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92-kDa Type IV Collagenase and TIMP-3, But Not 72-kDa Type IV Collagenase or TIMP-1 or TIMP-2, Are Highly Expressed During Mouse Embryo Implantation

PAULA REPONEN, ILMO LEIVO, CARIN SAHLBERG, SUNEEL S. APTE, BJÖRN R. OLSEN, IRMA THESLEFF, AND KARL TRYGGVASON

Biocenter Oulu and Department of Biochemistry, University of Oulu, FIN-90570 Oulu, Finland (P.R., K.T.); Department of Pedodontics and Orthodontics (C.S., I.T.) and Department of Pathology (I.L.), University of Helsinki, Helsinki, Finland; Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115 (S.S.A., B.R.O.)

ABSTRACT **Expression of 72 kDa and 92 kDa type IV collagenases and the metalloproteinase inhibitors TIMPs 1, 2, and 3 was studied by in situ hybridization in implanting mouse embryos of days 5.5 to 7.5. The 92 kDa type IV collagenase was strongly expressed in invading trophoblasts, signals above background not being observed in the embryonic proper or placental tissue. In contrast, signals above background were not seen for the 72 kDa enzyme in any cells of the implantation region, including trophoblasts and stromal cells of the decidual tissue. Only cells in the mucosal stroma outside the decidual region displayed some expression. TIMP-3 was intensily expressed in maternal cells in the area surrounding the invading embryonic tissue. No expression was observed for TIMP-1 or TIMP-2 in the embryo proper, trophoblasts, or the area of the uterine decidual reaction. Weak signals appeared for TIMP-1 only in the circular layer of myometrial smooth muscle and in some uterine stroma cells distant from the site of embryo implantation. The results suggest a central role for 92 kDa type IV collagenase and TIMP-3 in the extracellular proteolysis associated with implantation of the early embryo.**

Key words: Type IV collagenases, Tissue inhibitors of metalloproteinases (TIMPs), Trophoblast, Embryo implantation

INTRODUCTION

The implantation of the mammalian blastocyst requires extensively regulated tissue remodeling between maternal and fetal tissues. The embryonic trophoblast cells must cross the basement membranes of the uterine epithelium, connective tissue, and blood vessels to ensure successful implantation. Proteolytic enzymes and their inhibitors play an important role in this process. The nonmaternal extraembryonic trophoblast cells are reported to produce several proteolytic enzymes, including urokinase-type plasminogen activator (u-PA), stromelysin, and the 92 kDa type IV collagenase (Sappino et al., 1989; Brenner et al., 1989; Behrendtsen et al., 1992). Human trophoblast cells also express u-PA receptor which can bind active u-PA and thus confine proteolysis to a defined region (Zini et al., 1992).

The 72 kDa and 92 kDa type IV collagenases belong to a family of at least nine genetically distinct mammalian metalloproteinases (MMPs) which degrade extracellular matrix components. These enzymes are two interstitial collagenases (Goldberg et al., 1986; Hasty et al., 1990; Hibbs et al., 1985), three types of stromelysins (Matrisian et al., 1985; Breathnach et al., 1987; Whitham et al., 1986; Basset et al., 1990), the 72 kDa and 92 kDa type IV collagenases (MMP-2 and MMP-9) (Salo et al., 1983; Collier et al., 1988; Huhtala et al., 1990, 1991; Wilhelm et al., 1989), matrilysin (pump-1) (Quantin et al., 1989), and macrophage metalloelastase (Shapiro et al., 1992). The metalloproteinase family shares several structural and functional properties but its individual members differ to some extent with regard to substrate specificity. The type IV collagenases have been shown to cleave native type IV collagen at a single site into two 1/4- and 3/4-size fragments (Fessler et al., 1984; Murphy et al., 1989), but they also have high activity against denatured collagen (gelatin) and, therefore, are also referred to as gelatinases (Woessner, 1991). The metalloproteinases are secreted as proenzymes, which are activated by proteolytic processing to remove about 80 amino acid propeptide from the N-terminus. They have at least one Zn^{2+} binding site and they can be inhibited by chelating agents, α2-macroglobulin, and specific tissue inhibitors of metalloproteinases, TIMPs (for review, see Woessner, 1991; Matrisian, 1992; Birkedal-Hansen et al., 1993). TIMP-1 is a glycoprotein with a molecular mass of about 28 kDa and it can form a complex with activated interstitial collagenase, stromelysin, and 92 kDa type IV collagenase. TIMP-2 is a 21 kDa protein capable of binding to both the latent and activated forms of 72 kDa type IV collagenase (Carmichael et al., 1986; Stetler-Stevenson

