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REGULAR ARTICLE

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Immunohistochemistry of matrix metalloproteinases (MMP), their substrates, and their inhibitors (TIMP) during trophoblast invasion in the human placenta

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Abstract The invasion of extravillous trophoblast cells into the maternal endometrium is one of the key events in human placentation. The ability of these cells to infiltrate the uterine wall and to anchor the placenta to it as well as their ability to infiltrate and to adjust utero-placental vessels to pregnancy depends, among other things, on their ability to secrete enzymes that degrade the extracellular matrix. Most of the latter enzymes belong to the family of matrix metalloproteinases. Their activity is regulated by the tissue inhibitors of matrix metalloproteinases. We have studied the distribution patterns of matrix metalloproteinases-1, -2, -3, and -9 and their inhibitors TIMP-1 and TIMP-2 as compared to the distribution of their substrates along the invasive pathway of extravillous trophoblast of 1st, 2nd, and 3rd trimester placentas by means of light microscopy on paraffin and cryostat sections as well as at the ultrastructural level (only 3rd trimester placenta). The comparison of different methods proved to be necessary, since the immunohistochemical distribution patterns of these soluble enzymes are considerably influenced by the pretreatment of tissues. All three methods revealed immunoreactivities of both, proteinases and their inhibitors, not only intracellularly in the extravillous trophoblast but also extracellularly in its surrounding matrix, the distribution patterns depending on the stage of pregnancy and on the degree of differentiation of trophoblast cells along their invasive pathway. Within the extracellular matrix, immunolocalization of matrix metalloproteinases as well as their inhibitors showed a specific relation to certain extracellular matrix molecules.

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Introduction

The development of the human fetus depends on the ability of extravillous trophoblast cells to invade the maternal uterine tissues in order to anchor placenta and fetus to the maternal endometrium and to gain access to and control of the maternal circulation (Fisher and Damsky 1993; Benirschke and Kaufmann 1995; Kaufmann and Castellucci 1997). Extravillous trophoblast cells start their migration from the basement membrane of anchoring villi and invade deeply into the maternal uterus, finally reaching the myometrium.

Along this invasive pathway, extravillous trophoblast cells change their phenotype. Adjacent to the basement membrane of anchoring villi, proliferating stem cells of the extravillous trophoblast prevail (Bulmer et al. 1988; Mühlhauser et al. 1993). Their daughter cells achieve an invasive phenotype and migrate into deeper layers of the materno-fetal junctional zone. Only these latter cells start secreting matrix-type fibrinoid as its highly characteristic extracellular matrix in a seemingly unpolarized fashion (Frank et al. 1994, 1995; Lang et al. 1994; Huppertz et al. 1996). In doing this, they separate from each other, leaving large amounts of this peculiar fetal extracellular matrix around and behind them.

The ability of invasive extravillous trophoblast cells to invade maternal tissues involves at least three main factors: (1) binding of cells to the extracellular matrix, (2) polar degradation of extracellular matrix (ECM) in the direction of migration, and (3) active movement through the matrix. The first and also the third factor require the presence of cell surface receptors for matrix proteins, e.g. integrins. Their expression patterns change throughout differentiation of extravillous trophoblast from the proliferative to the invasive phenotype (Damsky et al. 1992; Aplin 1993; Bischof et al. 1995).

The second factor, degradation of extracellular matrix, requires respective enzymes. Different families of matrix-degrading proteases were found to be involved in trophoblast invasion into endometrial tissues. These include serine proteases of the plasmin system (Strickland et al. 1976; Castellucci et al. 1994; Hofmann et al. 1994; Multhaupt et al. 1994) and the matrix metalloproteinases (MMPs) (Fisher et al. 1985; Moll and Lane 1990; Librach et al. 1991; Autio-Harmainen et al. 1992; Fernandez et al. 1992; Polette et al. 1994; Hurskainen et al. 1996; Nawrocki et al. 1996, 1997; Vettraino et al. 1996).

MMPs are a still growing family of enzymes known to be involved in the degradation of extracellular matrices. This family is divided into four groups according to their substrate specificities and localizations:

1. Collagenases degrade mostly fibrillar collagens I, II, and III and include interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), and a recently isolated tumor-associated collagenase-3 (Freije et al. 1994).
2. Gelatinases degrade mostly denatured collagens (gelatins) and native collagen IV (Murphy et al. 1985) and include gelatinase A, a 72 kDa type IV collagenase (MMP-2), and gelatinase B, a 92 kDa type IV collagenase (MMP-9).
3. Stromelysins have the broadest substrate range (fibronectins, laminin, collagens III, IV, V, elastin, proteoglycan core proteins) and include stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), stromelysin-3 (MMP-11), matrilysin (MMP-7), and a metallo-elastase from human lung alveolar macrophages (Shapiro et al. 1993).
4. Membrane-type matrix metalloproteinases (MT-MMPs) make up a new subclass and include MT-MMP-1, -2, and -3 (Sato et al. 1994; Will and Hinzmann 1995; Takino et al. 1995). These MT-MMPs are localized at cell surfaces and their substrate specificities are not yet clear. MT-MMP-1 is known to be involved in the transformation of the latent to the active form of MMP-2 (Atkinson et al. 1995; Cao et al. 1995).

All MMPs are secreted as inactive proforms and must be cleaved to become active. Therefore latent and activated forms of MMPs can be found in extracellular matrices. Since the various members of the family may activate themselves or each other, with the activation of one single enzyme it might be possible to start a chain-reaction leading to the complete destruction of even a complex matrix.

The activity of matrix metalloproteinases is tightly regulated, among other things, by specific tissue inhibitors (TIMPs). These have been described to be expressed by human decidual cells in vitro (Graham and Lala 1991) and in vivo (Marzusch et al. 1996), by murine decidual cells in vivo (Nomura et al. 1989; Werb et al. 1992), and recently by human extravillous trophoblast cells in vivo (Hurskainen et al. 1996; Ruck et al. 1996). Up to now, three different TIMPs have been described. TIMP-1 inhibits all MMPs in activated form and, moreover, MMP-9 (gelatinase B) in both the latent and the active form (Goldberg et al. 1992). TIMP-2 binds to both forms of gelatinase A (MMP-2), its inhibitory activity to other MMPs is clearly lower (De Clerck et al. 1989; Stetler-Stevenson et al. 1989). Also, TIMP-3, recently discovered in the mouse (Leco et al. 1994) and the human (Apte et al. 1994; Uria et al. 1994), shows inhibition of MMPs.

Several groups have published data on the distribution patterns of MMPs and TIMPs in extravillous trophoblast by means of light-microscopical immunohistochemistry and in situ hybridization (Moll and Lane 1990; Autio-Harmainen et al. 1992; Fernandez et al. 1992; Polette et al. 1994; Hurskainen et al. 1996; Nawrocki et al. 1996, 1997; Ruck et al. 1996; Vettraino et al. 1996). All of these studies focussed either on selected stages of pregnancy or on certain levels of the invasive pathway. Moreover, different protocols of tissue preparation have been used. As a consequence, the data are barely comparable. Moreover, in the course of this study, we realized that the fixation protocols and tissue preparation considerably influenced the immunohistochemical distribution patterns.

We have studied the immunolocalizations of MMP-1, -2, -3, and -9 as well as TIMP-1 and -2 in order to provide a set of methodologically comparable immunohistochemical data for those matrix metalloproteinases and their inhibitors that are most likely to be involved in trophoblast invasion. To achieve this goal, the reactions were performed both at the light-microscopical (first to third trimester) and at the ultrastructural level (third trimester) using immunohistochemistry on unfixed cryostat sections, immunohistochemistry on formaldehyde-fixed paraffin sections, and ultrastructural immunocytochemistry on resin sections of paraformaldehyde-fixed specimens.

The extracellular matrix of the invasive trophoblast (matrix-type fibrinoid) is a highly heterogeneous mixture of various matrix molecules (Huppertz et al. 1996). We were interested whether the spatial localization of the enzymes can be correlated with the distribution of their substrates. For this purpose, the ultrastructural immunocytochemical data on MMPs were compared with those concerning the distribution of relevant extracellular matrix molecules.

