

Expression of Stromelysin-3 in the Human Placenta and Placental Bed

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Human placentation is mediated by fetal trophoblastic cells which penetrate into the decidualized uterine endometrium. Trophoblast invasion requires the precisely regulated secretion of specific proteinases able to degrade the endometrial basement membranes and extracellular matrix. To document further the involvement of these proteinases during human placentation, we evaluated *in vivo* the expression of stromelysin-3, a member of the metalloproteinase family, during the first and third trimesters of pregnancy, by means of immunohistochemistry, *in situ* hybridization and Northern blot analysis. Human extravillous trophoblasts invading the maternal decidua produced stromelysin-3 during both, the first and third trimesters of pregnancy, but to a lesser extent during the latter. In floating villi, stromelysin-3 expression was restricted to the syncytiotrophoblasts that line intervillous vascular spaces. In conclusion, stromelysin-3 is expressed by differentiated, non-proliferative villous and extravillous trophoblastic cells in early and late placental beds and villi, and its pattern of expression evolves during pregnancy. Our observations suggest that stromelysin-3 could play a role in human placentation. © 1997 W. B. Saunders Company Ltd *Placenta* (1997), 18, 277–285

INTRODUCTION

Implantation of the human blastocyst and the subsequent placental development are dependent on trophoblast invasion of the decidualized endometrium. As implantation proceeds, undifferentiated villous cytotrophoblast stem cells (CTB) originating from the outer cell layer of the blastocyst divide and differentiate into morphologically and functionally distinct cell populations. In the villi, they fuse to form hormonally active villous syncytiotrophoblast (STB) which controls the transfer of solutes between maternal and fetal blood. Where villi contact the uterine wall, multilayered trophoblast cell columns (TCC) physically anchor the embryo to the decidua. In these columns, CTB differentiate progressively into non-proliferative, invasive and migratory intermediate trophoblasts (IT) (Damsky et al., 1992; Kliman and Feinberg, 1992; Damsky, Sutherland and Fisher, 1993). During the first 4 months of gestation [Figure 1(a)], these IT sprout from the TCC and migrate through the decidua and the inner third of the myometrium. They infiltrate the walls of the spiral arteries and replace the endothelial lining as far as the myometrial segment of the vessels. This invasive trophoblastic activity

is characterized by the breaching of multiple basement membranes (including those of the endometrial glands, blood vessels and decidual cells) and the degradation of the interstitial extracellular matrix (ECM) of the decidua (Boyd and Hamilton, 1967; Brosens, Robertson and Dixon, 1967; Pijnenborg et al., 1980; Tittel et al., 1985; Aplin, 1991; Foidart et al., 1992).

The human placenta thus functions as an invasive tissue, analogous to a locally invasive tumour (Liotta, 1986). However, unlike tumour invasion, trophoblast penetration is regulated precisely, both spatially and temporally, during gestation.

Several *in vitro* and *in vivo* studies have revealed that trophoblast cells have a regulated capacity to produce different matrix-degrading enzymes (Lala and Graham, 1990; Bischof and Martelli, 1992; Graham and Lala, 1992). Production of serine proteinases, such as urokinase-type plasminogen activator (u-PA) by trophoblastic cells correlates temporally with blastocyst invasion (Sherman, Strickland and Reich, 1976; Strickland, Reich and Sherman, 1976; Sappino et al., 1989; Behrendtsen, Alexander and Werb, 1992). The presence of u-PA, plasminogen activator inhibitor type 1 and u-PA receptor has been associated with infiltrating trophoblasts