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Address reprint requests/correspondence to Dr. Karl Tryggvason, Biocenter Oulu and Department of Biochemistry, University of Oulu, FIN-90570 Oulu, Finland.

et al., 1989; Goldberg et al., 1989). A novel member of the TIMP family, TIMP-3, has recently been cloned from chick (Yang and Hawkes, 1992), mouse (Apte et al., 1994b; Leco et al., 1994), and man (Apte et al., 1994a). TIMP-3 has been shown to have inhibitory activity against 72 kDa type IV collagenase (Staskus et al., 1991), but it is not known if it inhibits the 92 kDa enzyme or other metalloproteinases. The expression of TIMP-3 has been studied during the latter half of mouse development where prominant expression was seen in developing epithelial tubes, myocardium, muscle and placental trophoblasts (Apte et al., 1994b).

Metalloproteinases are required for the normal turnover of extracellular matrix proteins, e.g., during embryonic development and morphogenesis. Thus, transcripts for collagenase, stromelysin, and TIMP mRNAs have been described in mouse unfertilized eggs and they are present also at the zygote and cleavage stages (Brenner et al., 1989). Also, stromelysin-3 and 72 kDa type IV collagenases have been shown to be expressed in human uterus and placenta (Basset et al., 1990; Autio-Harmainen et al., 1992). The implantation of the embryo is an interesting phenomenon which bears striking similarity to tumor cell invasion and metastasis because tumor cells must penetrate extracellular matrix barriers several times during the formation of metastases. A number of cultured tumor cells secrete both type IV collagenases, and immunostaining and in situ hybridization studies have demonstrated activation of the 92 kDa type IV collagenase gene in tumor tissues and in macrophages located at the invading tumor front of some carcinomas (Pyke et al., 1992, 1993; Tryggvason et al., 1993). In contrast, expression of 72 kDa type IV collagenase occurs in fibroblasts adjacent to the tumor front (Pyke et al., 1992, 1993; Tryggvason et al., 1993; Poulsom et al., 1992), while the antigen is mainly localized by immunostaining to the tumor cells at the front of the invading tumor mass (Monteagudo et al., 1990). There is, however, a distinct difference between tumor invasion and trophoblast invasion as the latter is precisely regulated, confined spatially to the uterus and temporally to early pregnancy. During the first trimester the decidua produces molecules which apparently can control trophoblast invasion. These molecules include TGF-β and TIMPs. For example, TGF-β downregulates interstitial collagenase and stromelysin-1 expression and upregulates TIMP-1 expression (Lala and Graham, 1990).

In order to further elucidate the proteolytic process involved in embryonic implantation we used in situ hybridization with homologous probes to examine the expression of 72 kDa and 92 kDa type IV collagenases and TIMPs 1, 2, and 3 during mouse embryo implantation. The appearance and tissue distribution of cells expressing these genes, studied from the early egg cylinder stage at 5.5 days of gestation to the late primitive streak stage at 7.5 days of gestation, demonstrated a distinct and cell specific expression pattern.

