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Codistribution of basic fibroblast growth factor and heparan sulfate proteoglycan in the growth zones of the human placenta

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Abstract. In order to obtain an insight into morphogenetic processes such as angiogenesis, cell proliferation, and tissue remodeling we have studied the localization of basic fibroblast growth factor (bFGF) and heparan sulfate proteoglycan (HSPG) in the human placenta by immunohistochemistry. Positive reaction product for bFGF is found mainly in the villous trophoblastic covering and for HSPG in the villous basement membranes. A codistribution of the two molecules is detectable in first trimester placental tissue, in areas previously identified as being responsible for the growth of the villous tree, i.e., in the mesenchymal villi and the cytotrophoblastic cell islands and cell columns, both consisting of extravillous trophoblast. HSPG and bFGF are codistributed in the distal half of the villous stroma in the mesenchymal villi. In cell islands and cell columns, bFGF is detectable in the cytoplasm of the extravillous cytotrophoblastic cells, whereas HSPG is localized between the extravillous cytotrophoblastic cells and in their cytoplasm. HSPG-bFGF codistribution in term placenta is confined to the walls of fetal vessels and to some extravillous cytotrophoblastic cells in the basal plate. The codistribution of bFGF and HSPG in first trimester placental tissue suggests that these two molecules play a pivotal role in the morphogenetic processes mentioned above in early stages of gestation.

Key words: Heparan sulfate proteoglycan – Growth factors – Placenta – Basic fibroblast growth factor – Immunohistochemistry – Human

Introduction

Enlightenment of the mechanisms and controls of morphogenesis forms the basis for the interpretation of developmental and pathological processes. Growth factors and extracellular matrix molecules play a pivotal role in morphogenesis, and the activity of these factors is in turn regulated by their interactions with the extracellular matrix (for reviews, see Klagsbrun 1989; Gospodarowicz 1990; Mason 1994). Studies on the localization of such interactions in developing organs can facilitate the understanding of developmental stages in organogenesis. The human placenta is a rapidly growing organ and can thus be considered as a useful model for investigating such morphogenetic processes. Recent studies on the growth and development of the human placenta suggest that three structures are responsible for the growth of the villous tree, particularly in the first half of gestation, viz., the mesenchymal villi, the extravillous cytotrophoblastic cell islands, and cell columns (Castellucci and Kaufmann 1982; Castellucci et al. 1990; Kosanke 1994; Benirschke and Kaufmann 1995). The mesenchymal villi are the forerunners of all other villous types and are responsible for the growth and differentiation of the villous tree (Castellucci and Kaufmann 1982; Castellucci et al. 1990; Kosanke et al. 1993; Kosanke 1994; Benirschke and Kaufmann 1995). The mesenchymal villi show the highest proliferation rates of cytotrophoblastic and mesenchymal cells (Kosanke 1994; Benirschke and Kaufmann 1995). They are the only tertiary villi that are continuously newly formed and are directly derived from trophoblastic and villous sprouts throughout pregnancy (Castellucci et al. 1990). Therefore, important angiogenic processes occur in these villi (Demir et al. 1989; Benirschke and Kaufmann 1995). Neovessel formation is fundamental for villous morphogenesis and differentiation, and for fetal nutrition.

Another important mode of placental growth is achieved through the cell islands and cell columns (Bulmer et al. 1988; Castellucci et al. 1991; Mühlhauser et al. 1993, 1995; Benirschke and Kaufmann 1995). Cell

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islands are large accumulations of predominantly extravillous cytotrophoblast attached to the tips of some villi. Cell columns are structurally comparable to cell islands and are responsible for the attachment of large caliber villi, the so-called anchoring villi, to the basal plate. It has been pointed out that the juxtastromal cytotrophoblast in cell columns and cell islands is highly proliferative (Bulmer et al. 1988; Castellucci et al. 1991). Thus, these two epithelial structures represent centers of trophoblastic proliferation situated at the periphery of the villous tree supporting villous growth.