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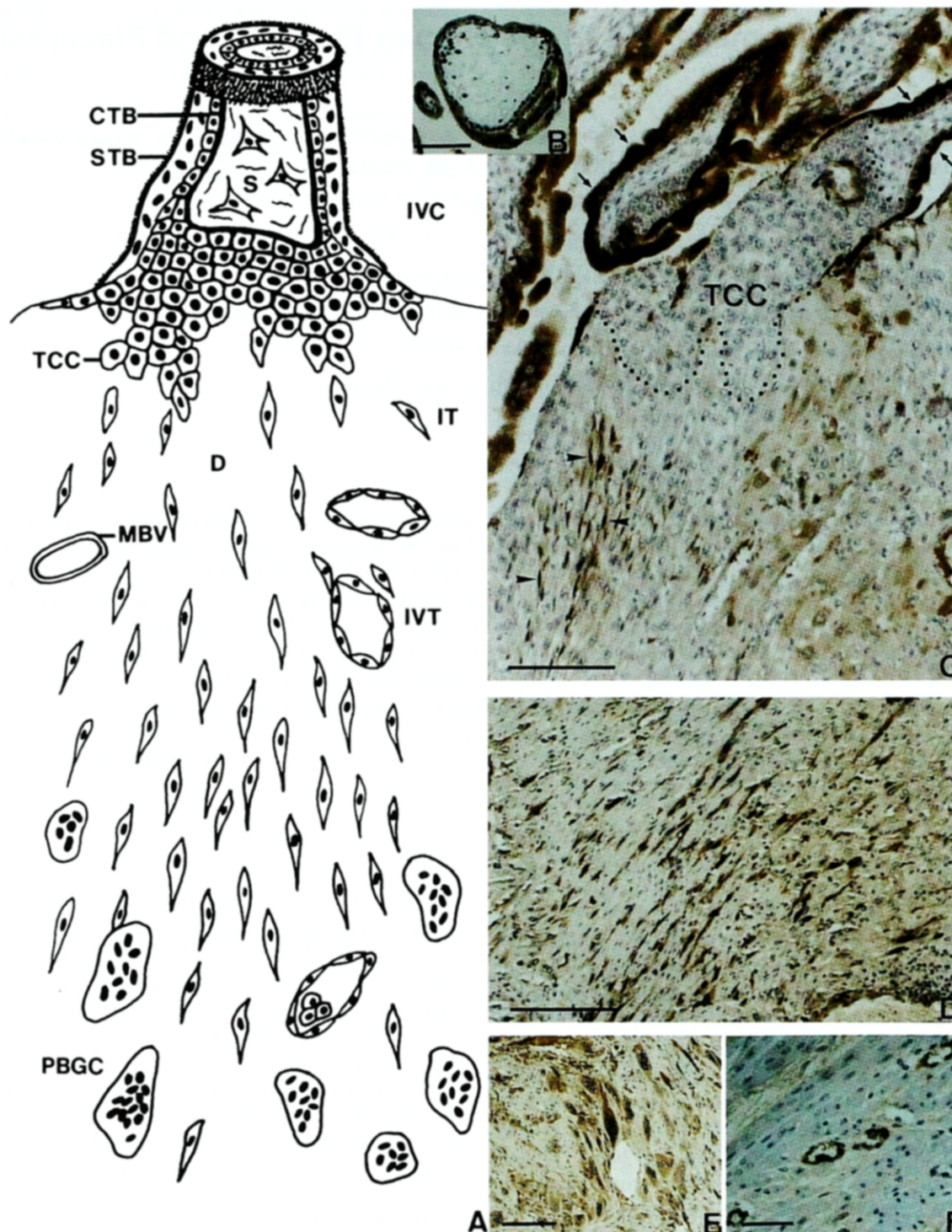


Figure 1. Localization of ST3 in a first trimester (11 weeks) placental bed. (A) Diagram showing the spatial organization of the fetal-maternal interface at the end of the first trimester. D, decidua; CTB, cytotrophoblast; IT, intermediate trophoblast; IVC, intervillous chamber; IVT, intravascular trophoblast; MBV, maternal blood vessel; PBGC, placental-bed giant cell; S, stroma; STB, syncytiotrophoblast bordered by sinusoids; TCC, trophoblastic cell column. (B) Transverse section through a villus showing STB strongly labelled by the anti-ST3 antibody. Scale bar=100 μ m. (C) Longitudinal section through the placental bed. Strong STB positivity (arrows) for ST3 is observed. ST3 is also expressed by some spindle-shaped IT (arrowheads) that are derived from negative TCC, become detached and infiltrate the maternal decidua. Scale bar=150 μ m. (D) Deeper in the decidua, numerous elongated IT, interspersed among negative decidual cells, were recognized by the anti-ST3 antibody. Scale bar=200 μ m. (E) Some IT fused to form PBGC which also express ST3. Scale bar=150 μ m. (F) The endothelial lining of the maternal blood vessels has been replaced by ST3-positive trophoblasts. Scale bar=80 μ m.

(Feinberg et al., 1989; Zini et al., 1992; Hoffman et al., 1994). Members of the matrix metalloproteinases (MMP) family also appear to play a pivotal role during placentation. The acquisition of an invasive phenotype by early trophoblasts has been correlated with the ability of these cells to synthesize a large array of MMP: interstitial collagenase (Yagel et al., 1988; Moll and Lane, 1990), gelatinase A (Bischof et al., 1991; Autio-Harminen et al., 1992; Fernandez et al., 1992; Blankenship and King, 1994; Polette et al., 1994), gelatinase B (Fisher et al., 1985; Emonard et al., 1990; Bischof et al., 1991; Librach et al., 1991; Behrendtsen, Alexander and Werb, 1992; Emonard et al., 1993; Polette et al., 1994), stromelysin-1 and stromelysin-2 (Brenner et al., 1989), as well as a membrane-type MMP potentially involved in gelatinase A activation (Nawrocki et al., 1996). The invasive trophoblasts and the maternal decidual cells also secrete specific inhibitors of these proteinases: plasminogen-activator inhibitors 1 and 2 (Feinberg et al., 1989; Hofmann et al., 1994), and tissue inhibitors of metalloproteinases 1 and 2 (Lala, and Graham, 1990; Polette et al., 1994). Thus, trophoblast invasiveness appears to be regulated tightly by the balance between the activated enzymes and their inhibitors.