Materials and methods

Material Human placentas (n=14) from clinically normal pregnancies of 9, 10, 12, 16, and 38–41 weeks of gestation were collected. Pregnancies were interrupted by curretage for psychosocial reasons or terminated by cesarean section for medical reasons, which were unlikely to affect placental structure and function. All placentas were investigated by light-microscopical immunohistochemistry. Three placentas from 38–40 weeks of gestation were examined by electron-microscopical immunocytochemistry.

Tissue preparation For cryostat sectioning, pieces of placental tissues with a maximum edge length of 20×20×5 mm were frozen in liquid nitrogen. Serial cryostat sections (5–8 µm) were cut at –21°C and mounted on glass slides. The sections were fixed either in absolute acetone (10 min at 4°C) or in 4% neutrally buffered formalin (4 min at room temperature).

For paraffin embedding, cubes of placental tissues with a maximum edge length of 20×20×5 mm were fixed in a neutral, phos-

Table 1 Primary antibodies used in immunohisto- and -cytochemistry. All MMP antibodies used for this study recognize the active as well as the proforms of the enzymes

Antibody	Type of antibody	Dilution	Source
anti-TIMP-1	Monoclonal, clone 7–6C1	1:2	Dianova
anti-TIMP-2	Monoclonal, clone T2-101	1:1	Dianova
anti-MMP-1	Monoclonal, clone 41–1E5	1:200	Dianova
	Polyclonal (RP2C1)	1:500	Triple Point Biologics
anti-MMP-2	Monoclonal, clone 75–7F7	1:200	Dianova
	Polyclonal (RP2G72)	1:500	Triple Point Biologics
anti-MMP-3	Monoclonal, clone 55–2A5 (B)	1:200	Dianova
	Polyclonal (RP2S1)	1:500	Triple Point Biologics
	Polyclonal (RP3S1)	1:1000	Triple Point Biologics
anti-MMP-9	Monoclonal, clone 6–6B	1:200	Dianova
	Polyclonal (RP2G92)	1:500	Triple Point Biologics
anti-Collagen IV	Monoclonal, clone CIV 22	1:50	Dako
anti-Laminin (B-1 portion)	Monoclonal, clone C1	1:100	Telios
anti-Heparan sulfate	Monoclonal, clone 7E12	1:10	Boehringer Mannheim
anti-Vitronectin	Monoclonal, clone 8E6	1:10	Boehringer Mannheim
Cellular fibronectin (ED-A)	Monoclonal, clone IST-9	1:4	Carnemolla et al. 1987
Oncofetal fibronectin (III-7 domain followed by ED-B)	Monoclonal, clone BC-1	1:2	Carnemolla et al. 1989, 1992
anti-Cytokeratin	Monoclonal, clone MNF 116	1:500	Dako
anti-Vimentin	Monoclonal, clone V9	1:20	Dako

phate-buffered 4% formaldehyde solution for maximally 24 h at 4°C. The samples were dehydrated in a graded series of ethanol and embedded in paraffin (melting point: 52°C, Merck, Germany) using xylene as an intermedium. During embedding, the temperature did not exceed 58°C. Serial sections (3–5 µm) were cut and mounted on glass slides. The sections were deparaffinized using xylene and a graded series of ethanol (10 min each step).

For ultrastructural immunocytochemical analysis, samples from different parts of the basal plates of three term human placentas were used. Specimens with an average edge length of 2×1×1 mm were gently dissected and fixed at 4°C for 18 h in 4% paraformaldehyde [in phosphate-buffered saline (PBS), pH 7.4, with 6.5% (w/v) sucrose]. After fixation, the samples were rinsed in PBS at 4°C (3×10 min). Dehydration took place in an increasing ethanol gradient with a decreasing temperature gradient using a CS Auto (Reichert-Jung, Austria). Dehydration was completed in 100% ethanol at –35°C. The samples were embedded in Lowicryl K4M or LR-Gold (Polysciences Ltd., Germany) at –35°C using monomer concentration steps of 33%, 66%, and three times 100%. Polymerization took place under UV light for 24 h, raising the temperature by 3°C/h, and was completed at room temperature for an additional 48 h. The accomplished blocks were stored in an exsiccator at room temperature.

Light-microscopical immunohistochemistry The data concerning the primary antibodies are summarized in Table 1. According to the producer's descriptions, the MMP-antibodies used for our study are thought to show no cross-reactivities with other MMPs. In order to test this assumption, we have applied at least one monoclonal and one polyclonal antibody for each MMP. The immunohistochemical results were always consistent. Moreover, the antibodies have been tested in Western blots of placental extracellular matrix extracts prepared as described earlier (Frank et al. 1995). The Western blots for MMP-2 and MMP-3 recognized proteins with apparent molecular weights in the ranges of about 66–72 and 52–60 kDa, respectively, corresponding to the molecular weights of the enzymes (pro- and active forms), thus demonstrating the single-protein specificity of both antibodies (data not shown). Extraction of MMP-1 and MMP-9 from extracellular matrices proved to be difficult and no reliable results could be obtained; this is in agreement with the experience published by Woessner (1991). Because of this, for the latter two enzymes, our assumption of monospecificity is based only on the consistency of immunohistochemical data obtained with monoclonal as compared to polyclonal antibodies. For TIMP-1 and TIMP-2, only monoclonal antibodies were available, which were only tested by Western blotting. Here, we did not find any evidence of cross reactivity (data not shown) suggesting that these antibodies are monospecific.

Immunohistochemical reactions with monoclonal and polyclonal primary antibodies were performed using a standardized sequence (Aachen-Kit) based on the streptavidin-biotin technique for detection of a biotinylated link antibody as previously described (Frank et al. 1994).

Electron-microscopical immunocytochemistry Ultrathin sections (0.1 µm) were mounted on nickel grids (300 mesh) and treated with undiluted bromelin solution (Biotest, Germany) for 20 min at 37°C (Frank et al. 1994). Unspecific binding was blocked by incubation in 50 mM glycin in PBS (pH 7.6) for 15 min and subsequent immersion in incubation buffer (PBS, pH 7.6, 0.5% BSA) with 5% goat serum for 30 min. After washing (1×5 min in incubation buffer, 2×5 min in incubation buffer containing 0.1% Tween-20), the first antibody was used at 4°C overnight. For antibodies and dilutions, see Table 1. After a second washing step, the grids were incubated for 2 h with the secondary antibody (goat anti-mouse or goat anti-rabbit, 15/25 nm gold labelled, Polysciences Ltd., Germany), diluted 1:30 in incubation buffer. The grids were washed again as described above and fixed for 5 min in 2% glutaraldehyde (EM-grade, Polysciences Ltd., Germany) in PBS (pH 7.6), afterwards immersed in aqua bidest. (2×5 min), and stained with lead citrate and uranyl acetate. Subsequently, all grids were examined in a Philips 300 electron microscope.

Results

Definition of structures

Anchoring villi are all those villi that are connected to the basal plate of the placenta by means of a trophoblastic cell