It is well known that interactions between growth factors and extracellular matrix molecules play a fundamental role in angiogenesis and in other morphogenic processes (Vlodavsky et al. 1991; Folkman and Shing 1992). In the present study, we have investigated the expression of basic fibroblast growth factor (bFGF) and heparan sulfate proteoglycan (HSPG) in the placental villous tree by immunohistochemistry. bFGF is involved in the modulation of cell motility and differentiation, and is a recognized potent stimulator of different cell types, particularly fibroblasts and endothelial cells. It is involved in neovessel formation in vitro and in vivo, and accumulates in areas of enhanced capillary growth (Montesano et al. 1986; Folkman et al. 1988; Rifkin and Moscatelli 1989; Folkman and Shing 1992; Montesano 1992; Aviezer et al. 1994; Mason 1994). It has been established that HSPG binding to bFGF provides protection against proteolytic degradation, thus creating a reservoir of the growth factor in the extracellular matrix (Saksela et al. 1988; Ruoslahti 1989; Flaumenhaft et al. 1990; Coltrini et al. 1993; Nurcombe et al. 1993).

Materials and methods

Materials

Fifteen first-trimester placentas were obtained from legal abortions at 7–12 weeks of pregnancy. Ten full-term placentas were obtained from spontaneous deliveries after uncomplicated pregnancies. To the best of our knowledge, there were no pathological problems affecting the placentas used in this study.

Tissue preparation

Placental tissue was cut into small blocks. For immunohistochemistry, some tissue blocks were snap-frozen in liquid nitrogen and stored at –20° C. The blocks were sectioned on a cryostat. Parallel cryostat sections (5 μm) were mounted on uncoated glass slides and were air-dried at room temperature for 10 min. The sections were fixed in 100% acetone at 4° C for 10 min and stored at 4° C until used (up to one night after sectioning).

Other tissue blocks were fixed in 4% neutral buffered formalin or Bouin solution for 8 h, rapidly dehydrated, and embedded in paraffin at temperatures not exceeding 56° C. Serial paraffin sections (4 μm) were cut and stretched at 45° C, allowed to dry, and stored at 4° C until used.

Primary antibodies

The following primary antibodies were used: (a) a rabbit polyclonal antibody against natural bovine brain bFGF (British Bio-technology, Oxford, U.K.), dilution 1:100 v/v; (b) a mouse monoclonal antibody (mAb) against the protein core of HSPG (Chemicon, Temecula, Calif., USA), dilution1:20 v/v; (c) MIB 1, a mouse mAb recognizing the Ki-67 nuclear cell-proliferation-associated antigen (Immunotech, Marseille, France), dilution 1:20 v/v.

Immunohistochemistry

Immunohistochemical reactions were performed using indirect immunofluorescence, the streptavidin-biotin-peroxidase technique, or the avidin-biotin-peroxidase complex. Frozen sections were processed without pre-treatment. Paraffin sections were deparaffinized and rehydrated in xylene and a graded series of ethyl alcohol. When using the mAb MIB 1 as the primary antibody, paraffin sections were additionally processed in a microwave oven (Cattoretti et al. 1992) before starting the immunoreaction.

For immunofluorescence, frozen sections were pre-incubated with non-immune swine serum or with non-immune rabbit serum in the case of primary monoclonal antibodies for 20 min at room temperature. After tapping off the serum, sections were incubated with polyclonal or monoclonal primary antibody for 1 h at room temperature in a humid chamber. After the sections were rinsed in three changes of phosphate-buffered saline (PBS), the second antibody F205, viz., fluorescein-isothiocyanate (FITC)-conjugated swine anti-rabbit, or F232 (FITC-conjugated rabbit anti-mouse; Dakopatts Glostrup, Denmark) was applied at a dilution of 1:20 in PBS with 1.5% bovine serum albumin (BSA; pH 7.4). After being further rinsed in PBS, the sections were mounted under coverslips with n-propyl gallate (Giloh and Sedat 1982).