A few years ago, another member of the MMP family, stromelysin-3 (ST3), was identified (Basset, Wolf and Chambon, 1990). ST3 exhibits the same general features as previously described MMP, including a typical zinc-binding site and a conserved cysteine residue characteristic of the MMP prodomain (Basset, Wolf and Chambon, 1993). Although its physiological substrate is still unknown, ST3 is probably a proteinase, because the purified protein exhibits proteolytic activities (Murphy et al., 1993). However, ST3 displays functional properties and an activation mechanism different from that of other MMP (Birkedal-Hansen et al., 1993; Murphy et al., 1993; Pei, Majmudar and Weiss, 1994; Noël et al., 1995; Santavica et al., 1996). Wolf and co-workers demonstrated that st3 transcription in embryonic fibroblasts can be induced by 12-*o*-tetradecanoyl phorbol 13-acetate and growth factors, thereby suggesting a transcriptional regulation similar to that of interstitial collagenase, stromelysin-1 and gelatinase B (Wolf et al., 1992). According to those data, ST3 appears to belong to a new MMP subfamily that exhibits its proper enzymatic characteristics and narrow substrate specificity (Basset et al., 1993; Murphy et al., 1993; Pei, Majmudar and Weiss, 1994; Noël et al., 1995). The st3 gene is known to be expressed in the stromal compartment of most invasive human carcinomas and the highest levels of ST3 transcripts are found in tumours demonstrating high local invasiveness (Urbanski et al., 1992; Wolf et al., 1992; Basset, Wolf and Chambon, 1993; Birkedal-Hansen et al., 1993; Hähnel et al., 1993; Kawami et al., 1993; Muller et al., 1993; Polette et al., 1993; Segain et al., 1993). However, ST3 transcripts are also present in healthy situations in which extensive ECM remodelling occurs: embryonic development (Basset et al., 1990), frog metamorphosis (Patterson, Pär Hayes and Shi, 1995), mammary gland involution (Lefebvre et al., 1992), normal uterus and, interestingly, at

very high levels in human term placenta (Basset et al., 1990).

To localize ST3 in placental tissues and to investigate the potential role of this enzyme during human placentation, immunohistochemical labelling, in situ hybridization and Northern blot analysis were used to evaluate the expression of ST3 in first and third trimester placental tissues from normal pregnancies.

MATERIALS AND METHODS

Tissue preparation

Samples of human placental villi obtained from 16 first trimester therapeutic abortions and 10 term third trimester placentae immediately after delivery were examined. Twelve placental-bed biopsies were obtained in situ with echoguided sampling forceps during the first and third trimesters of normal pregnancies. This procedure allows precise anatomical localization (placental bed) of the sample and prevents the tissue disruption observed during abortion or delivery (Hustin and Franchimont, 1992). Finally, four full-thickness implantation sites in hysterectomy specimens taken at 8.5–13 weeks of pregnancy were also studied. One part of each sample was frozen in liquid nitrogen for Northern blot analysis, while the remainder was fixed in formalin and embedded in paraffin for in situ hybridization and immunohistochemical studies.

Immunohistochemistry

Immunolabelling of ST3 was accomplished according to the following procedure. Endogenous peroxidase activity was quenched by a 10-min incubation with 3 per cent hydrogen peroxide in phosphate-buffered saline (PBS). Tissues sections were subjected to three 5-min heating cycles in citric acid buffer (pH 6) in a microwave oven (Philips, power setting: 750 W). Non-specific antibody binding was blocked by incubation in 7 per cent bovine serum albumin-PBS (BSA-PBS) for 30 min. Sections were incubated overnight at room temperature with a mouse monoclonal antibody raised against the haemopexin domain of ST3 (5ST-4A9) (Wolf et al., 1993; Santavica et al., 1995), grown as an ascites fluid and used diluted 1/4000 in 3 per cent BSA-PBS. A biotinylated secondary antibody (LSAB 2 Kit, Dako, Denmark) was applied for 30 min prior to incubation with streptavidin-horseradish peroxidase (LSAB 2 Kit) for an additional 30 min. Antibody localization was visualized with a solution of diaminobenzidine and 0.03 per cent hydrogen peroxide. Each step of the procedure was followed by three washes in PBS.