EXPERIMENTAL PROCEDURES

In Situ Hybridization

cDNA clones for mouse 72 kDa and 92 kDa type IV collagenases (Reponen et al., 1992, 1994), TIMP-1 (Edwards et al., 1986), TIMP-2 (Shimizu et al., 1992), and TIMP-3 (Apte et al., 1994b) have previously been isolated. For the preparation of RNA probes, the 72 kDa type IV collagenase cDNA was cut with *Hae III* enzyme and a 335 bp fragment from the 3′ end of the cDNA (bases 2051–2386) was ligated to the pGEM-3Z vector. cDNA for mouse TIMP-1 was made by PCR using the sequence data available and the two RNA probes of sizes 256 bp (bases 215–471) and 296 (bases 472–768) were generated by *Pst I* digestion, and ligated into the pSP64/65 vectors. The mouse TIMP-2 cDNA, kindly provided by Dr. S. Shimizu, Japan, was digested with *Hae III* resulting in a fragment of 289 bp (bases 498–787) in length and with *Ava I* and *EcoRI* for a fragment of 240 bp (bases 1438–1678) in length. These fragments were ligated to the pGEM-3Z vector. The mouse TIMP-3 cDNA clone pSAmT39 was digested with *EcoRI* and *Hinc II* and a 328 bp fragment (bases 1–328) from the 5′ and a 175 bp fragment from the 3′ end (bases 1306–1481) were ligated to the same sites in pGEM-3Z vector. The M92KD-2 cDNA clone (Reponen et al., 1994) was cut with *Sma I* and *EcoRI* restriction enzymes and the 324 bp fragment (bases 1915–2239) was cloned into pSP64 and pSP65 plasmid vectors (Promega, Madison, WI). All the vectors were linearized with suitable restriction enzymes, and the [^{35}S]-uridine S′-triphosphate (=1,000 Ci/nmol, Amersham, Arlington Heights, IL) labeled RNA-probes were transcribed using a transcription kit (Promega). The labeled probes were precipitated with ethanol, dissolved in hybridization buffer, and used at 50,000–60,000 cpm/μl. The in situ hybridization was carried out according to Wilkinson and Green (1990). In brief, the deparaffinized sections were pretreated with proteinase K (Sigma, Chemical Co., St. Louis, MO), hybridized with the labeled probes in a humid chamber overnight at 50°C, and washed under high-stringency conditions. The dried slides were dipped in autoradiographic emulsion (Kodak NTB2) and exposed for 10 days at 10°C. After development of the sections, they were stained in hematoxylin and mounted.

Preparation of Tissues Sections

The embryonic age of the hybrid mouse embryos (CBA × C57BL) F1 was set according to the day of the vaginal plug, which was designated as day 0 of pregnancy. Early postimplantation embryos (5.5, 6.5, and 7.5 days) were obtained by dissecting uteri of appropriate gestational age, and the removed tissues were immediately transferred to 4% fresh paraformaldehyde in PBS (pH 7.2) at +4°C, and fixed overnight. The tissues were then dehydrated in ethanol and xylene, embedded in paraffin, and serially sectioned. Sections of 7 μm thickness were placed on silanized glass slides, dried