Fig. 1. Mesenchymal villus (*MV*) branching off from an immature intermediate villus (IIV) and protruding into the intervillous space. Sprouting of a new mesenchymal villus is observable (*arrow*). Numerous nuclei of cytotrophoblastic cells and mesenchymal cells are stained with MIB 1. Ninth week of gestation. Paraffin section. ×400. *Bar:* 20 μm

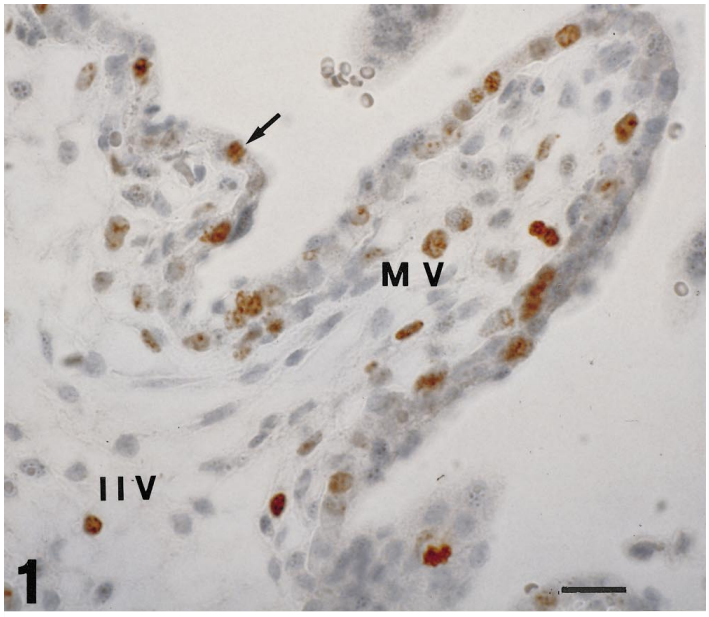
Fig. 2. Chorionic villi stained with the MIB 1 antibody. A cross-section through a mesenchymal villus (*arrow*) is shown. The reaction product in this villus is localized in the nuclei of numerous cells. Few cells are stained in the adjacent villi. Ninth week of gestation. Paraffin section. ×160. *Bar:* 40 μm

Fig. 3. Paraffin section of chorionic villi stained with the antibody against bFGF. Immunostaining is present in the trophoblastic covering. Positive reaction product is also localized in the distal stroma (*arrows*) of the mesenchymal villi, whereas their proximal part (*arrowheads*) and the extracellular matrix of the immature intermediate villus (*IIV*) show weak or negative immunostaining. Twelfth week of gestation. ×150. *Bar:* 40 μm

Fig. 4. Control section. No staining is observable either in the trophoblast or in the stroma of the various types of chorionic villi. Twelfth week of gestation. Paraffin section. ×170. *Bar:* 50 μm

Fig. 5. Mesenchymal villus (*MV*) branching off from an immature intermediate villus (*IIV*) immunostained for bFGF. Strong immunoreactivity is present in the trophoblastic covering. The villous stroma shows positive reaction product only in the distal half of the mesenchymal villus. Some mesenchymal cells are also immunostained. Twelfth week of gestation. Paraffin section. ×300. *Bar:* 20 μm