To evaluate the specificity of immunolabelling, the primary antibody was replaced by either an ascites fluid containing 5ST-4A9 which was preincubated with immobilized recombinant ST3 (Santavica et al., 1995) or an isotype-matched

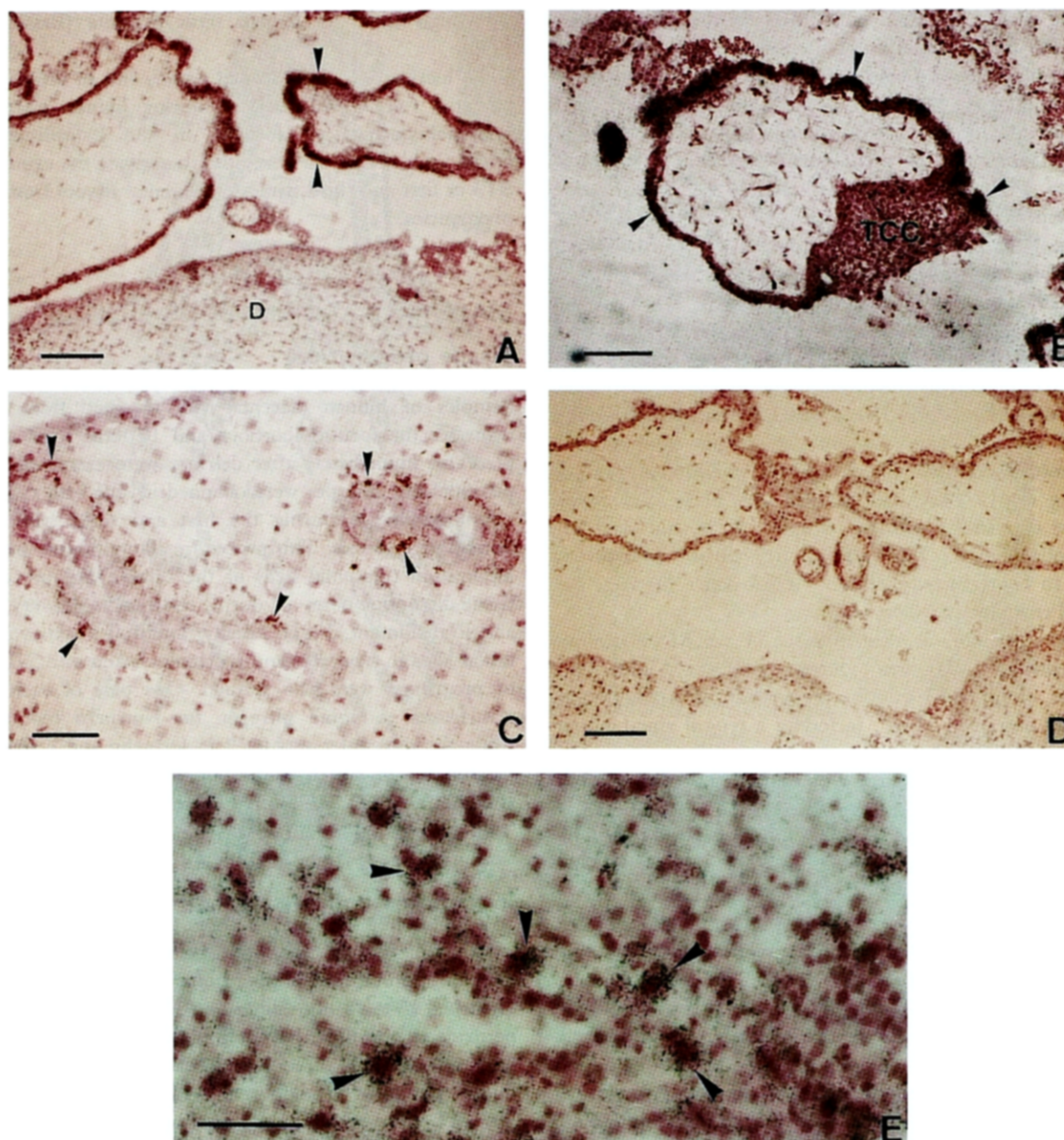


Figure 2. Localization of ST3 mRNA in first trimester villi and placental beds. (A) mRNA transcripts of ST3 are detected in the STB (arrowheads) of villi. The decidua (D) does not express ST3. Scale bar=200 μ m. (B) TCC arising from the villus do not contain ST3 mRNA while the STB (arrowheads) is clearly labelled. Scale bar=100 μ m. (C) IT (arrowheads) expressing ST3 mRNA have replaced the endothelial lining of MBV. Scale bar=50 μ m. (D) A section incubated with the sense RNA probe. Scale bar=200 μ m. (E) IT (arrowheads) expressing ST3 mRNA are observed among decidual cells. Scale bar=25 μ m.

irrelevant antibody (a mouse ascites fluid raised against hamster CD-3). No immunoreactivity was observed in the controls.