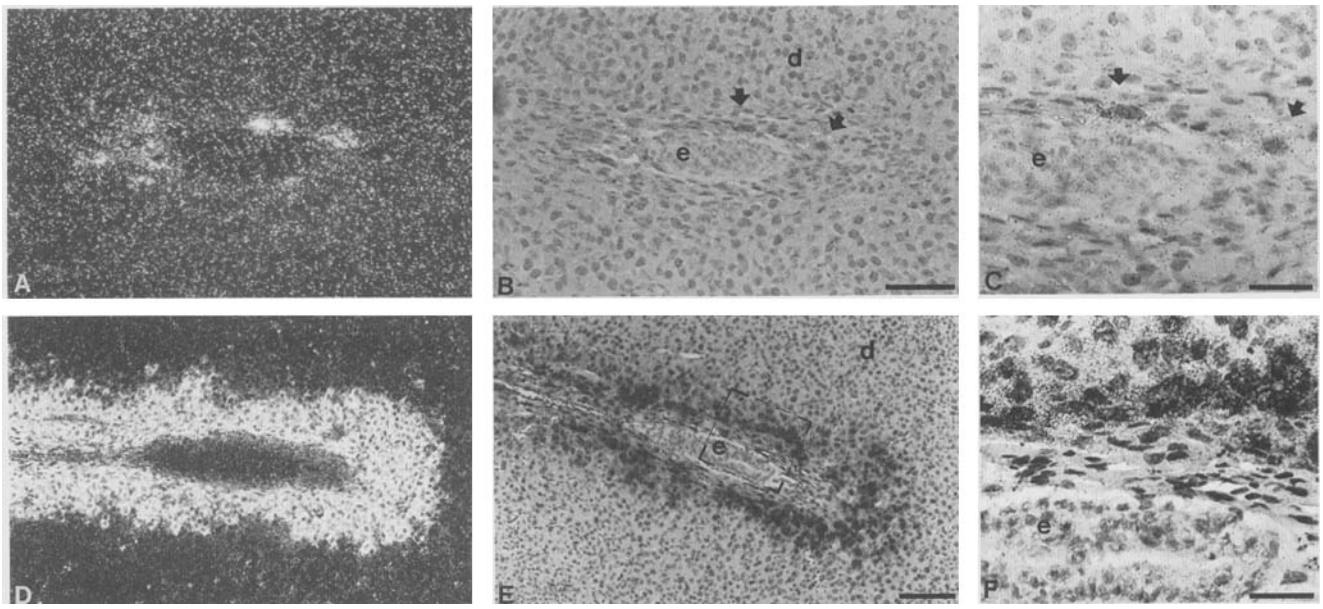

Fig. 1. Localization of 92 kDa type IV collagenase and TIMP-3 transcripts at 5.5 days of gestation by in situ hybridization. **A–C:** 92 kDa type IV collagenase mRNA is seen in some rounded trophoblast cells (arrows) located around the embryo, while cells of the embryo proper (e) are negative. No distinct signals above background are observed in the region of the decidual stroma (d) surrounding the embryo. A and B are dark and bright fields, respectively, of same magnification. Bar in B: 100 μm. High magnification of a region of B is shown in C (bar: 50 μm). **D–F:** The expression of TIMP-3 mRNA is intense in maternal cells of the area of the decidual reaction surrounding the 5.5 day early egg cylinder. The embryo itself is negative at this stage. D and E are dark and bright fields, respectively, of same magnification; bar: 200 μm. High magnification (bar: 50 μm) of a region of E (box) is shown in F.

overnight at +37°C, and stored in tight boxes at +4°C until use.

RESULTS

In situ hybridization was used to identify the cells in early postimplantation mouse embryos expressing the genes for 92 kDa and 72 kDa collagenases, and for tissue inhibitors of the metalloproteinases TIMP-1, TIMP-2, and TIMP-3. The appearance and tissue distribution of cells expressing these genes were studied from the early egg cylinder stage at 5.5 days of gestation to the late primitive streak stage at 7.5 days of gestation.

Localization of 92 kDa Type IV Collagenase in Invading Trophoblasts

In the early egg cylinder stage at 5.5 days of gestation, a weak signal for 92 kDa type IV collagenase mRNA was seen in some rounded trophoblast cells emerging from the flat layer of trophectoderm at points of contact with the uterine wall (Fig. 1A,B,C). Cells of the inner cell mass constituting the embryo proper and the trophectoderm surrounding the embryo were negative (Fig. 1A,B,C). Also, all uterine tissues, including the epithelium, stroma, and the uterine muscle coat, were negative for 92 kDa type IV collagenase mRNA. At 6.5 days of gestation, the multinucleated trophoblast giant cells emerging around the embryo and penetrating the uterine surface epithelium at points of implantation displayed an intense reaction for 92 kDa collagenase mRNA (Fig. 2A,B,C). Also, smaller trophoblast cells in contact with the uterine surface gave clear signals for the 92 kDa enzyme mRNA. In the late primitive streak stage at 7.5 days, numerous trophoblast giant cells surrounding the embryo and invading the uterine stroma adjacent to the growing embryo showed strong positive signals (Fig. 3A,B,C). The embryo proper, uterine decidua, and other uterine tissues were negative for 92 kDa collagenase mRNA throughout the observation period. Also, non-pregnant uterine tissues were negative. In control experiments using the sense strand of 92 kDa type IV collagenase mRNA, no labeling of trophoblasts or other cells was detected (not shown).