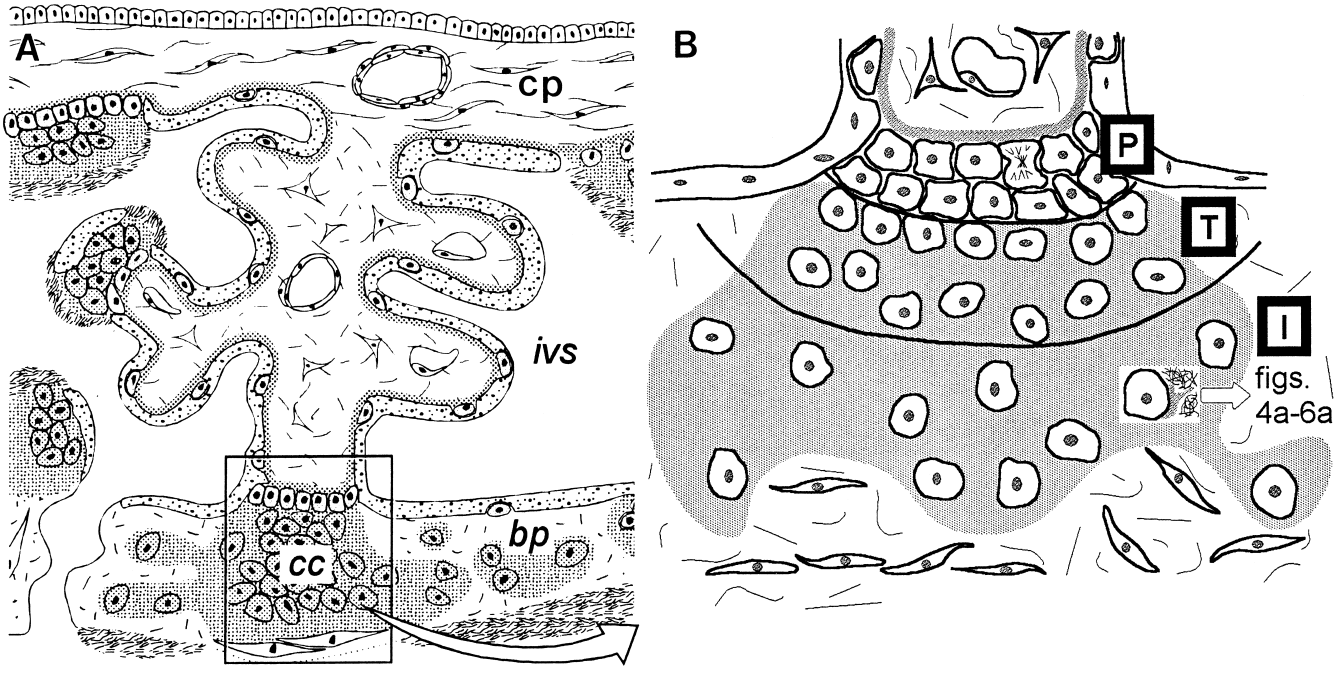


Fig. 1A, B **A** Scheme of an anchoring villus reaching from the chorionic plate into the basal plate. Extravillous trophoblast cells (*shaded*) can be found in different locations, but are always embedded in their self-secreted extracellular matrix, the matrix-type fibrinoid (*point shaded*). *bp* Basal plate, *cc* cell column, *cp* chorionic plate, *ivs* intervillous space. **B** Higher magnification of a connecting anchoring villus to the basal plate. The first cell layers facing the basement membrane between villous stroma and trophoblast represent the proliferating stem cell population (*P*). Clearly separated from the basement membrane in the transitional zone (*T*), the cells change their phenotype; they stop proliferation and start secreting ample extracellular matrix in a seemingly unpolarized fashion. After leaving the transitional zone, the cells invade maternal tissues, now achieving an invasive phenotype (*I*)

column (Fig. 1). The trophoblast cells of the cell columns (extravillous trophoblast) are composed of two different phenotypes (for reviews, see Fisher and Damsky 1993; Benirschke and Kaufmann 1995; Kaufmann and Castellucci 1997):

1. The *proliferative phenotype* (proximal part of cell columns, P in Fig. 1B) is represented by one to several compact layers of cells, which are attached to each other by intercellular junctions without light-microscopically detectable intercellular accumulations of extracellular matrix. The basal layer of this cluster of stem cells, directly connected to the stroma of the anchoring villi, is clearly polar and rests on a basal lamina that separates the trophoblastic cell column from the villous stroma. In contrast, the following, deeper layers of proliferating cells have lost polarity.
2. One to five cellular layers peripherally from the basal lamina, in the transitional zone, (T in Fig. 1B) trophoblast cells leave the cell cycle and start differentiating. First spots of identifiable extracellular matrix dilate the intercellular spaces among these post-proliferative, early-invasive trophoblast cells. The *invasive phenotype* (I in Fig. 1B) comprises the later post-proliferative extravillous trophoblast cells that no longer form intercellular junctions. They become separated from each other by increasing amounts of extracellular matrix (matrix-type fibrinoid) and spread from the cell columns into the surrounding basal plate and into the endometrium.

This differentiation pathway is accompanied by respective changes in the integrin-expression patterns (integrin shift: Damsky et al. 1994). Also without application of proliferation markers and integrin immunohistochemistry, the different phenotypes can be easily discriminated by their topographical relations (only in perpendicular sections of cell columns) and by the absence or presence of extracellular matrix embedding the cells (Kaufmann and Castellucci 1997).

With decreasing proliferative activity of the stem cells in later gestation, the proximal, proliferating cellular layers of cell columns become rarified. Rather, most of the remaining extravillous trophoblast is represented by deeply invasive, extravillous trophoblast cells, which are evenly spread over the entire materno-fetal junctional zone.

Light-microscopical immunohistochemistry

The results obtained from light-microscopical immunohistochemistry cover extravillous trophoblast cells of the cell columns, the attached parts of the basal plate, as well as the neighboring basal lamina of anchoring villi of placentas from all three trimesters. In the junctional zone (zone I in Fig. 1B), identity of trophoblast cells was proven by cytokeratin immunoreactivity, whereas

decidual cells were marked by anti-vimentin staining (cf. Fig. 3A,B). Our light-microscopical data on MMPs presented here are based on paraffin and cryostat sections incubated with polyclonal antibodies. Being different from the electron-microscopical immunocytochemistry, in light microscopy the monoclonal antibodies gave some background staining and thus were not further evaluated. The monoclonal antibodies against TIMP-1 and TIMP-2 did not show immunoreactivities on paraffin sections, so that only respective cryostat sections were evaluated. The data for both methods and all three trimesters of pregnancy are synoptically schematized in Fig. 2.

MMP-1

In first trimester placentas, the basal lamina between villous stroma and the proximal parts of cell columns showed a stronger reactivity on cryostat than on paraffin sections. Proliferating stem cells of the cell columns were negative, while some cells of the transitional zone were stained only on paraffin sections. The invasive trophoblast cells showed uniform reactivity mostly extracellularly in the surrounding matrix on paraffin sections, while on cryostat sections reactivity was heterogeneously distributed, leaving some of the invasive cells unstained (Fig. 2A,B). The only second trimester case available showed a slightly changed but generally comparable reaction (Fig. 2C,D): on cryostat sections the basal lamina at the proximal part of the cell columns lost reactivity, while on paraffin sections the weak reactivity of the transitional zone now appeared in the proliferative zone. In third trimester placentas, all invasive extravillous trophoblast cells expressed MMP-1, most of the spot-like staining being visible in the extracellular matrix around the invasive cells (Fig. 2E,F). Cells of the proliferative phenotype were so rare that evaluation was impossible.

MMP-2

In cryostat sections from first trimester, weak reactivity of gelatinase A in the proliferative layer and largely absent reactivity in the transitional zone could be detected. Among the invasive extravillous trophoblast cells, groups of highly reactive cells were observed, which also showed accumulation of immunoreactivity in the surrounding ECM (Fig. 2A). In paraffin sections, cell columns showed a clear gradient with absent reactivity in the proliferative parts and a strong, homogeneous immunoreaction in the distal, invasive parts of cell columns (Fig. 2B). In the second trimester, this enzyme showed a staining pattern resembling that of the first trimester (Fig. 2C,D). In term placentas, all extravillous trophoblast cells together with their surrounding matrix were clearly immunoreactive (Fig. 2E,F) with the decidual cells being even more reactive (Fig. 3D).