Fig. 6. Mesenchymal villus (*MV*) branching off from an immature intermediate villus (*IIV*) and stained with the monoclonal antibody against HSPG. This section is serial to that depicted in Fig. 5. The antibody reacts with the stromal connective tissue of the mesenchymal villus in its distal half. Twelfth week of gestation. Paraffin section. ×300. *Bar:* 20 μm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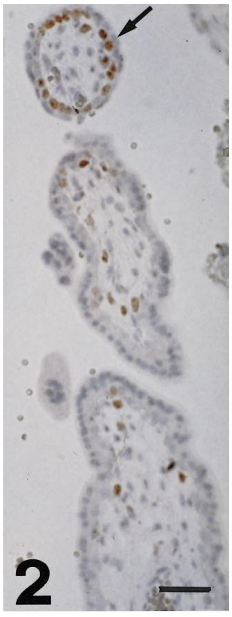
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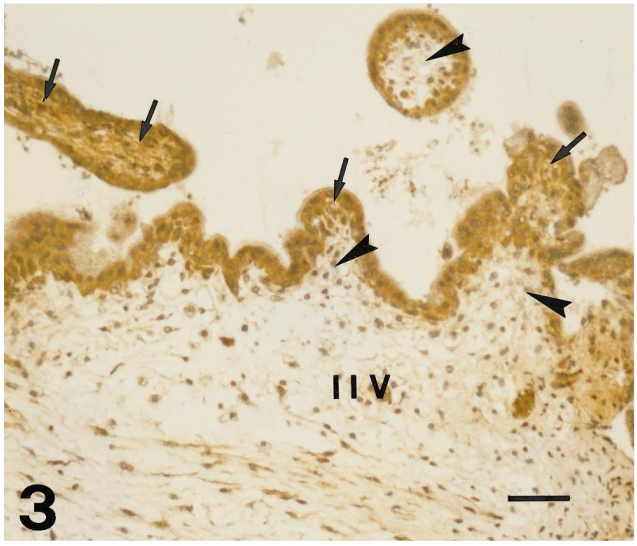
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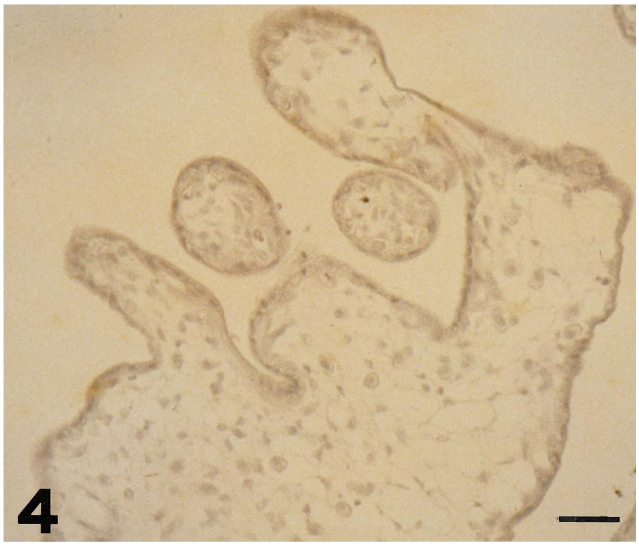
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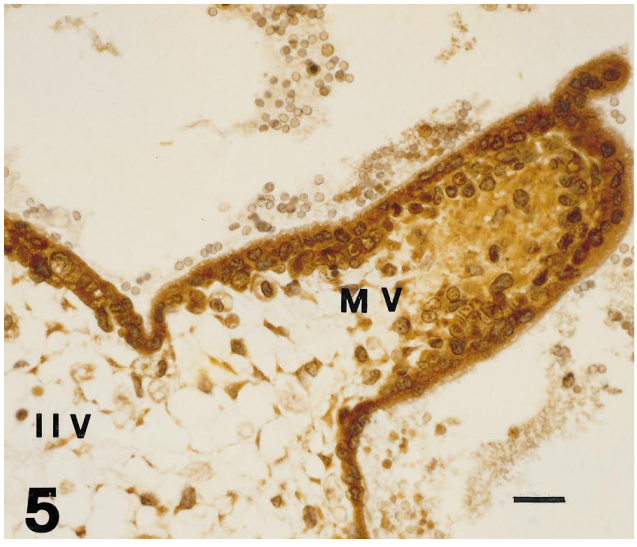
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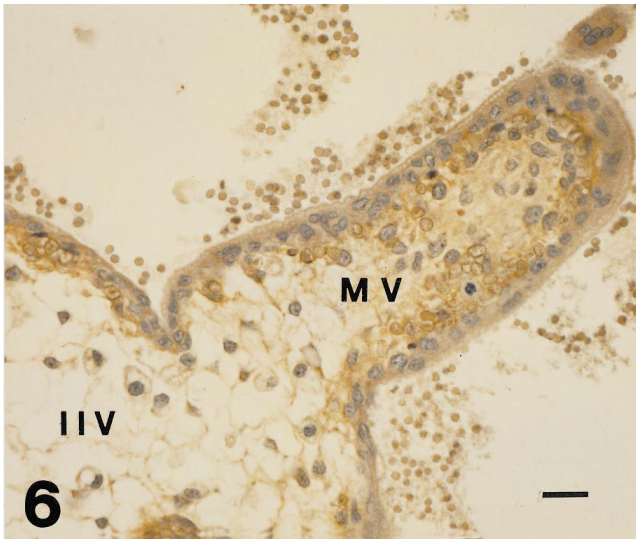
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For the avidin-biotin-peroxidase complex and streptavidin-biotin method, frozen and paraffin sections were incubated for 15 min with 1% hydrogen peroxide in methanol to inhibit endogenous peroxidases. Sections were then incubated for 20 min at room temperature with non-immune goat serum (or with non-immune rabbit serum in the case of primary monoclonal antibodies). Afterwards, the sections were incubated with one of the three primary antibodies diluted in PBS/BSA for 45 min at room temperature or overnight at 4° C. Sections were then washed in PBS before further treatment for the avidin-biotin-peroxidase complex.

