref); Peak II (0.5<Kav<0.8) was identified as degradation products of HS side chains (Ishai-Michaeli *et al.*, 1990, 1992). Recoveries of labelled material applied on the columns ranged from 85 to 95% in different experiments. Each experiment was performed at least three times and the variation in elution positions (Kav values) did not exceed ±15%.

Analysis of heparanase activity

Microtitre 96-well plates coated with sulphate-labelled ECM were used for screening of heparanase activity in the large number of fractions eluted in the course of enzyme purification. Samples (20–100 μl) of column fractions were added to each well, followed by incubation of the plate for 3 h at 37°C and measurements for the released radioactivity. Samples yielding the highest counts were subjected to Sepharose 6B gel filtration chromatography in order to verify that the released radioactivity consists mostly of HS degradation products.

Displacement of ECM-bound bFGF

Recombinant bFGF was iodinated using chloramine T, as described (Ishai-Michaeli *et al.*, 1990, 1992). The specific activity was $0.8–1.2\times10^5$ c.p.m./ng bFGF and the labelled preparation was kept for up to 3 weeks at −70°C. ECM was incubated for 3 h at 24°C with [^{125}I]-bFGF (2.5 ng/ml in PBS containing 0.02% gelatin). Unbound bFGF was washed away and the bFGF-labelled ECM incubated at 37°C for 3 h with third trimester cytotrophoblasts ($5.0–7.0\times10^5$ cells/cm^2) that were initially maintained in culture for 48 h, dissociated with EDTA and suspended in PBS. At the end of the incubation period, media were removed for determination of released iodinated material. The ECM subshare was incubated at 37°C for 3 h with 1 N NaOH to release the soluble iodinated material. Radioactivity was determined in a γ-counter. The percentage of released [^{125}I]-bFGF was calculated from the total ECM-associated radioactivity (Ishai-Michaeli *et al.*, 1992). The experiment was performed three times with different third trimester placentas.

Results

Isolation and purification of placental heparanase

Incubation (12 h, 37°C) of the material eluted from the ConA-Sepharose column (2.5 μl, ~ 0.1 μg/ml) with sulphate-labelled ECM resulted in release of low relative molecular weight (Mr)-labelled degradation fragments (Figure 1C, peak II, Sepharose 6B) which was inhibited in the presence of 5.0 μg/ml heparin (Figure 1C, peak II). Material eluted from ConA-Sepharose was also subjected to FPLC gel filtration (Superdex 75). Each fraction was analysed for heparanase activity (Figure 2A) and subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and silver nitrate staining (Figure 2B). Maximal activity was obtained in fractions 19 and 20, corresponding to a ~45 kDa doublet protein, the most abundant band within these fractions (Figure 2B). Studies with *N*- and *O*-glycanases indicate that the doublet proteins represent glycosylated forms of heparanase. Based on measurements of heparanase activity and protein content following each step of the purification process, we estimated the overall purification of the enzyme to exceed 2.5×10^5-fold, relative to the initial activity determined in the 55% ammonium sulphate precipitate.

Expression of heparanase activity in a primary culture of cytotrophoblasts

Freshly isolated cytotrophoblasts were seeded on top of sulphate-labelled ECM, washed free of cellular debris and blood constituents and incubated for another 48 h in the absence and presence of 5 mg/ml heparin. Sulphate-labelled material released into the incubation medium was analysed by gel filtration on Sepharose 6B. In the presence of incubation medium alone, there was a constant release of labelled material that consisted almost entirely (>90%) of large Mr fragments eluted with or next to V_o. We have previously shown that a proteolytic activity residing in the ECM itself (Korner *et al.*, 1993) and/or expressed by cells, is responsible for release of the high Mr material. On the other hand, incubation of the