MMP-3

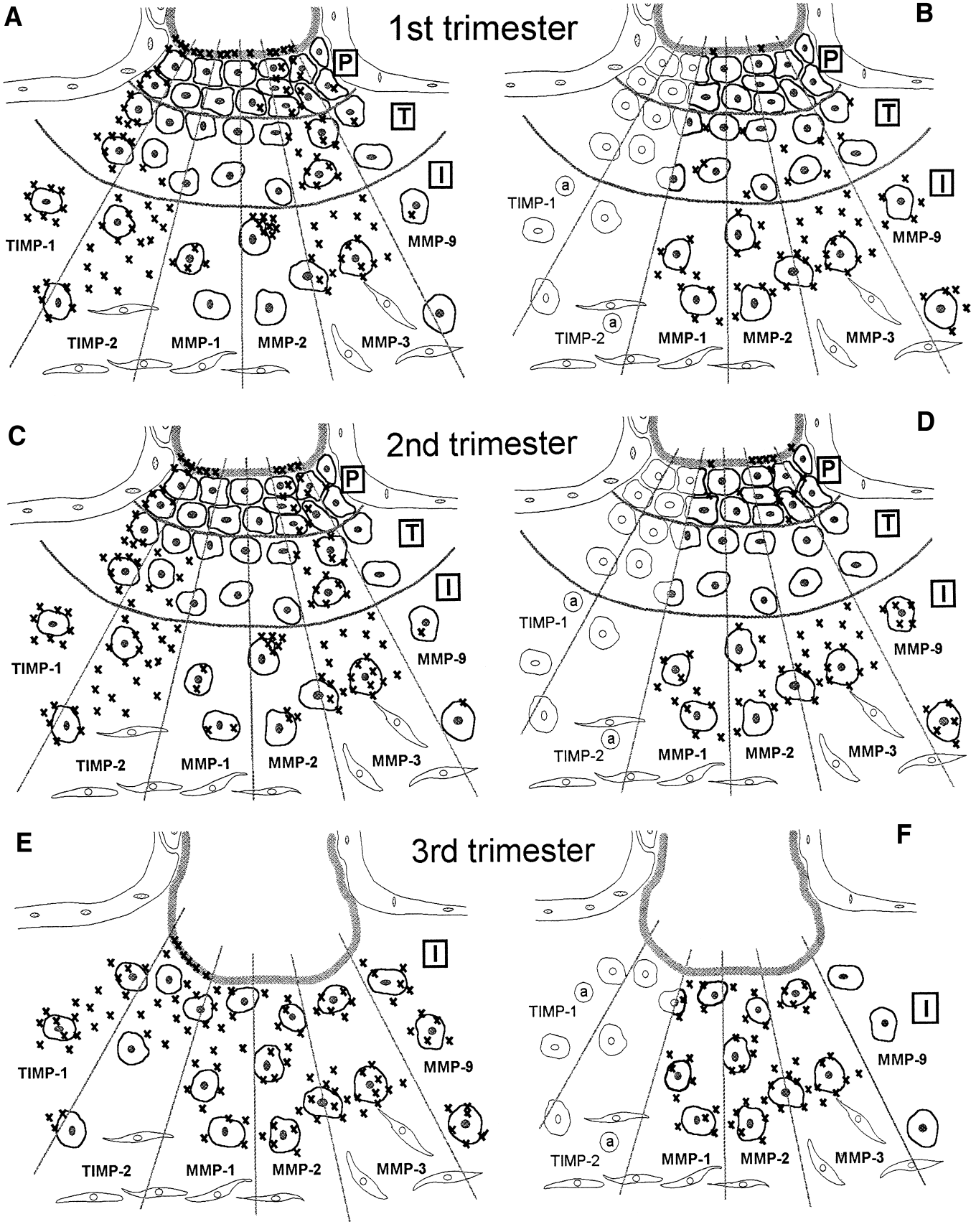
In cryostat sections, stromelysin-1 showed a very constant immunoreaction pattern throughout pregnancy. In all three trimesters of pregnancy, extravillous trophoblast cells showed immunoreactivities over the entire invasive pathway both intra- and extracellularly (Fig. 2A,C,E). In cryostat sections of third trimester placentas, only the invasive extravillous trophoblast cells were strongly immunoreactive, while the decidual cells were immunonegative (Fig. 3E). In paraffin sections, the proliferative cells from first trimester cell columns were negative, while the reactivity of non-proliferative cells of the columns resembled that of cryostat sections (Fig. 2B). In second trimester placentas, the picture of the first trimester changed in that the transitional zone was now negative, while the basal lamina at the proximal part of the column was strongly positive (Fig. 2D). Third trimester placentas showed the same intense staining in paraffin sections observed in cryostat sections (Fig. 2F).

MMP-9

In first trimester placentas with both methods, a rather faint staining of cell columns for gelatinase B was found throughout the entire invasive pathway (Fig. 2A,B). In the second trimester specimen, immunoreactivities on cryostat sections were reduced (Fig. 2C), while on paraffin sections the reactivity of the invasive trophoblast cells remained constant (Fig. 2D). The situation at the end of pregnancy was not clear. On cryostat sections, all extravillous trophoblast cells belonging to the invasive phenotype showed strong intra- and extracellular immunoreactivities (Fig. 2E). Surprisingly, on paraffin sections, immunoreactivities of the extravillous trophoblast were completely lost (Fig. 2F).

TIMP-1

On cryostat sections of first and second trimester placentas, the trophoblastic basal lamina and all extravillous trophoblast cells and their extracellular matrix were immunopositive (Fig. 2A,C). Immunostaining for vimentin-expressing decidual cells and cytokeratin-expressing trophoblast cells in parallel sections revealed that not only decidual cells, but also extravillous trophoblast cells were clearly immunoreactive. And even in areas void of decidua, TIMP-1 immunoreactivities were found in and around extravillous trophoblast cells. In term placentas, the extravillous trophoblast cells, now exclusively belonging to the invasive phenotype, showed uniformly strong expression of TIMP-1 in and around the cells (Fig. 2E).


A
1st trimester
B
P
T
I
TIMP-1
MMP-9
TIMP-2
MMP-1
MMP-2
MMP-3
a
C
2nd trimester
D
E
3rd trimester
F

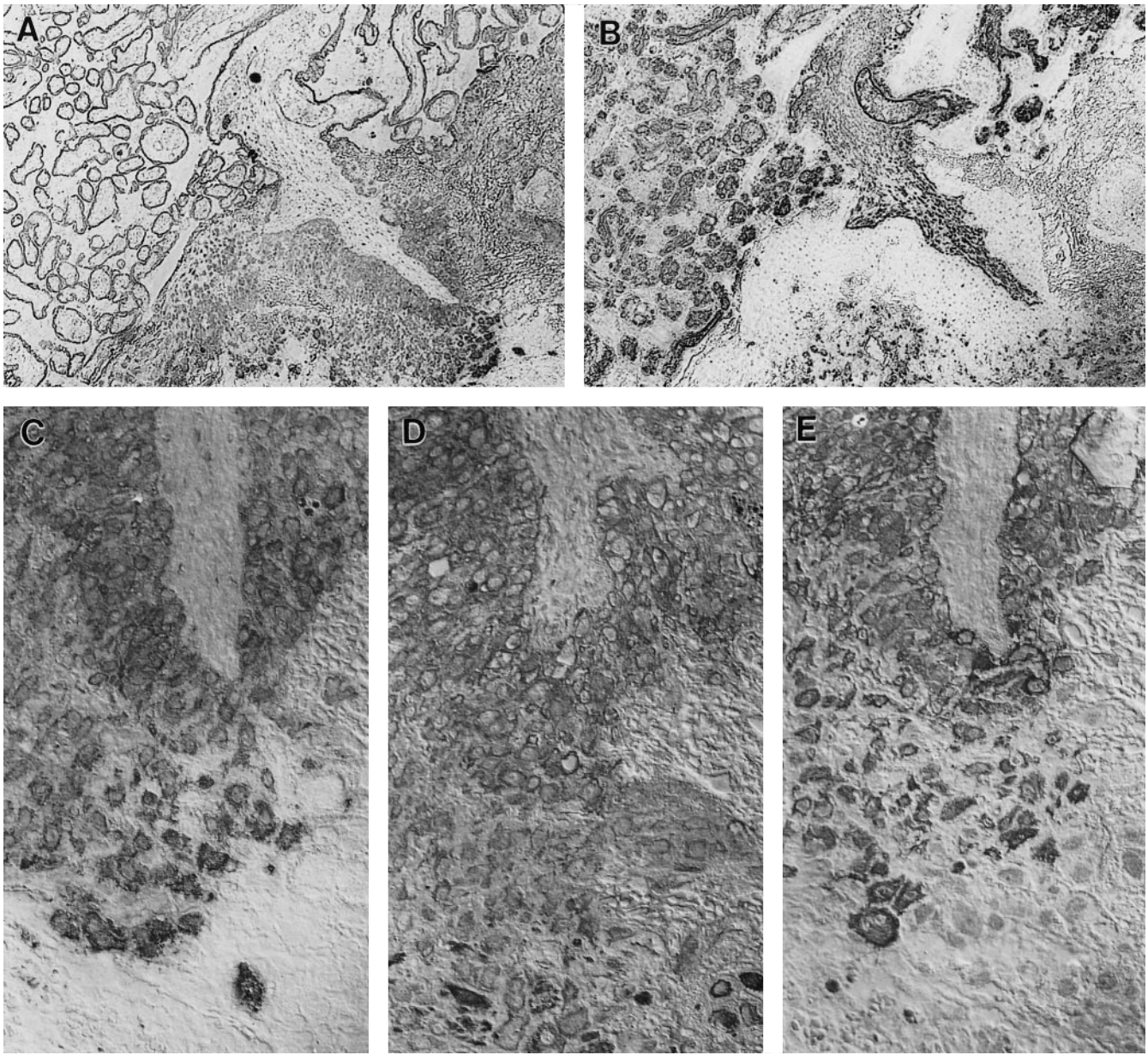


Fig. 3A–E Cryostat sections of term placenta. Distribution of trophoblast cells (**A**) and decidua cells (**B**) could be demonstrated using antibodies against cytokeratin (**A**) and vimentin (**B**) ×12.5. **C** represents a higher magnification of **A** (cytokeratin); **D** and **E** represent subsequent parallel sections showing immunoreactivities for MMP-2 (**D**) and MMP-3 (**E**). Note that MMP-2 is stronger in decidua as compared to trophoblast, whereas MMP-3 can only be found in and around trophoblast cells. ×50