Expression of 72 kDa Type IV Collagenase

Expression of 72 kDa type IV collagenase mRNA differed significantly from that of 92 kDa type IV collagenase. It was not detected in embryonic or extraembryonic tissues including the various types of trophoblast cells. Also, deciduous uterine stroma was negative. Some positive reactions were, however, seen in uterine mucosal stroma cells outside the decidualized area and in the layer of myometrial smooth muscle (Fig. 4A,B). Furthermore, there was some positivity in the non-pregnant uterine stroma, especially immediately subjacent to the surface epithelium. The uterine

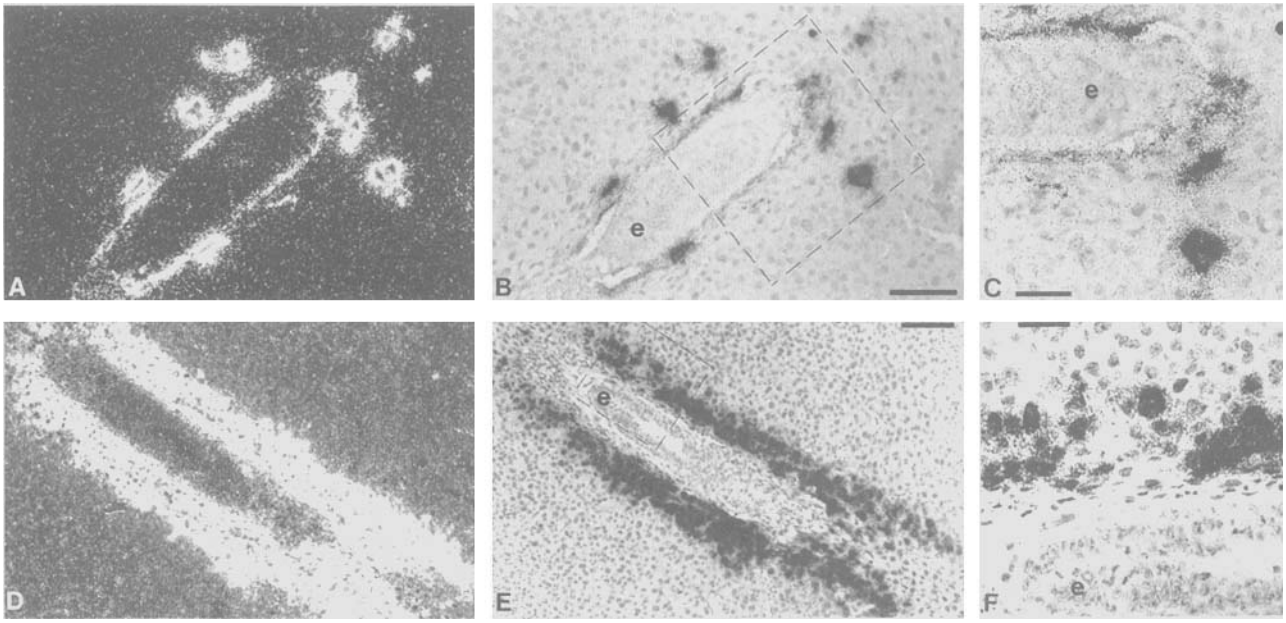

Fig. 2. Expression of the 92 kDa type IV collagenase and TIMP-3 mRNA by in situ hybridization at 6.5 days of gestation. **A–C:** Intense reaction of 92 kDa type IV collagenase is seen in the multinucleated trophoblast giant cells emerging around the embryo and penetrating the uterine surface epithelium into the decidual stroma. The embryo (e) and uterine tissues are negative. A and B are dark and bright fields, respectively, of same magnification. Bar in B: 100 μm. High magnification (bar: 50 μm) of a region of B (box) is shown in C. **D–F:** Expression of the TIMP-3 gene is still intense in maternal cells of the area of the decidual reaction. D and E are dark and bright fields, respectively, of same magnification; bar: 200 μm. High magnification (bar: 50 μm) of a region of E (box) is shown in F.

surface epithelium itself was, however, negative (data not shown).