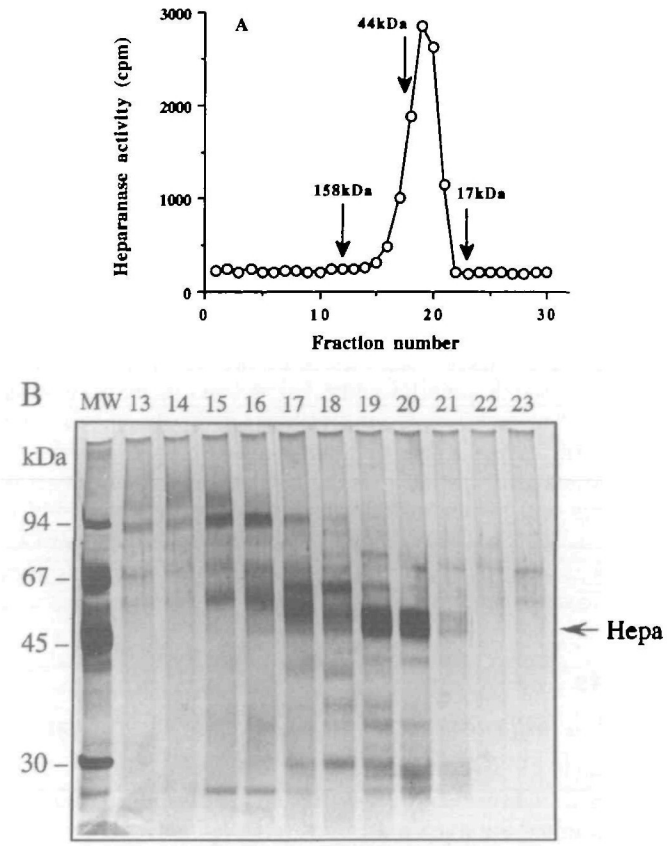

Figure 2. Gel filtration analysis and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of placental heparanase. (**A**) Gel filtration. Active fractions eluted from ConA-Sepharose were pooled and subjected to FPLC gel filtration on a Superdex 75 column (the MW markers were γ-globin 158 kDa, ovalbumin 44 kDa and myoglobin 17 kDa). Heparanase activity (2 h, 37°C, pH 6.2) in each fraction was measured using sulphate-labelled extracellular matrix (ECM)-coated wells of a 96-well plate. (**B**) SDS–PAGE. Aliquots of fractions 13–23 were subjected to electrophoresis on a 7.5–12% gradient polyacrylamide gel followed by silver nitrate staining. The molecular weight markers used were phosphorylase b (94 000), bovine serum albumin (67 000), ovalbumin (44 000) and carbonic anhydrase (31 000). The position of heparanase is marked by an arrow (Hepa).

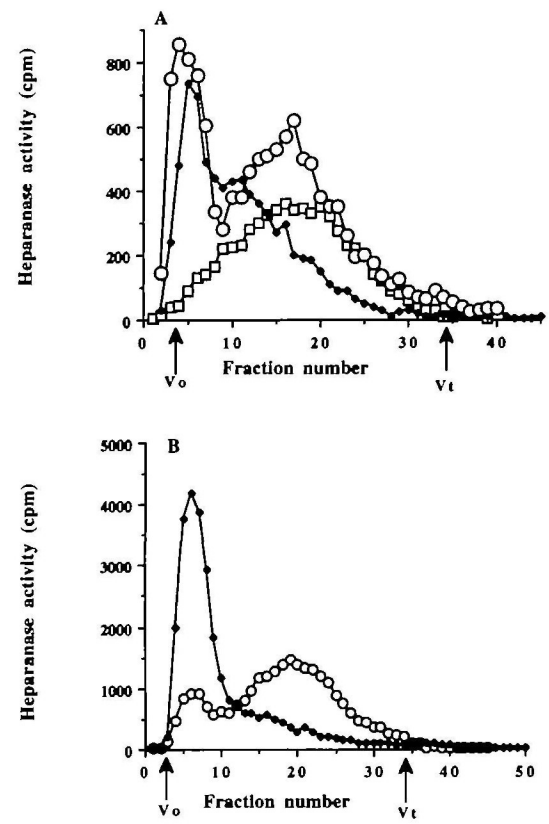

Figure 3. Expression of heparanase by cultured cytotrophoblasts. Third trimester cytotrophoblasts were isolated and plated on tissue culture dishes. The medium was collected 48 h after seeding, the cells dissociated with trypsin-EDTA and either lysed (three cycles of freezing and thawing) or seeded onto sulphate-labelled extracellular matrix (ECM). (**A**) Intact cells (○), cell lysates (□) and conditioned medium (◆) were incubated (48 h, pH 6.2, 37°C) with labelled ECM and the released radioactive material analysed by gel filtration on Sepharose 6B. The elution profile of labelled material released from ECM by conditioned medium was similar to that obtained with fresh medium alone. (**B**) Intact cytotrophoblasts were incubated (48 h, pH 6.2, 37°C) with sulphate-labelled ECM in the absence (○) and presence (□) of 10 μg/ml heparin. Labelled degradation fragments were subjected to gel filtration on a Sepharose 6B column as described in the Methods.