◀ **Fig. 2A–F** Schematic summary of immunohistochemical reaction patterns of antibodies directed against different MMPs and TIMPs in all three stages of pregnancy; *x* represents localization and density of immunoreactivities. **A, C, E** Immunohistochemical reactivities on cryostat sections; **B, D, F** immunohistochemical reactivities on paraffin sections; **A, B** placentas from 9–12 weeks of gestation; **C, D** one specimen from the 16th week of gestation; **E, F** placentas from 38–41 weeks of gestation. Immunoreactivities of villous syncytiotrophoblast and maternal decidua are not listed here. @ No convincing data were obtained

TIMP-2

On cryostat sections of first and second trimester placental material, the basement membrane separating cell columns from stromal cells was positive. Within the extravillous trophoblast, we did not find immunoreactivity in the proliferative phenotype, whereas more distally staining increased with increasing depth of invasion (Fig. 2A,C). In this area of maximal TIMP-2 activities in cell columns, no reactivity for vimentin could be found indicating the absence of any maternal decidual cells (cf. Fig. 3A,B); rather cytokeratin-positive trophoblast cells were immunoreactive for TIMP-2. In third trimester placentas, TIMP-2 reactivity was detected in the basement membrane dividing extravillous trophoblast from villous stroma, whereas the reactivity of extracellular matrix of extravillous trophoblast decreased with increasing depth of invasion (Fig. 2E).

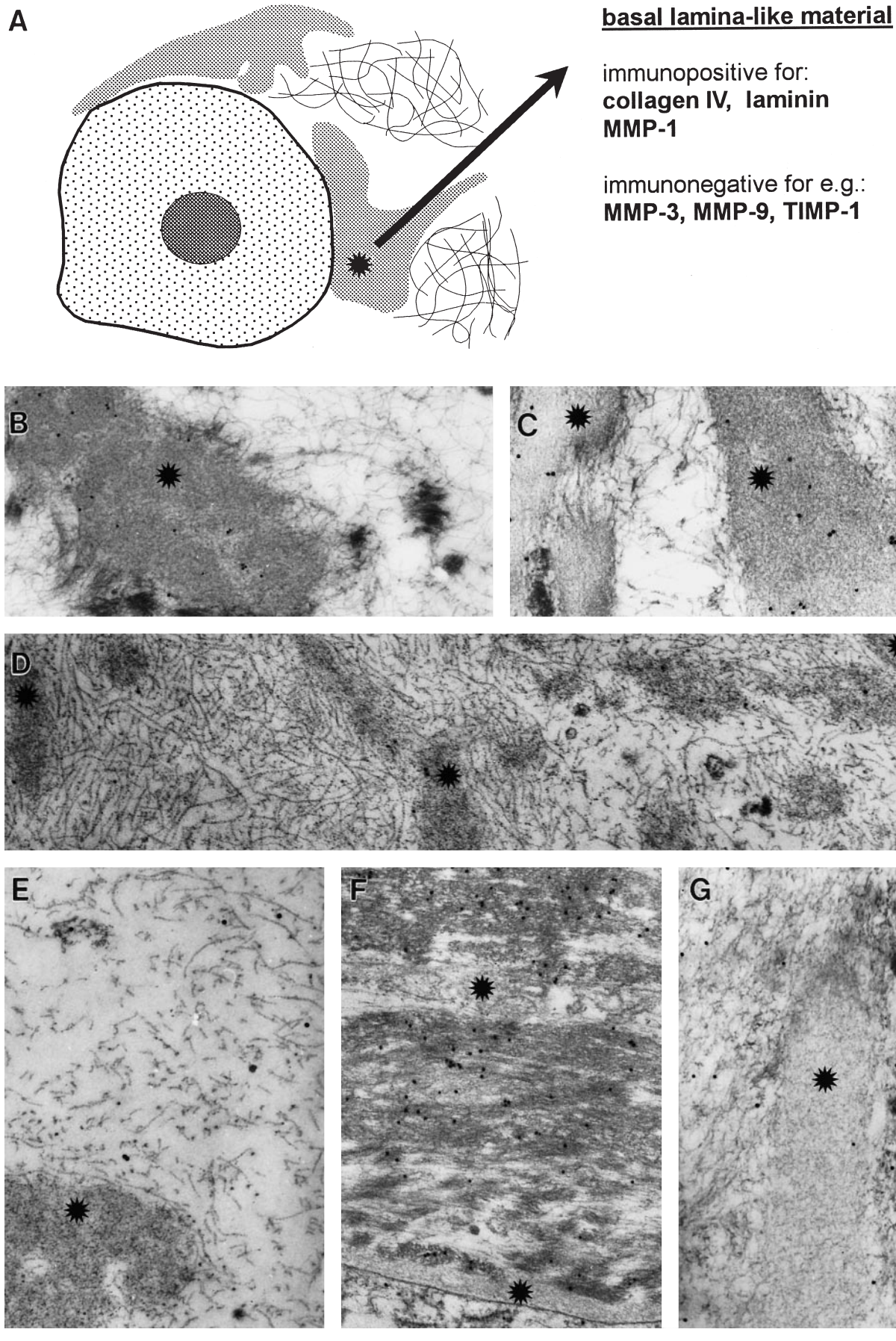

A
basal lamina-like material
immunopositive for:
collagen IV, laminin
MMP-1
immunonegative for e.g.:
MMP-3, MMP-9, TIMP-1
B
C
D
E
F
G

Table 2 Immunocytochemical distribution of MMPs and TIMPs in different areas of matrix in the junctional zone of the placenta. + clearly present, (+) weak staining, – no or very few gold particles

	Matrix-type fibrinoid			Fibrin-type fibrinoid
	Collagen IV/laminin	Fibronectin fibrils	Heparan sulfate/vitronectin	
TIMP-1	–	–	+	–
TIMP-2	–	–	+	–
MMP-1	(+)	–	–	–
MMP-2	–	+	–	–
MMP-3	–	+	–	+
MMP-9	–	+	+	+

Electron-microscopical immunocytochemistry

In order to correlate the extracellular localization of proteinases and their inhibitors with the localization of extracellular matrix molecules as analyzed in previous publications (Frank et al. 1994; Huppertz et al. 1996), ultrastructural studies were performed on material from basal plates of term placentas. Application of monoclonal and polyclonal antibodies (cf. Table 1) resulted in identical staining patterns, so that the results can be described together. We cannot exclude that diffusion of the enzymes and their inhibitors took place during the preparation procedure. Therefore, it has to be cautioned that some of the immunoreactivities may have been dislodged or even lost. However, for most enzymes, the staining patterns resembled those already observed at the light microscopical level.

Immunoreactivity for interstitial collagenase (MMP-1) was rarely found within the extracellular matrix (matrix-type fibrinoid) that surrounded invasive extravillous trophoblast cells. The few gold particles representing MMP-1 (Fig. 4D) were usually associated with granular basal lamina-like material (Fig. 4A) binding laminin and collagen IV antibodies (Fig. 4B,C). Other subtypes of matrix-type fibrinoid as well as fibrin-type fibrinoid remained unstained (Table 2).

The immunocytochemical distribution pattern of MMP-2 (gelatinase A) was more prominent than that of MMP-1. Gold particles were restricted to areas with fine-fibrillar networks (Fig. 6A) and lined the 10–14 nm light-core fibrils of matrix-type fibrinoid (Fig. 6D), which specifically bind to antibodies against cellular and oncofetal fibronectins (Fig. 6B,C). Several matrix subtypes remained completely unlabelled. These comprise homogeneous, glossy ground substance composed of vitronectin and heparan sulfate (Fig. 5A–C), granular, basal lamina-like material composed of collagen IV and laminin (Fig. 4–C), and fibrin-type fibrinoid (Table 2).