Epithelial cells and trophoblasts were identified by immunohistochemical labelling with a monoclonal antibody to cytokeratins 8/18 (CAM 5.2, Becton-Dickinson) revealed with a rabbit anti-mouse antibody conjugated to horseradish peroxidase (1/50 in PBS; Dako). All sections were counterstained with hematoxylin, mounted, and examined under an Olympus AH3 microscope.

In situ hybridization

Paraffin was removed from 5- μ m thick tissue sections which were then rehydrated and treated with 0.2 M HCl for 20 min at room temperature, followed by a 15-min incubation at 37°C with proteinase K (1 μ g/ml in Tris-ethylenediaminetetraacetic acid-NaCl; Sigma Chemical Co., St Louis, MO, USA) to remove basic proteins. The sections were washed in 2 \times SSC (saline-sodium citrate), acetylated in 0.25 per cent acetic anhydride in 0.1 M triethanolamine for 10 min and hybridized

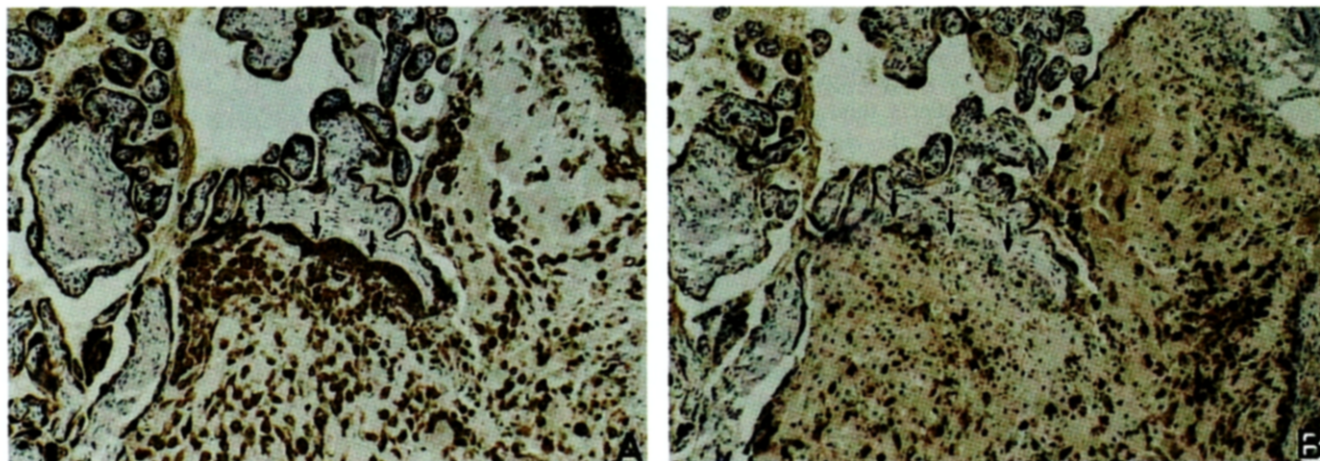


Figure 3. Term placental bed. (A) Immunolabelling of cytokeratins revealed their presence in STB, a few CTB, TCC (arrows) and IT. Scale bar=150 µm. (B) The STB and a subpopulation of the IT in this serial section reacted positively with the anti-ST3 antibody. The TCC are negative (arrows). Scale bar=150 µm.

overnight with ^{35}S -labelled antisense RNA transcripts. ST3 cDNA insert (1600-bp Z IV probe; Basset et al., 1993) was subcloned into the Bluescript plasmid (Stratagene, La Jolla, CA, USA) and used to prepare ^{35}S -labelled RNA probes. Hybridizations were followed by RNase treatment (20 µg/ml, 1 h, 37°C) to remove unhybridized probes. Four stringent washes (2 × SSC and 1 × SSC, 15 min at room temperature) were performed and autoradiography was carried out by using D19 emulsion (Kodak, Vilvorde, Belgium). Slides were exposed for 21 days prior to development. The controls were performed under the same conditions using ^{35}S -labelled sense RNA probes. All slides were counterstained with haematoxylin, mounted and examined under an Olympus AH3 microscope. Control slides were negative.