labelled ECM with intact cytotrophoblasts resulted in release of 60–70% of the ECM-associated radioactivity in the form of low Mr sulphate-labelled fragments (peak II, $0.5<Kav<0.75$) (Figure 3A). Degradation fragments eluted in peak II were shown to be degradation products of heparan sulphate as they were: (i) five- to six-fold smaller than intact heparan sulphate side-chains (Kav ~0.33) released from ECM by treatment with either alkaline borohydride or papain; and (ii) resistant to further digestion with papain or chondroitinase ABC and susceptible to deamination by nitrous acid (Vlodavsky *et al.*, 1983). High levels of heparanase were found in lysates of cytotrophoblasts, but there was no detectable activity in medium conditioned by the cytotrophoblasts, regardless of whether the cells were maintained on plastic or ECM. Results shown in Figure 3 were obtained from third trimester cytotrophoblasts; first trimester cells gave similar results (not shown). Accumulation in the medium of low Mr sulphate-labelled HS degradation fragments was inhibited by heparin (Figure 3B), a potent inhibitor of heparanase-mediated HS degradation (Bar-Ner *et al.*, 1987).

Similar results were obtained when freshly isolated cytotrophoblasts were either seeded directly onto sulphate-labelled ECM or were first maintained in primary cultures on tissue culture dishes, trypsinized and then seeded onto sulphate-labelled ECM. The process of trypsinization and reseeding was undertaken in order to eliminate any residual blood cells which may have contaminated the cytotrophoblast preparation despite the preceding Percoll gradient centrifugation. Since the subcultured cells were free of blood cells (i.e. platelets, lymphocytes, neutrophils), as evaluated by haematoxylin and eosin staining and light microscopy, the observed heparanase activity can be clearly attributed to the cytotrophoblasts.

Release of ECM-bound bFGF by cytotrophoblasts

Heparanase mediated release of active bFGF from its storage in basement membranes and ECM may promote an angiogenic

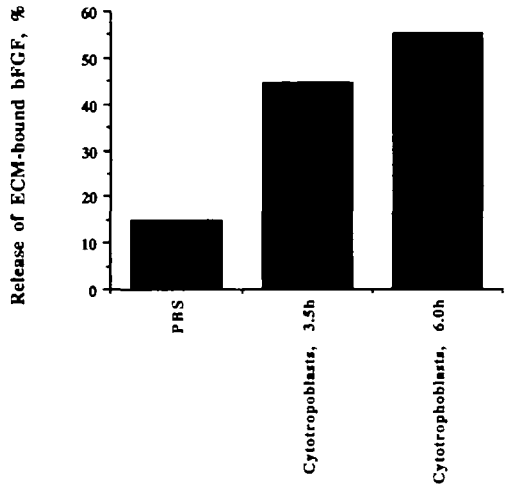

Figure 4. Release of extracellular matrix (ECM) bound by cytotrophoblasts. ECM was preincubated with 2.5 ng/ml [^{125}I]-basic fibroblast growth factor (bFGF) (10^4 c.p.m./well) and washed free of unbound FGF. The ECM was then incubated (3.5 and 6 h, 37°C) with phosphate-buffered saline (PBS) or with third trimester cytotrophoblasts suspended in PBS. Released [^{125}I]-bFGF, expressed as percent of total ECM-bound [^{125}I]-bFGF (5.6×10^4 c.p.m./well), was counted in γ counter. Each data point was the mean of 4 wells and the variation did not exceed 15%.

response (Ishai-Michaeli *et al.*, 1990; Vlodavsky *et al.*, 1990, 1991). Freshly isolated third trimester cytotrophoblasts were suspended in PBS for their ability to release [^{125}I]-bFGF from ECM. As demonstrated in Figure 4, incubation of ECM with $5.0–7.0\times10^5$ cytotrophoblasts/cm^2 resulted in release of 50% of the ECM-bound [^{125}I]-bFGF. A similar incubation of the ECM with PBS alone resulted in release of 10–15% of the ECM-bound [^{125}I]-bFGF. This basal release of FGF was attributed to an endogenous proteolytic activity residing in the ECM (Korner *et al.*, 1993).