The most prominent labelling occurred with the antibodies directed against stromelysin-1 (MMP-3) (Fig. 6E) and gelatinase B (MMP-9) (Fig. 6F). Immunoreactivities for both enzymes were found on the light-core fibrils that bind to various fibronectin antibodies (Fig. 6A–C). In addition, MMP-3 antibodies immunostained fibrin-type fibrinoid (not shown) and MMP-9 antibodies stained the amorphous ground substance of matrix-type fibrinoid (Fig. 5F), which is composed of heparan sulfate and vitronectin. The basal lamina-like material remained completely unstained with antibodies directed against MMP-3 and MMP-9 (Fig. 4E,F).

Immunoreactivities for the inhibitors of the matrix metalloproteinases, TIMP-1 (Fig. 5D) and TIMP-2 (Fig. 5E), were mostly concentrated over the amorphous ground substance (Fig. 5A), which is immunoreactive for heparan sulfate (Fig. 5B) and vitronectin (Fig. 5C). TIMP-2 immunoreactivities were generally weaker than those of TIMP-1. Again, the basal lamina-like material composed of collagen IV and laminin remained unstained for TIMP-1 (Fig. 4G) and TIMP-2.

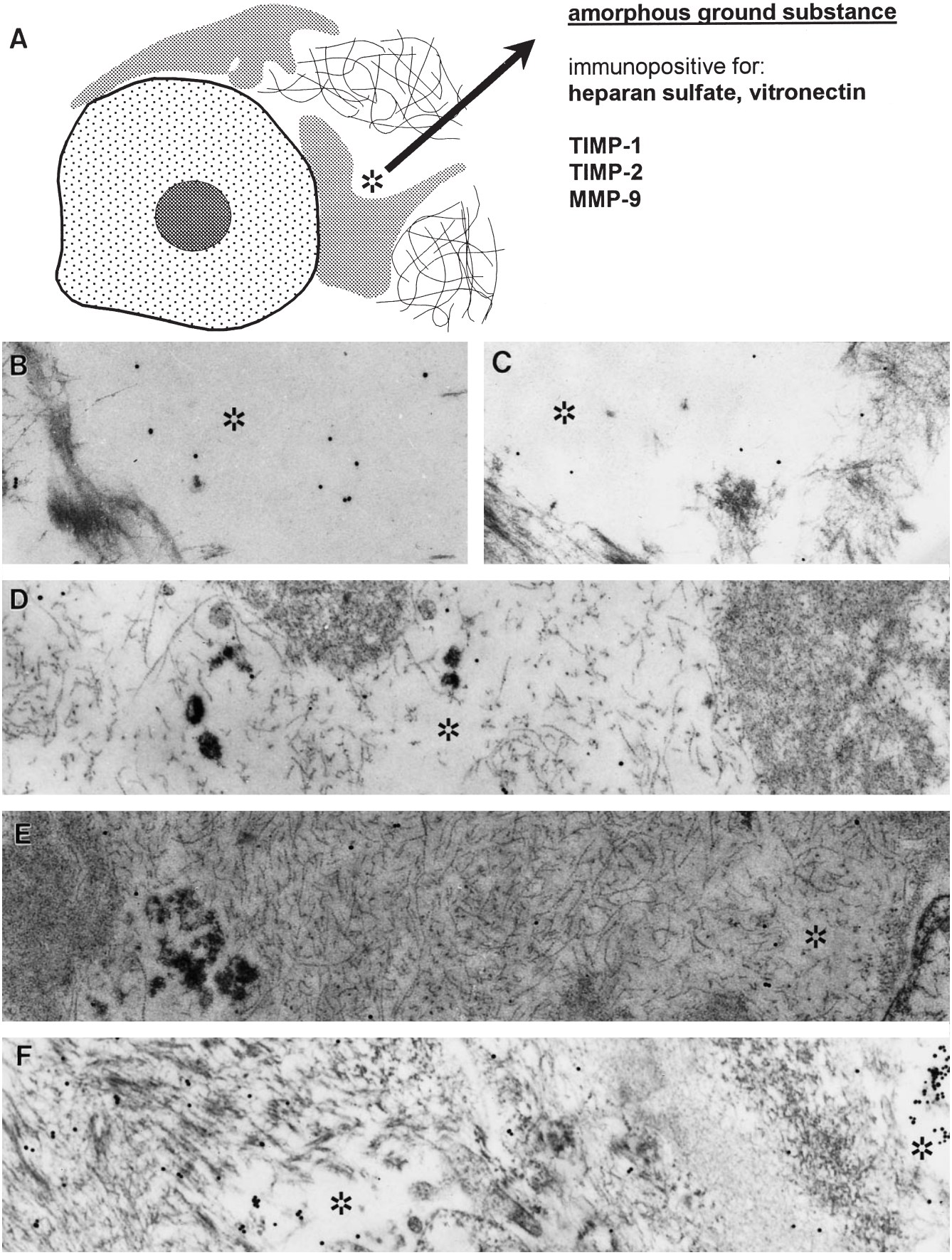
Discussion

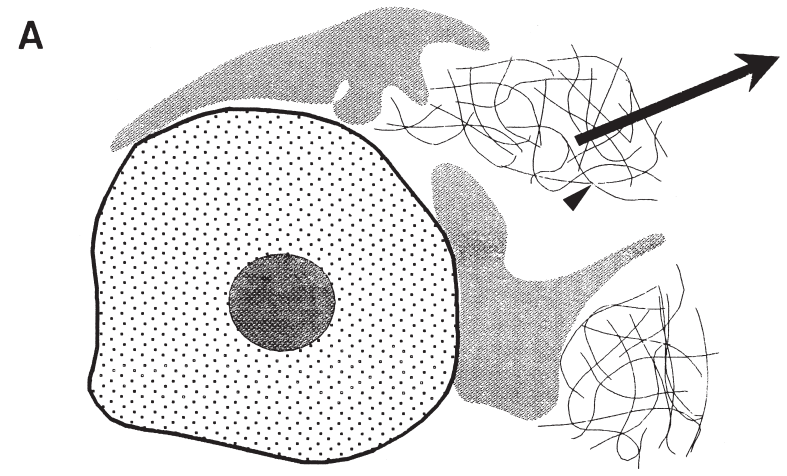
Methodological aspects

When performing immunohistochemistry of secretory, diffusible enzymes, one, generally, has the choice among: (1) unfixed cryostat sections, which guarantee undestroyed immunoreactivities of the antigen, but are usually hampered by loss of enzyme due to diffusion, or (2) prefixed paraffin sections, which reduce the danger of diffusion of macromolecules, but may be handicapped by reduction of antigenicity. It is to be expected that not only the general decision between these two methods, but also finer details of their performance influence the results. The contradictory findings on MMP expression (Autio-Harmainen et al. 1992; Polette et al. 1994; Vettraino et al. 1996; Nawrocki et al. 1997) and TIMP expression (Damsky et al. 1993; Polette et al. 1994; Hurskainen et al. 1996; Ruck et al. 1996; Nawrocki et al. 1997) published by various groups are likely to be caused by these methodological problems.