Avidin-biotin complex method. For frozen and paraffin sections, two kits were used, the first containing avidin (rabbit anti-mouse ABC kit; Dakopatts), and the other streptavidin (S-ABC goat anti-rabbit streptavidin-biotin system; Zymed Laboratories, South San Francisco, Calif., USA). Both kits used peroxidase enzyme as a label, and peroxidase activity was revealed by diaminobenzidine (Sigma; St. Louis, Mo., USA). Kit instructions were followed with regard to dilutions and incubation times. The sections were then mounted with an aqueous mounting solution.

Enzymatic treatment. Before incubation with the primary antibody against bFGF, some cryosections and deparaffinized sections were incubated with 2.5 mU heparitinase (Sigma) in 0.5 ml buffer for 60–120 min at 37° C (Silbert et al. 1990; David et al. 1992).

Controls. For the above immunohistochemical procedures, controls were performed by replacing the primary antibody by 10% non-immune serum or by PBS/BSA. Further controls were performed by omitting the secondary antibody. The control sections showed negative results. Incubations were also performed by preabsorbing antibodies with bFGF peptides (RD System, Minneapolis, USA) or with HSPG (Sigma) for 24–48 h at 4° C; this attenuated or abolished the staining.

Results

Identical results where obtained by using different immunohistochemical techniques and when the same antibody was used on paraffin and cryostat sections. Therefore, no separate descriptions will be provided.

First trimester placenta

MIB 1 stained the nucleus of almost every cytotrophoblastic cell of the mesenchymal villi (Figs. 1, 2). Connective tissue cells of the villous stroma were also positive (Figs. 1, 2). Fewer cytotrophoblastic cells of other villous types were stained (Fig. 2; see also Kosanke 1994). The proximal extravillous cytotrophoblastic cells of cell islands and cell columns were positive for MIB 1, whereas the distally located cells were negative (data not shown).

bFGF was localized in the trophoblastic covering of the various types of chorionic villi (Fig. 3). Positive reaction product for bFGF was also observed in the connective tissue stroma of mesenchymal villi, particularly in their distal halves (Figs. 3, 5). Such staining of the stroma was weak or abolished in sections previously treated by heparitinase (data not shown). The extracellular matrix of the other villous types, e.g., the immature intermediate villi, was negative (Figs. 3, 5), but fibroblasts, some Hofbauer cells, and blood vessel walls showed a slightly positive reaction in some villi.

Sections stained for HSPG showed immunoreactivity in the trophoblast and vessel basement membranes (Fig. 6). Positive staining for HSPG was also present in the distal stromal part of mesenchymal villi (Fig. 6). Such staining was absent from control sections (Fig. 4). Thus, the stromal reactions for HSPG and bFGF overlapped in corresponding sections (compare Figs. 5, 6).