Discussion

Developing trophoblasts simultaneously exhibit two distinct properties. On the one hand, they express cell–cell and cell–matrix binding capacity and on the other hand, consistent with an invasive behaviour, they exhibit the ability to degrade various components of the ECM (Cross *et al.*, 1994). While proteases such as gelatinase B (Librach *et al.*, 1991) and urokinase-type plasminogen activator (Queenan *et al.*, 1987) were shown to be involved in trophoblast invasion, little is known about the involvement of enzymes which degrade HSPG, a central component of cell surfaces and ECM.

In the present study we report that cytotrophoblasts isolated from first and third trimester placentas are capable of degrading HS when placed in contact with a naturally produced ECM. Heparanse activity expressed by the cytotrophoblasts exhibited characteristics (i.e. size of degradation fragments, pH dependence, inhibition of heparin, molecular weight of degradation fragments and their susceptibility to deamination by nitrous acid) similar to those of heparanase enzymes expressed by highly invasive tumour cells and activated cells of the immune system (Nakajima *et al.*, 1988; Lider *et al.*, 1989; Vlodavsky *et al.*, 1990, 1992). Moreover, we have purified a ~45 kDa heparanase enzyme from human placentas and demonstrated that the enzyme is highly enriched in this tissue.

The finding that heparanase activity was the same for the two stages of pregnancy may surprise, given the fact that term placentas do not possess invasive characteristics. However, the characteristics of cytotrophoblast cells isolated from placentas of different gestational age tissues do not strictly follow this pattern of differential invasive properties. Purified human cytotrophoblasts, not only from first trimester but also from term placentas, have already been shown to provide a unique in-vitro model system for studying ECM attachment, proteolysis and invasion (Kliman and Feinberg, 1990). These findings, although in dispute with some other groups (Fisher *et al.*, 1989), were further discussed and supported recently (Shimonovitz *et al.*, 1994). The ability of isolated third trimester cytotrophoblasts to maintain the invasive properties may be explained by the fact that cytotrophoblasts isolated by trypsinization and Percoll gradient centrifugation are neither homogeneous in their appearance (Gileadi *et al.*, 1986) nor in their biochemical and hormonal characteristics (Rachmilewitz *et al.*, 1993). Thus, the fact that the enzyme activity is present throughout pregnancy may result from the relative contribution of different cytotrophoblast subpopulations maintaining the placenta. Moreover, as already stated, the heparanase assay is a semiquantitative one, and as such any conclusion concerning its developmental regulation could be neither substantiated nor nullified.

Heparanase activity was not detected in medium conditioned by cultured cytotrophoblasts, nor in the incubation medium of cytotrophoblasts seeded on top of intact ECM. This result indicates that the enzyme is not released by the cells, but may rather be expressed on the cell surface and/or preferentially released in a polar fashion toward the ECM when the appropriate cell-ECM contacts are formed. Likewise, we have previously demonstrated that although human platelets degrade the ECM-HS when placed in contact with the ECM, there is little or no release of the platelet heparanase into the incubation medium (Yahalom *et al.*, 1984).

Heparanase-mediated degradation of HS is likely to be involved in trophoblast invasion through the decidual basement membrane and ECM. This invasion is accompanied by formation of new blood vessels which provide a bridge between the maternal and fetal circulation. Heparanase may contribute to this process, by means of releasing active heparin binding growth factors (i.e. bFGF) that are sequestered as a complex with HS in basement membranes and ECMs (Vlodavsky *et al.*, 1987, 1991; Ishai-Michaeli *et al.*, 1990, 1992). In this study, release of ECM-bound bFGF was observed during incubation of cytotrophoblasts with intact ECM, suggesting an indirect involvement in neovascularization. Members of the FGF family have been shown to be involved in the development of the post-implantation mouse embryo (Feldman *et al.*, 1995). HSPG-bound bFGF may also be released by other ECM-degrading enzymes such as plasmin (Saksela and Rifkin, 1990) and thrombin (Benezra *et al.*, 1993).

We postulate that at the initial stage of hatching, heparanase remains in its intracellular localization and thus does not interfere with the attachment of the trophoblast's surface HS

to its uterine binding proteins. In the second stage, the trophoblast must invade the ECM, utilizing its heparanase activity, among other enzymes. In future studies we plan to investigate the regulation of heparanase gene expression and to further analyse its involvement in implantation and placental development, based on recent sequence information and progress in cloning the heparanase gene.