Expression of TIMP-1 and TIMP-2

During gestation days 5.5–7.5, no expression of TIMP-1 or TIMP-2 mRNAs was observed above background in the embryo proper, trophoblasts, or the area of the uterine decidual reaction (Fig. 4C–F). However, weak signals for TIMP-1 mRNA appeared in the circular layer of myometrial smooth muscle, in some uterine stromal cells distant from the site of implantation, and adjacent to the uterine cavity in a pattern similar to the signals observed for the 72 kDa type IV collagenase mRNA (Fig. 4C,D). The uterine surface epithelium was negative.

Expression of TIMP-3

Intense expression of TIMP-3 mRNA was observed in maternal cells in the area of the decidual reaction surrounding the 5.5 day early egg cylinder (Fig. 1D,E,F). The embryo itself was negative, as well as the uterine stroma further away from the implantation site. By 6.5 days of gestation, no change in the expression of TIMP-3 mRNA was observed (Fig. 2D,E,F). At day 7.5, however, the number of positive cells as well as the intensity of the signal had reduced considerably. At this late primitive streak stage, only a few positive cells were observed in the immediate vicinity of the implanted embryo (Fig. 3D,E,F). No reaction for TIMP-3 mRNA was seen in the uterine stroma close to the original lumen away from the implantation site. Also, non-pregnant uterine tissues were negative.

DISCUSSION

The results of the present study demonstrated distinct differences between the expression of the two type IV collagenases and also between TIMPs 1, 2, and 3 during mouse embryonic implantation. The 92 kDa type IV collagenase and the metalloproteinase inhibitor TIMP-3 were both expressed in cells at the site of early embryo implantation, while the 72 kDa type IV collagenase and TIMPs 1 and 2 do not appear to have a major role in this process, as they were not expressed in the implantation region on days 5.5 to 7.5 based on in situ hybridization analyses.

Expression of 72 kDa and 92 kDa Type IV Collagenases

The observed strong expression of 92 kDa type IV collagenase in the trophoblasts at days 5.5 and 7.5 implies a crucial role for this enzyme in the implantation/invasion process of the early embryo. Expression of the enzyme has previously been shown in cultured trophoblast cells, both by RNA analysis and zymography, and the inhibition of enzyme activity with specific antibodies inhibited trophoblast invasion in an in vitro extracellular matrix invasion assay (Librach et al., 1991). The enzyme probably has a role in the degradation of