The extravillous cytotrophoblastic cells of cell islands and cell columns showed an intense staining in their cytoplasm for bFGF at all stages of proliferation and invasion (Fig. 7). In such epithelial structures, HSPG was detected between extravillous cytotrophoblastic cells, strongly increasing in expression in between those cells distally located from the stroma of the adherent villus

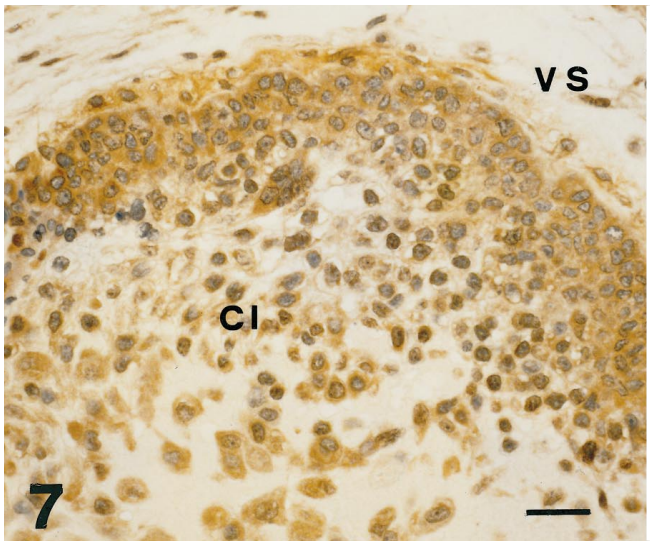

Fig. 7. Paraffin section of an extravillous cytotrophoblastic cell island (*CI*) immunostained for bFGF. Cytotrophoblastic cells show an intense reaction product in their cytoplasm. *VS*, Villous stroma. Eleventh week of gestation. ×300. *Bar:* 20 μm

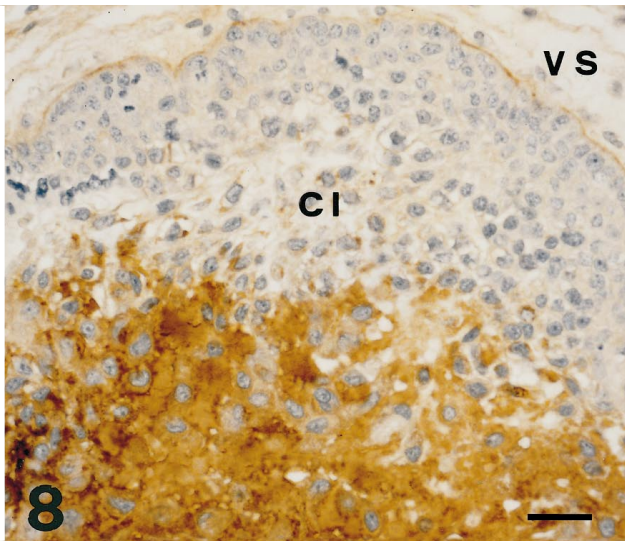

Fig. 8. Cell island (*CI*) stained with the monoclonal antibody against HSPG. This section is serial to that depicted in Fig. 7. HSPG can be detected in the cytoplasm of extravillous cytotrophoblastic cells and in the intercellular space. Note the heavy reaction product in the distal part of the cell island. *VS*, Villous stroma. Eleventh week of gestation. Paraffin section. ×300. *Bar:* 20 μm