References

Bar-Ner, M., Eldor, A., Wasserman, L. *et al.* (1987) Inhibition of heparanase mediated degradation of extracellular matrix heparan sulfate by modified and non-anticoagulant heparin species. *Blood*, **70**, 551–557.

Benezra, M., Vlodavsky, I., Neufeld, G. and Bar-Shavit, R. (1993) Thrombin-induced release of active basic fibroblast growth factor-heparan sulfate complexes from subendothelial extracellular matrix. *Blood*, **81**, 3324–3332.

Carson, D.D., Tang, J.-P. and Julian, J. (1993) Heparan sulphate proteoglycan expression by mouse embryos during acquisition of attachment competence. *Dev. Biol.*, **155**, 97–106.

Cross, J.C., Werb, Z. and Fisher, S.J. (1994) Implantation and the placenta: key pieces of the development puzzle. *Science*, **266**, 1508–1518.

Farach, M.C., Tang, J.P, Decker, G.L. and Carson, D.D. (1987) Heparin/heparan sulphate is involved in attachment and spreading of mouse embryos *in vitro*. *Dev. Biol.*, **123**, 401–410.

Feldman, B., Poueymirou, W., Papaioannou, V.E. *et al.* (1995) Requirement of FGF-4 for postimplantation mouse development. *Science*, **267**, 246–249.

Fisher, S.J., Cui, T., Zhang, L. *et al.* (1989) Adhesive and degradative properties of human placental cytotrophoblast cells *in vitro*. *J. Cell Biol.*, **109**, 891–902.

Gileadi, O., Schneider, T., Chebath, J. *et al.* (1988) The expression of proto-oncogenes and 2′-5′ oligoadenylate synthetase in differentiating human trophoblasts *in vitro*. In Mochizuki, M. and Russa, R. (eds), *Placental Protein Hormones*. Elsevier, Amsterdam/New-York, pp. 251–260.

Gospodarowicz, D., Mescher, A.R. and Birdwell, C.R. (1977) Stimulation of corneal endothelial cell proliferation *in vitro* by fibroblast and epidermal growth factors. *Exp. Eye Res.*, **25**, 75–82.

Hassell, J.R., Kimura, J.H. and Hascall, V.C. (1986) Proteoglycan cor protein families. *Annu. Rev. Biochem.*, **55**, 539–567.

Hook, M., Kjellen, L., Johansson, S. and Robinson, J. (1984) Cell surface glycosaminoglycans. *Annu. Rev. Biochem.*, **53**, 847–869.

Ishai-Michaeli, R., Eldor, A. and Vlodavsky, I. (1990) Heparanase activity expressed by platelets, neutrophils and lymphoma cells releases active fibroblast growth factor from extracellular matrix. *Cell Reg.*, **1**, 833–842.

Ishai-Michaeli, R., Svaln, C.M., Chajek-Shaul, T. *et al.* (1992) Importance of size and sulfation of heparin in release of basic fibroblast growth factor from the vascular endothelium and extracellular matrix. *Biochemistry*, **31**, 2080–2088.

Kjellen, L. and Lindhal, U. (1991) Proteoglycans—structure and interactions. *Annu. Rev. Biochem.*, **60**, 443–475.

Kliman, H.J. and Feinberg, R.F. (1990) Human trophoblast-extracellular matrix (ECM) interactions *in vitro*: ECM thickness modulates morphology and proteolytic activity. *Proc. Natl. Acad. Sci. USA*, **87**, 3057–3061.

Korner, G., Bar-Ner, M., Bjornsson, T. *et al.* (1993) Active plasminogen activator is deposited into subendothelial extracellular matrix. *J. Cell Physiol.*, **151**, 456–465.

Librach, C.L, Werb, Z., Fitzgerald, M.L. *et al.* (1991) 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. *J. Cell Biol.*, **113**, 437–449.

Lider, O., Baharav, E., Mekori, Y. *et al.* (1989) Suppression of experimental autoimmune diseases and prolongation of allograft survival by treatment of animals with heparinoid inhibitors of T lymphocyte heparanase. *J. Clin. Invest.*, **83**, 752–756.

Nakajima, M., Irimura, T. and Nicolson, G.L. (1988) Heparanase and tumor metastasis. *J. Cell Biochem.*, **36**, 157–167.