Northern blot analysis

Total RNA was extracted from tissues by RNAzol treatment (Biogenesis, Bournemouth, UK). An aliquot of RNA (15 µg) was electrophoresed on 1 per cent agarose gels, containing 10 per cent formaldehyde and transferred onto a nylon membrane (Hybond[™]-N; Amersham, Gent, Belgium). The membrane was hybridized with a cDNA probe (as described above), that had been labelled with ^{32}P using random priming synthesis (Boehringer Mannheim, Mannheim, Germany). The filter was exposed for 1 day for floating villi and 7 days for placental beds (six independent samples were analysed for each gestational stage). The membrane was rehybridized to an oligonucleotide probe of human 28 S rRNA (Clontech, Palo Alto, CA, USA), which served as a control. The amounts of ST3 transcripts were quantified by densitometric analysis of autoradiographs of the Northern blots. All results were corrected for RNA loading by densitometric data obtained for the 28 S rRNA signals.

RESULTS

Immunolabelling of the first trimester placental-bed biopsies and implantation-site sections with anti-cytokeratin antibody

revealed mainly two different trophoblastic populations: the anchoring TCC (composed of rounded uniform cohesive cells) that sprouted from the base of the villi and penetrated into the decidua; and fusiform IT that rose from the tips of the TCC and infiltrated the placental bed as deep as the myometrium. Some of these IT were located in the maternal arterial walls and lumen as intravascular trophoblasts (IVT), while others fused with each other to form multinucleated placental-bed giant cells (PBGC) (Loke, 1990). When sections were incubated with anti-ST3 antibody, the villous CTB and their developing TCC anchors were unreactive. In contrast, clearly positive IT and PBGC were interspersed among negative decidual cells throughout the placental-bed thickness [Figure 1(C), (D) and (E)]. In some maternal blood vessels, the endothelial lining had been replaced by ST3-positive trophoblasts [Figure 1(F)]. Deeper in the myometrium, very few ST3-positive trophoblastic cells were observed (data not shown).

In situ hybridization data clearly indicated that ST3 mRNA was localized in the same immunoreactive cell populations (i.e. IT, PBGC and IVT) in the first trimester placental beds (Figure 2).

Analysis of first trimester floating villi by both immunohistochemistry and in situ hybridization revealed that the STB layer, which borders the villous surface, was strongly positive for ST3 protein and mRNA transcripts. In contrast, no detectable label was observed in the underlying villous CTB or in the stromal core (including the fetal fibroblasts, Hofbauer's cells and vascular endothelial cells) [(Figures 1(B) and 2(A)].

As gestation progresses, placental morphology changes. In floating villi, the villous surface expands and the CTB become widely separated. When term floating villi were examined, only STB were positive for ST3; CTB and the stromal core were negative [Figure 3(B)]. These findings were confirmed by hybridization studies (data not shown). In term placental beds, the TCC had frequently lost their structure and were limited to a few cell layers. Their precise delimitation required cytokeratin immunolabelling [Figure 3(A)]. As illustrated

in Figure 3(B), the TCC were negative for ST3. In the decidua, cytokeratin-positive IT were present [Figure 3(A)] but they had assumed a more rounded shape than during the first trimester, an observation that reflects their diminished migratory capacity. Immunolabelling of the third trimester placental beds did not reveal any significant modification in the expression pattern of ST3. No immunoreactivity was observed in TCC, while positivity was only seen in IT [Figure 3(B)]. ST3 mRNA localization was restricted to IT (data not shown).

These histological studies were complemented by Northern blot analysis in order to obtain a semiquantitative evaluation of ST3 mRNA expression [Figure 4(A)]. Densitometric scanning of the blots, after standardization for the total amount of mRNA deposited on the gel, indicated that in floating villi, the level of ST3 mRNA increased threefold from the first trimester to term [Figure 4(B)]. In contrast, the level of ST3 mRNA measured in placental beds was 4.5-fold lower at term than during the first trimester [Figure 4(B)].

DISCUSSION

In this study, the *in vivo* expression of ST3, a member of the MMP family, was analysed in human placenta during the first and third trimesters of gestation. To the best of our knowledge, this is the first demonstration of the spatiotemporal distribution of this enzyme during human pregnancy.