◀ **Fig. 4A–G** Electron-microscopical immunocytochemistry of the densely granular, basal lamina-like material (✶) embedding extravillous trophoblast (**A**). This matrix subtype is composed of collagen IV (**B**) and laminin (**C**). MMP-1 showed a weak but specific staining of these areas (**D**), whereas MMP-3 (**F**), MMP-9 (**G**), and their inhibitors, e.g., TIMP-1 (**E**), showed no immunoreactivities in the densely granular material. ×29 400

Fig. 5A–F Electron-microscopical immunocytochemistry of the amorphous ground substance (✶) of matrix-type fibrinoid, embedding extravillous trophoblast (**A**). Only matrix component immunoreactivities for heparan sulfate (**B**) and vitronectin (**C**) were found. In addition, TIMP-1 (**D**), TIMP-2 (**E**), and MMP-9 (**F**) were immunoreactive in this matrix subtype. ×29 400 ▶

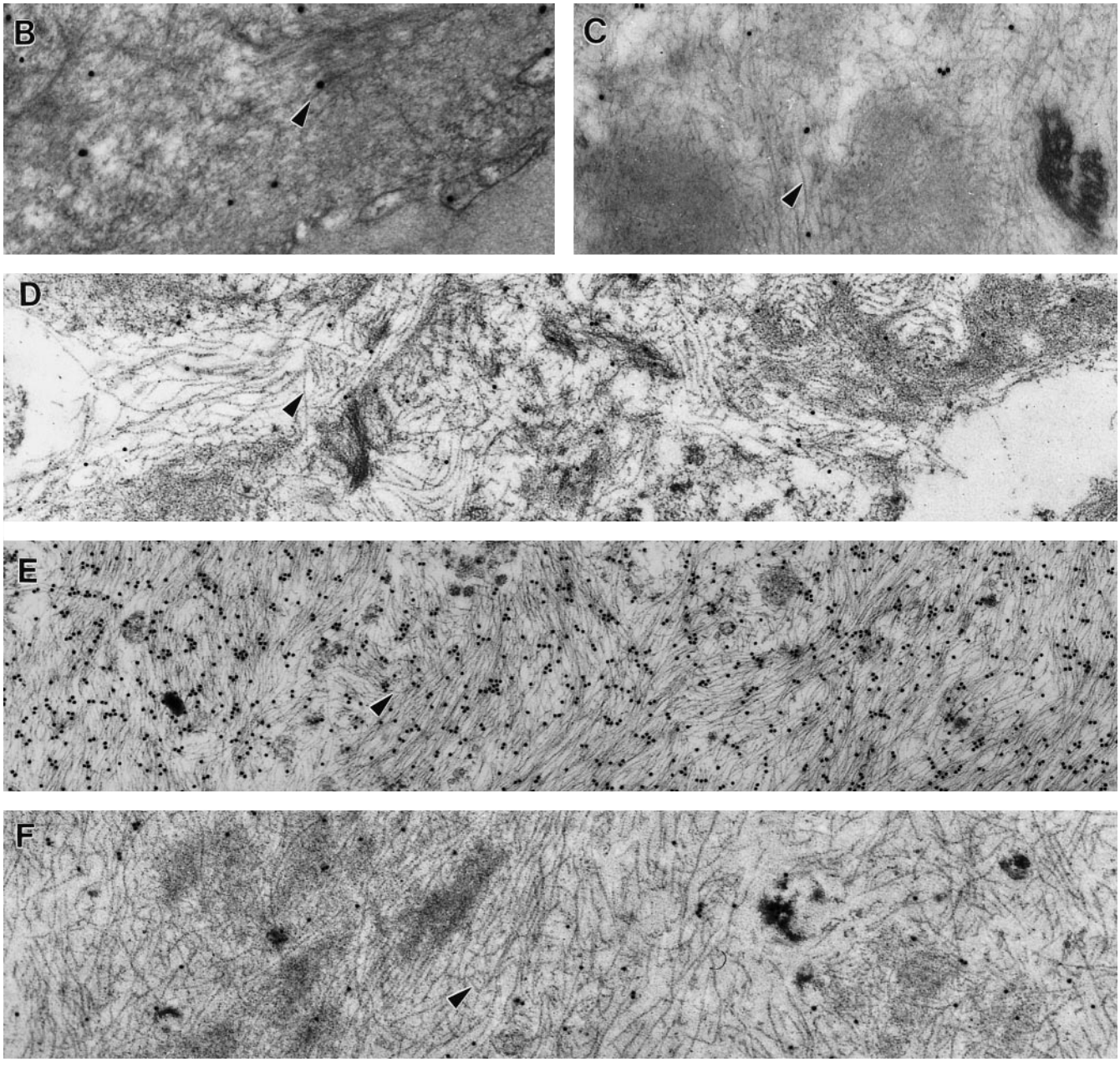

A
amorphous ground substance
immunopositive for:
heparan sulfate, vitronectin
TIMP-1
TIMP-2
MMP-9
B
C
D
E
F



fine fibrillar networks

immunopositive for:
fibronectin domains
ED-A, ED-B

MMP-2
MMP-3
MMP-9



Applying cryostat and paraffin technology in parallel partly helps to interpret the contradictions. Unfortunately, this was possible only for the MMPs, which seemed to be stable enough to maintain immunogenicity at least partly after formaldehyde fixation and paraffin embedding. TIMPs, however, lost their antigenicity nearly totally during preparation for paraffin methodology. This could partly be compensated by the fact that TIMP immunoreactivities were preserved in immunocytochemical preparations for electron microscopy after Lowicryl and LR-Gold embedding.

The second trimester findings caused particular problems since only one placenta was available, the samples of which were about four years old, so that the differences between cryostat and paraffin sections may have been due to the age of the material. We tend to trust only those 2nd trimester findings that fit into the pattern of first and third trimester findings.

As speculated above, missing reactivities in cryostat sections may be due to loss by diffusion, whereas in paraffin sections missing accessibility of the antibody to the sterically altered antigen is the most likely explanation. The latter may be valid, e.g., in the case of complete absence of MMP-9 reactivity from term paraffin samples, and the higher activities in the cryostat sections are likely to be the more reliable ones. In contrast, it is difficult to decide whether clearly positive MMP-2 and MMP-9 immunoreactivities of invasive trophoblast in first trimester paraffin probes as compared to largely negative results in cryostat sections are a consequence of loss by diffusion in the cryostat section or of formaldehyde-induced unspecific reaction in the paraffin material. Western blotting is not helpful since the immunohistochemical patterns are not a question of presence or absence, but rather a question of micro-distribution in this tissue.

Immunohistochemical distribution of MMPs and TIMPs in extravillous trophoblast and its ECM

The interstitial collagenase, MMP-1, mainly digests interstitial collagens, such as collagens I and III. It was found to be secreted by trophoblast cells in vitro by Emonard et al. (1990). In placental sections, MMP-1 reactivities were not only found in first trimester (Moll and Lane 1990), but throughout gestation (Librach et al. 1991). In agreement with the latter result, we found weak MMP-1 immunoreactivities throughout pregnancy, preferably in the invasive phenotype of extravillous trophoblast and its extracellular matrix. This matrix is void of interstitial collagens (Frank et al. 1994). Therefore it is not surprising to find only limited reactivity for MMP-1 during the first invasive steps of extravillous trophoblast. Only in deeply invasive stages, when the extravillous trophoblast cells establish more intense contact with the decidual tissues that are rich in interstitial collagens, does secretion of MMP-1 make functional sense.

The 72 kDa collagenase, MMP-2 (gelatinase A), the main target of which is collagen IV, was found to be secreted by trophoblast cells from first trimester placentas in in-vitro cultures (Emonard et al. 1990; Bischof et al. 1991, 1995; Librach et al. 1991). In histological sections of primate placentas, the presence and distribution of MMP-2 both at the protein and at the mRNA level have been described by Autio-Harmainen et al. (1992), Fernandez et al. (1992), Blankenship and King (1994), Polette et al. (1994), Vettraino et al. (1996), and Nawrocki et al. (1997). Most of the publications cited above described the expression of MMP-2 in trophoblastic cell columns of early pregnancy. This is in agreement with our present data, which, moreover, suggest that this expression does not change considerably in the later course of pregnancy. Again, preferably the invasive phenotype (Blankenship and King 1994) and its surrounding extracellular matrix are the main sites of immunoreactivities.