Nicolson, G.L. (1988) Organ specificity of tumor metastasis: role of preferential adhesion, invasion and growth of malignant cells at specific secondary sites. *Cancer Met. Rev.*, **7**, 143–188.

Parish, C.R., Coombe, D.R., Jakobsen, K.B. and Underwood, P.A. (1987) Evidence that sulphated polysaccharides inhibit tumor metastasis by blocking tumor-cell-derived heparanases. *Int. J. Cancer*, **40**, 511–518.

Queenan, J.T.J., Kao, L.-C., Arboleda, C.E. *et al.* (1987) Regulation of urokinase-type plasminogen activator production by culture human cytotrophoblasts. *J. Cell Biol.*, **262**, 10903–10906.

Raboudi, N., Julian, J., Rohde, L.H. and Carson, D.D. (1992) Identification of cell-surface heparin/heparan sulfate-binding proteins of human uterine epithelial cell line (RL95). *J. Biol. Chem.*, **267**, 11930–11939.

Rachmilewitz, J., Gonik, B., Goshen, R. *et al.* (1993) Intermediate cells during cytotrophoblast differentiation *in vitro*. *Cell Growth Diff.*, **4**, 395–402.

Saksela, O. and Rifkin, D.B. (1990). Release of basic fibroblast growth factor-heparan sulfate complexes from endothelial cells by plasminogen activator-mediated proteolytic activity. *J. Cell Biol.*, **110**, 767–775.

Shimonovitz, S., Hurwitz, A., Dushnik, M. *et al.* (1994) Developmental regulation of the expression of 72 and 92 kDa type IV collagenases in human trophoblasts: a possible mechanism for control of trophoblast invasion. *Am. J. Obstet. Gynecol.*, **171**, 832–838.

Vlodavsky, I., Liu, G.M. and Gospodarowicz, D. (1980) Morphological appearance, growth behavior and migratory activity of human tumor cells maintained on extracellular matrix vs plastic. *Cell*, **19**, 607–616.

Vlodavsky, I., Fuks, Z., Bar-Ner, M. *et al.* (1983) Lymphoma cell mediated degradation of sulfated proteoglycans in the subendothelial extracellular matrix: relationship to tumor cell metastasis. *Cancer Res.*, **43**, 2704–2711.

Vlodavsky, I., Folkman, J., Sullivan, R. *et al.* (1987) Endothelial-cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. *Proc. Nat. Acad. Sci. USA*, **84**, 2292–2296.

Vlodavsky, I., Korner, G., Ishai-Michaeli, R. *et al.* (1990) Extracellular matrix-resident growth factors and enzymes: possible involvement in tumor metastasis and angiogenesis. *Cancer Met. Rev.*, **9**, 203–226.

Vlodavsky, I., Bar-Shavit, R., Ishai-Michaeli, R. *et al.* (1991). Extracellular sequestration and release of fibroblast growth factor: a regulatory mechanism? *Trends Biochem. Sci.*, **16**, 268–271.

Vlodavsky, I , Eldor, A., Haimovitz-Friedman, A. *et al.* (1992) Expression of heparanase by platelets and circulating cells of the immune system: possible involvement in diapedesis and extravasation. *Invasion Metast.*, **12**, 112–127.

Vlodavsky, I , Mohsen, M., Lider, O. *et al.* (1995) Inhibition of tumor metastasis by heparanase inhibiting species of heparin. *Invasion Metast.*, **14**, 290–302.

Wight, T.N., Kinsella, M.G. and Qwarnsrom, E.E. (1992) The role of proteoglycans in cell adhesion, migration and proliferation. *Curr. Opin. Cell Biol.*, **4**, 793–801.

Wilson, O., Jacobs, A.L., Stewart, S. and Carson, D.D. (1990) Expression of externally-disposed heparin/heparan sulfate binding sites by uterine epithelial cells. *J. Cell. Physiol.*, **143**, 60–67.

Yagel, S., Parhar, R.S., Jeffrey, J. and Lala, P. (1988) Normal and non-metastatic human trophoblast cells share *in-vitro* invasive properties of malignant cells. *J. Cell Physiol.*, **136**, 455–462.

Yahalom, J., Eldor, A., Fuks, Z. and Vlodavsky, I. (1984) Degradation of sulfated proteoglycans in the subendothelial extracellular matrix by human platelet heparitinase. *J. Clin. Invest.*, **74**, 1842–1849.

Received on February 12, 1996; accepted on July 10, 1996