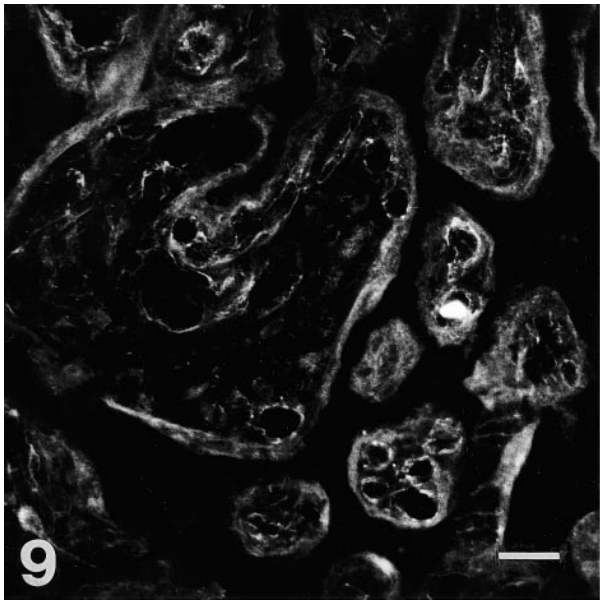

Fig. 9. Immunofluorescence staining for bFGF. Term placental villi. The trophoblast, vessel walls, and some stromal cells are positive. Cryostat section. ×160. *Bar:* 50 μm

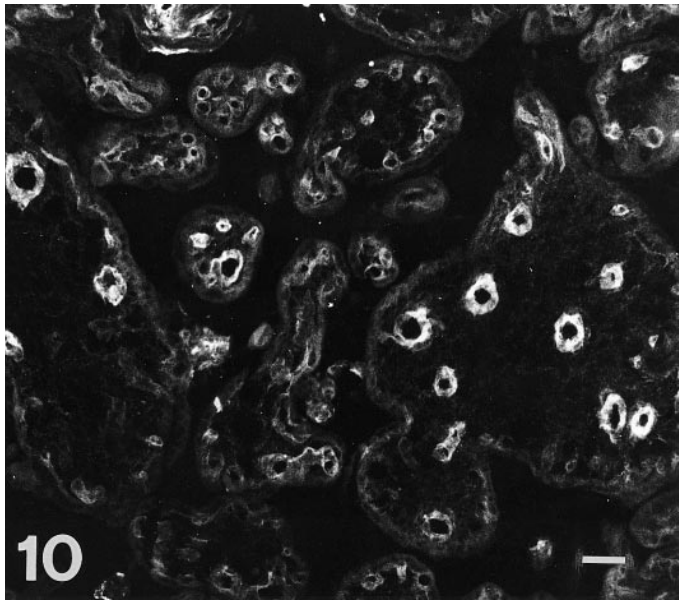

Fig. 10. HSPG immunofluorescence staining pattern. Term placental villi. The trophoblast basement membrane and vessel walls are positive. Cryostat section. ×100. *Bar* 50 μm

(Fig. 8). In addition, some of these cells showed positive staining for HSPG in their cytoplasm (Fig. 8).

Term placenta

MIB-1-positive reaction product was present in some cytotrophoblastic cells of the mesenchymal villi and less frequently in the cytotrophoblast of other villous types (Kosanke 1994). Few stromal cells of the mesenchymal villi and other villous types were positive for MIB 1 (data not shown).

bFGF reaction product was observable in the villous trophoblast. Moreover, a slightly positive immunoreactivity was present in some stromal cells and vessel walls (Fig. 9). No consistent differences in immunostaining for bFGF were detectable in the stroma of the various villous types, unlike those observed in the first trimester. HSPG staining was mainly located in the walls of the fetal vessels, whereas weaker immunoreactivity for HSPG was present in the basement membrane of the villous trophoblast (Fig. 10).

In the basal plate, bFGF was expressed in the extravillous cytotrophoblastic cells, whereas HSPG was localized around and/or in the cytoplasm of such cells. The positive staining reaction for MIB 1 was confined to some extravillous trophoblastic cells close to the anchoring villi.

Discussion

Although biochemical studies have demonstrated that HSPG and bFGF interact, to the best of our knowledge, this is the first study showing that these two molecules are codistributed in delimited areas that play a key role in the development of an organ. The mechanism by which bFGF is released remains unresolved, since bFGF does not contain the signal sequence for secretion (Klagsbrun 1992; Mason 1994). Opinion has recently moved toward the idea that bFGF is released by a novel secretory mechanism that does not involve the classical route of translocation through the endoplasmic reticulum and Golgi apparatus (Logan 1990; Klagsbrun 1992; Mason 1994). One of these mechanisms could be the passive release of bFGF from cells whose plasma membranes have become leaky or compromised (Klagsbrun 1992).