The processes of implantation and placentation both depend on the penetration and remodelling of the uterine endometrium and vasculature by invasive trophoblasts. The migratory nature of human trophoblasts resembles closely that of highly invasive tumours, so that the normal trophoblasts have been called 'pseudomalignant' (Strickland and Richards, 1992). In the pregnant endometrium, the decidual cells surround themselves with a sparse fibrillar network of different matrix proteins, including laminin, fibronectin, collagens I, III, IV, V, entactin, heparan sulfate proteoglycan, etc. (Wewer et al., 1985; Foidart et al., 1990; Damsky et al., 1992). To degrade these matrix components, human first trimester invasive trophoblasts have been shown to secrete a large array of proteolytic enzymes including u-PA (Hofmann et al., 1994), interstitial collagenase (Moll and Lane, 1990), gelatinase A (Fernandez et al., 1992; Polette et al., 1994), gelatinase B (Polette et al., 1994), and membrane-type MMP (Nawrocki et al., 1996).

Immunohistochemical labelling and *in situ* hybridization showed that, in addition to these previously described proteinases, extravillous invasive trophoblasts also express ST3 during the first and third trimesters of pregnancy. The level of ST3 expression was lower during the third trimester as revealed by Northern blot analysis. However, we can not completely exclude that this decreased ST3 mRNA level could result from a reduced number of IT present in term placental bed biopsies.

Interestingly, CTB which proliferate to form TCC are initially noninvasive villous cells that progressively differentiate

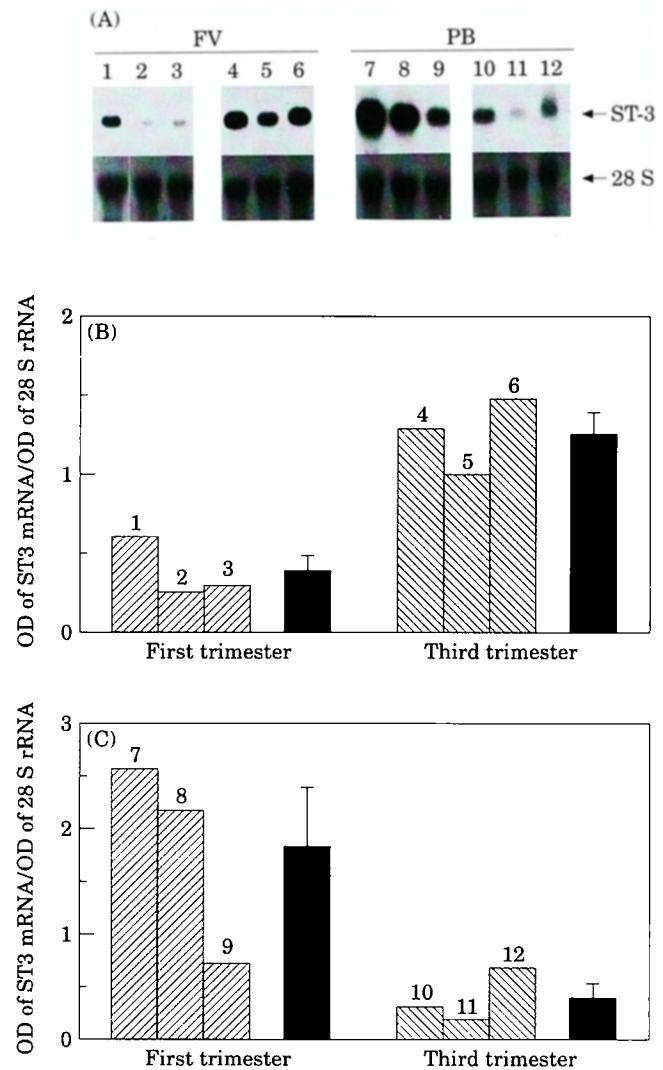


Figure 4. (A) Northern blot analysis of ST3 mRNA and 28 S rRNA in first and third trimester floating villi (FV) and placental beds (PB). Total RNA was isolated, electrophoresed and blot hybridized as described in 'Materials and Methods'. Lanes 1, 2, 3: first trimester FV (9, 8, and 10 weeks, respectively); lanes 4, 5, 6: third trimester FV (38, 40, and 40 weeks, respectively); lanes 7, 8, 9: first trimester PB (9, 8, and 10 weeks, respectively); lanes 10, 11, 12: third-trimester PB (38, 40, and 40 weeks, respectively). (B) and (C) Densitometric analysis of the Northern blot. Levels of ST3 transcripts in (B) floating villi and (C) placental beds of first and third trimesters were quantified by densitometric analysis of autoradiographs of the Northern blots. All results were corrected for RNA loading by densitometric data obtained for the 28 S rRNA signals. Results are expressed as optical density (OD) of ST3 signal/OD of 28 S rRNA. Relative OD of individual samples (hatched bars) and mean values for first and third trimesters (black bars) are presented. Gestation ages are identical to those described in (A).