MMP-3 (stromelysin-1) has a broad spectrum of matrix substrates including fibronectins and collagens (Cawston 1995). Moreover, it has been described to cleave pro-MMP-1 and pro-MMP-9 to the active forms of these enzymes (Woessner 1991; Goldberg et al. 1992; Ogata et al. 1992; Okada et al. 1992). Information on the distribution and localization of stromelysin-1 in the human placenta is scarce (Vettraino et al. 1996), and to the best of our knowledge no information is available about its distribution in cell columns and extravillous trophoblast. To our surprise, stromelysin-1 gave the strongest and most reproducible immunoreactivities in the present study. Interestingly, different from the other three MMPs studied, there was also evidence for MMP-3 immunoreactivity in the proliferative phenotype and in the transitional stages towards the invasive phenotype, with a slight tendency to increase with increasing invasive depth. Extracellular immunoreactivities were even more impressive than for the other proteases. This finding has interesting implications, since, with the exception of the basal lamina separating extravillous trophoblast and villous stroma, both the proliferative and the transitional stage of the invasive pathway are characterized by the structural absence or scarcity of intercellular ECM accumulations (Okudaira et al. 1991; Blankenship et al. 1992; Castellucci et al. 1993; Fisher and Damsky 1993; Frank et al. 1994, 1995; Huppertz et al. 1996). So far it is an open question whether this is a consequence of reduced or failing expression of ECM molecules during the transition from the polar stage of proliferating cells to the subsequent apolar stages, or whether this is a consequence of increased turnover of extracellularly deposited matrix molecules. The expression of the

◀ **Fig. 6A–F** Fine fibrillar networks of light-core fibrils (►) make up the third subtype of extracellular matrix embedding extravillous trophoblast (**A**). The fine fibrils are embedded in the amorphous ground substance, depicted in Fig. 5. Gold particles directly bound to the fibrils represent immunoreactivities for different fibronectin isoforms, e.g., cellular fibronectin (antibody IST-9; **B**) and oncofetal fibronectin (antibody BC-1; **C**). Moreover, antibodies against MMP-2 (**D**), MMP-3 (**E**), and MMP-9 (**F**) showed immunoreactivities along these fibrils. ×29 400

universally cleaving MMP-3 and its inhibitor TIMP-1 in just this zone (see below) lead us to favor the latter possibility. Respective in-situ hybridization and in-vitro studies are required.

MMP-9 (92 kDa collagenase, gelatinase B) cleaves mainly collagen IV and has been the subject of several publications based either on immunohistochemistry and in-situ hybridization (Polette et al. 1994; Hurskainen et al. 1996; Nawrocki et al. 1997) or on in-vitro assays (Fisher et al. 1989; Librach et al. 1991; Fisher and Damsky 1993). All authors agreed that this protease is expressed by extravillous trophoblast preferably in early pregnancy but is downregulated towards the end of gestation. Our results obtained with paraffin sections support this notion. Similar to MMP-1 and MMP-3, it is again mainly expressed by the invasive cells. In contrast to all these findings is the unexplained but reproducible immunoreactivity in cryostat sections of term placentas, which is supported by electron-microscopical immunocytochemistry of term placental extravillous trophoblast and its extracellular matrix. These data await confirmation.

TIMP-1 and TIMP-2 are the inhibitors of the four MMPs investigated in this study. Their importance for the regulation of the invasive process in the utero-placental junctional zone has been demonstrated by Librach et al. (1991) in vitro. Most data in the literature describe expression of TIMPs in the junctional zone to be restricted to the decidua cells (Graham and Lala 1991; Damsky et al. 1993; Polette et al. 1994; Nawrocki et al. 1997). Contradictory findings concerning an additional extravillous trophoblastic secretion were reported recently by Hurskainen et al. (1996) and Ruck et al. (1996). Our data based on cryostat immunohistochemistry and electron-microscopical immunocytochemistry support the findings of the latter groups and make an additional trophoblastic source for TIMP-1 and TIMP-2 rather likely. If this is correct, trophoblastic MMP-activities are not only regulated by the maternal host tissue in a paracrine manner, but, additionally, in autocrine loops by the invading trophoblast cells themselves.

It may be of interest that our specimens of term placenta that showed high TIMP-1 immunoreactivities in invasive trophoblast were collected after delivery. These data fit in the data by Bryant-Greenwood and Yamamoto (1995), who found increased levels of MMP-3 and MMP-9 mRNA shortly before delivery and an increase of TIMP-1 mRNA after delivery. These findings suggest that MMPs and TIMPs may not only be involved in the invasive process of extravillous trophoblast, but also in the separation of the placenta at birth.

Ultrastructural distribution of MMPs and TIMPs in matrix-type fibrinoid of the term human placenta

In a recent publication, we analyzed the spatial distribution of extracellular matrix molecules within the matrix of the invasive extravillous trophoblast (Huppertz et al. 1996). We were able to show that this special ECM is composed of three different subcompartments, which are arranged in a mosaic-like pattern, each compartment being composed only of special mixtures of matrix molecules (cf. Table 2). In order to correlate the micro-distribution of MMPs and TIMPs with that of their substrates, we have repeated the ultrastructural analysis of some selected matrix molecules parallel to that of MMPs and TIMPs.

First respective findings were provided by Moll and Lane (1990), who stained trophoblast cells from villous outgrowth cultures immunocytochemically with an antibody directed against MMP-1. They observed labelling on the trophoblast cell surfaces and on extracellular collagen-like fibrils.

The ultrastructural part of our present study is confined to the extracellular matrix of extravillous trophoblast in the basal plate of term placentas, i.e., the invasive parts of cell columns. In this region, trophoblast-derived matrix-type fibrinoid may be intermingled with decidual matrix components. Because of this, we restricted our study on areas that showed no evidence for the presence of decidual cells.

Most of the extracellular MMP- and TIMP-activities within matrix-type fibrinoid were found in the areas where fibronectin-positive fibrils prevailed. The labelling occurred either directly on the fibrils (MMP-2, -3, -9) or in the amorphous ground substance (heparan sulfate and vitronectin) embedding the fibrils (MMP-9, TIMP-1, -2).

These findings lead to several presumptions:

1. Matrix spots composed of collagen IV and laminin may have a rather low turnover, since only a weak direct contact to specific proteases is identifiable. On the other hand, we cannot exclude that, due to the absence of specific binding of proteases, considerable amounts of the enzymes may have been lost as a result of diffusion.
2. The areas composed of fibronectin-positive fibrils embedded in heparan sulfate and vitronectin may be of particular importance for the invasive process, since here at least three different proteases and their inhibitors could be demonstrated. Since the gold particles were found mostly in direct contact to the fibronectin fibrils, one can speculate about a specific binding of the proteases to their substrates. Moreover, this binding may prevent loss of activity by diffusion during tissue preparation. Since the antibodies we used recognize both the latent and the active forms of the proteases, we do not have any clues whether the gold particles represent the active and degrading proteases or the still inactive pro-enzymes.

   From several experimental studies it is known that the fibronectins are essentially involved in the migratory and invasive behavior of tumor cells (for a review, cf. Pasqualini et al. 1996). Incubation of tumor cells with superfibronectin (sFN), an in-vitro produced polymeric form of fibronectin which is not a normal

substrate of MMPs, reduced the invasive capacity of the tumor cells (Pasqualini et al. 1996). In this context, the co-localization of most MMPs including their inhibitors with the fibronectin fibrils may be of particular functional interest.

3. As compared to the strong immunoreactivities of TIMP-1, the weak immunoreactivities of TIMP-2, which is mainly an inhibitor of gelatinase A (MMP-2) (De Clerck et al. 1989; Stetler-Stevenson et al. 1989), corresponds to the weak immunoreactivity of the latter enzyme along the fibronectin fibrils of matrix-type fibrinoid. This coincidence makes it quite unlikely that the weakness of immunoreactivities represents diffusion artifacts.

Concluding remarks

Immunohistochemistry on paraffin and cryostat sections at the light-microscopical level clearly demonstrates the presence of the various MMPs and TIMPs along the invasive pathway extending from placental cell columns to maternal tissues. Light microscopy with either method does not allow more detailed statements due to the methodological problems of: (1) fixation-dependent denaturation of antigens, or (2) loss of enzyme as a result of diffusion. Immunocytochemistry at the electron-microscopical level shows a conspicuous co-distribution of several matrix substrates with their corresponding cleaving enzymes. This correlation seems to be more specific than the specificities of MMPs found in in-vitro systems: e.g., MMP-3 in vitro degrades nearly all extracellular matrix molecules, whereas in vivo it is specifically found associated with the fibronectin fibrils. Possibly the spectrum of substrates degraded in vivo is much smaller, due to binding of limited enzyme quantities to their preferential substrates, than found under in vitro conditions.

In the present study, we have focussed on the cell columns and their close vicinity, thus on the first stages of the invasive pathway, in which maternal tissue components are absent or rare and trophoblastic proteases are mainly opposed to trophoblast-derived substrates. To further elucidate understanding of MMP action during trophoblast invasion, future studies are required, which analyze the co-localization of extracellular matrix molecules with their proteases and respective inhibitors in deeper invasive zones where maternal extracellular matrix molecules are the natural substrate of the trophoblastic proteases.

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