Released bFGF can bind to cellular HSPG present at the cell surface and to HSPG present in the extracellular matrix (Yayon et al. 1991; Roghani and Moscatelli 1992; Coltrini et al. 1993; Aviezer et al. 1994). Cell surface HSPG constitutes the low affinity binding site for bFGF. Binding of bFGF to cell surface HSPG is necessary for its binding to the high affinity FGF receptor and for its mitogenic activity (Yayon et al. 1991; Roghani and Moscatelli 1992; Turnbull et al. 1992; David 1993). bFGF bound to HSPG in the extracellular matrix acts as a reservoir. This bFGF-heparan sulfate complex is protected from proteolytic degradation (Saksela et al. 1988; Roghani and Moscatelli 1992) and can be mobilized from the extracellular matrix by specific mechanisms when needed for biological activity (Flaumenhaft et al. 1990). Therefore, the bFGF-heparan sulfate complex, rather than bFGF alone, may stimulate angiogenesis in vivo (Flaumenhaft et al. 1990). Flaumenhaft and coworkers (1990) have demonstrated an additional property of the bFGF-heparan sulfate complex released from extracellular matrix, viz., the enhanced ability to diffuse in the extracellular environment. The ability of an angiogenic factor to diffuse to its target vascular supply is essential for the factor to be active. In addition to stimulating en-

dothelial cell proliferation, bFGF also induces the production of proteases whose action is required for neovessel penetration through the extracellular matrix (Pierce et al. 1992).

The immunohistochemical localization of bFGF in the chorionic villi supports recent in situ hybridization findings of Shams and Ahmed (1994). The immunostaining of bFGF in the stroma of the mesenchymal villi strongly suggests an important role of this growth factor in angiogenic, proliferative, and differentiative processes in these villi.

HSPG and bFGF molecules are extensively codistributed in the growth zones of the first trimester placenta and probably represent a reservoir of bFGF bound to HSPG in these areas. On the other hand, the absence or low expression of stromal-positive reaction products for these two molecules in the immature intermediate villi directly connected to the mesenchymal villi suggests that the main angiogenic and proliferative processes have been realized during the formation of the mesenchymal villi and their differentiation into immature intermediate villi. In term placentas, the codistribution of bFGF and HSPG is confined to the vessel walls. This suggests that these two molecules might also play a role in angiogenic processes in late gestation.

The observation that cytotrophoblastic cells of cell islands and cell columns show positive staining for bFGF in their cytoplasm has various functional implications. It has recently been established that cytoplasmic bFGF mediates increased cell migration and influences expression of integrins (Mason 1994). Damsky et al. (1992) and Zhou et al. (1993) have pointed out that migration of the extravillous cytotrophoblastic cells of cell columns into the basal plate is characterized by gradual alterations in the expression of different types of integrins, modifications of such expression patterns being related to pathological processes. bFGF may play an important role in modulating the migration of such cells. Moreover, the extravillous cytotrophoblastic cells that migrate distally no longer proliferate and, at least in part, degenerate when pregnancy advances (Frank et al. 1994; Benirschke and Kaufmann 1995). Such cells might release bFGF that could, in turn, stimulate the production of proteases and of extracellular matrix in these areas, and angiogenic processes at the maternal side of the basal plate. This could also explain the presence of large amounts of HSPG only in the distal part of cell columns.

In conclusion, our study shows that, during organogenesis of the human placenta, bFGF and HSPG are codistributed in delimited zones specifically involved in time-dependent differentiative processes. Alterations in the expression of bFGF and/or of its binding to HSPG may be responsible for abnormal development of the placenta at the fetal and/or at the maternal side and may consequently influence fetal nutrition.

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