into highly invasive IT (Damsky et al., 1992; Damsky, Sutherland and Fisher, 1993; Denker, 1993). They express ST3 only in their most distal regions, where they become embedded into the decidual compartment as IT. This spatially regulated onset of ST3 expression coincides with the down-regulation of the $\alpha_6\beta_4$ integrin and the concomitant appearance of the $\alpha_1\beta_1$ integrin, which mediates the interaction of trophoblasts with different ECM ligands (Damsky et al., 1992; Damsky, Sutherland and Fisher, 1993). This switch in

adhesion-molecule expression has been shown to contribute to the acquisition of an invasive phenotype by cultured CTB (Damsky et al., 1992; Damsky, Sutherland and Fisher, 1993; Denker, 1993). Moreover, integrin-ligand interactions have been reported previously to modulate the expression of MMP in several cell lines (Werb et al., 1989; Seftor et al., 1993; Seltzer et al., 1994). Therefore, we hypothesize that during the differentiation of CTB into invasive IT, ST3 expression could be induced by specific integrin-mediated interactions with ECM components. Our observations indicate that the expression of ST3 by these cells may contribute to the extensive basement membrane and stroma remodeling associated with first trimester trophoblast invasion.

In addition to the extravillous trophoblasts, we also localized ST3 in the villous STB which line the intervillous vascular spaces. Previous studies demonstrated the presence of u-PA, interstitial collagenase, gelatinase A and B and their corresponding inhibitors (plasminogen-activator inhibitors 1 and 2 and tissue inhibitor of metalloproteinase 1) in the villous STB (Feinberg et al., 1989; Moll and Lane, 1990; Fernandez et al., 1992; Hofmann et al., 1994; Polette et al., 1994). In this particular location, it is possible that ST3, in conjunction with the other proteinases, may play a role by preventing extensive perivillous deposition of fibrin. Such deposits would reduce the solute transfer between the maternal and fetal blood, thus dangerously limiting the exchange of nutrients and gases to the embryo.

It is interesting to note that the ST3 distribution pattern is very similar to that previously reported for u-PA (Hofmann et al., 1994). These two proteinases are expressed mostly in the same trophoblastic cells (STB, IT and IVT), suggesting that both enzymes may cooperate during placenta formation and functioning, possibly through a proteinase cascade. A similar colocalization of these two proteinases has already been reported in breast carcinomas (Wolf et al., 1993), as well as during mouse embryo implantation (Lefebvre et al., 1995). Recently, Pei, Majmudar and Weiss (1994) demonstrated that purified human ST3 is able to cleave α_2 -macroglobulin, an

inhibitor of all proteinases, as well as two serine proteinase inhibitors: α_1 -proteinase inhibitor and the α_2 -antiplasmin. This demonstration reinforces the hypothesis that ST3 and u-PA could be involved in a proteolytic cascade. Indeed, cell-bound u-PA is a key enzyme in the initiation of the plasminogen activation cascade, a major pathway of extracellular proteolysis. Also, u-PA can convert the widely occurring zymogen plasminogen into enzymatically active plasmin (Dano et al., 1985; Liotta, Steeg and Stetler-Stevenson, 1991; Pöllänen, Stephens and Vaheri, 1991), which can directly degrade various ECM glycoproteins (Montgomery et al., 1993) and activate other matrix-degrading enzymes, such as pro-interstitial collagenase (He et al., 1989; Murphy et al., 1994), pro-gelatinase B and pro-stromelysin-1 (Murphy et al., 1994) which mediate this matrix degradation. As ST3 is able to cleave and inactivate the proteinase inhibitors cited above, the concomitant production of ST3 and plasmin (via u-PA activity) by trophoblastic cells could be seen as a prerequisite for initiating a potent proteolytic cascade that enables the degradation of most ECM components. However, the capacity of other MMP to cleave plasmin inhibitors (Zhang et al., 1994; Noël et al., 1995) suggests that ST3 might also have another proteolytic activity against a more specific substrate, presently unknown (Noël et al., 1995). Our results also demonstrate that, in contrast to epithelial tumor cells which induce the synthesis of ST3 in adjacent fibroblasts (Basset, Wolf and Chambon, 1993), the invasive trophoblasts themselves produce ST3 and other proteinases which are involved in the infiltration of the maternal decidua.

From these different observations, we can conclude that ST3 expression is spatially restricted to differentiated, non-proliferative, villous (STB) and extravillous (IT, PBGC and IVT) trophoblastic populations, both of which are involved in important steps of placentation. This demonstration constitutes the first non-fibroblastic localization to be reported for ST3. All previous studies in human tissues indicated that ST3 was exclusively expressed by stromal cells (for review, see Basset, Wolf and Chambon, 1993).

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