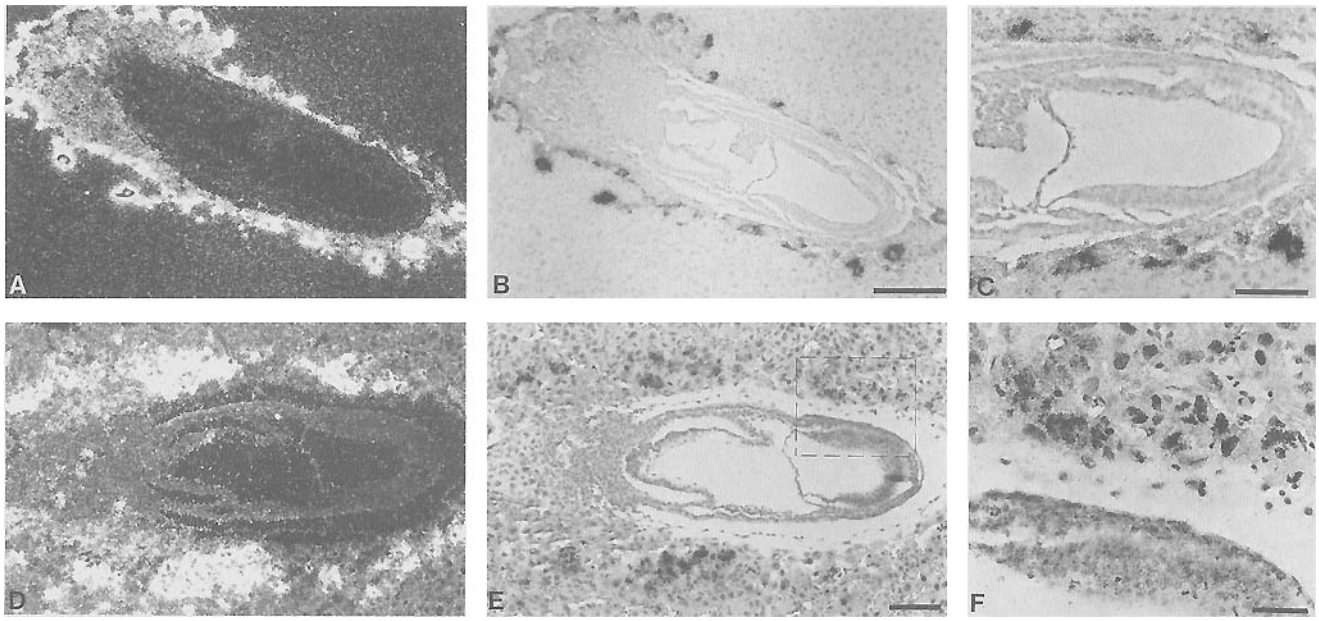

Fig. 3. Localization of 92 kDa type IV collagenase and TIMP-3 mRNA by in situ hybridization at 7.5 days of gestation. **A–C:** Numerous trophoblast giant cells surrounding the embryo and invading into the uterine stroma show strong positive signals for 92 kDa type IV collagenase. **D–F:** Maternal cells of the decidua still exhibit strong signals for TIMP-3 mRNA, but the intensity has apparently reduced compared with that of 6.5 days of gestation. A,D, dark field; B,C,E,F, bright field. Bar (A,B,D,E): 200 μm; C: 100 μm, F: 50 μm.

type IV collagen of the uterine epithelial basement membrane which is the first extracellular barrier to be penetrated by the embryonic cells, but it is also likely to have a major role in the removal of fibrillar collagen of the decidual stroma as well. The enzyme has been shown to have activity against native type IV, V, and VII collagens and also against gelatin in vitro (Murphy et al., 1989; Woessner, 1991), but the actual molecular substrates of 92 kDa type IV collagenase in vivo are not known with certainty. Based on studies with cultured cells, it was previously thought that the enzyme is mainly secreted by leukocytes, macrophages, and keratinocytes for the degradation of type IV collagen and denatured collagen (Woessner, 1991; Matrisian, 1992; Birkedal-Hansen et al., 1993). However, our recent in situ hybridization studies demonstrated that the enzyme is almost solely expressed in osteoclasts during development (Reponen et al., 1994) indicating that the enzyme has a major role in the degradation of bone collagens which include both fibrillar and non-fibrillar types. The actual substrate is not known, but it could be denatured fragments (gelatin) of degraded collagen molecules or even fibrillar collagen such as type V which is degraded by this enzyme in vitro (Woessner, 1991). In addition to trophoblasts and osteoclasts, expression of 92 kDa type IV collagenase has also been observed in macrophages which are derived from the same cell lineage as the latter. By in situ hybridization and immunolocalization, macrophages located at the invasive front of tumors, and sometimes also the tumor cells themselves, have been shown to contain mRNA for 92 kDa type IV collagenase (Pyke et al., 1992, 1993; Tryggvason et al., 1993). In summary, the currently available data show that the 92 kDa type IV collagenase has a highly restricted pattern in vivo, and that it is primarily expressed in cells with invasive properties, but the actual physiological substrates are not known for sure.

In the present study we did not observe signals above background for 72 kDa type IV collagenase in trophoblasts and, surprisingly, not even in stromal cells of the immediately adjacent decidual tissue. This enzyme has been shown to be primarily expressed in stromal fibroblast-like cells during development (Reponen et al., 1992), while, e.g., normal adult stromal tissues do usually not display expression. However, in stromal reaction to injury or tumor growth the fibroblasts have been reported to show strong expression of 72 kDa type IV collagenase (Pyke et al., 1992, 1993; Autio-Harmainen et al., 1993). Hence, the nature of the decidual stroma reaction to implantation and invasion of the embryo appears to differ from that of injury or tumor invasion.

Different Spatial Expression of TIMPs 1, 2, and 3

In this study we did not observe signals above background for TIMP-1 or TIMP-2 mRNAs in the embryo or embryonic trophoblasts, nor in the area of uterine decidual reaction at stages E 5.5 to E 7.5. Signals above background for TIMP-1 could only be observed in uter-

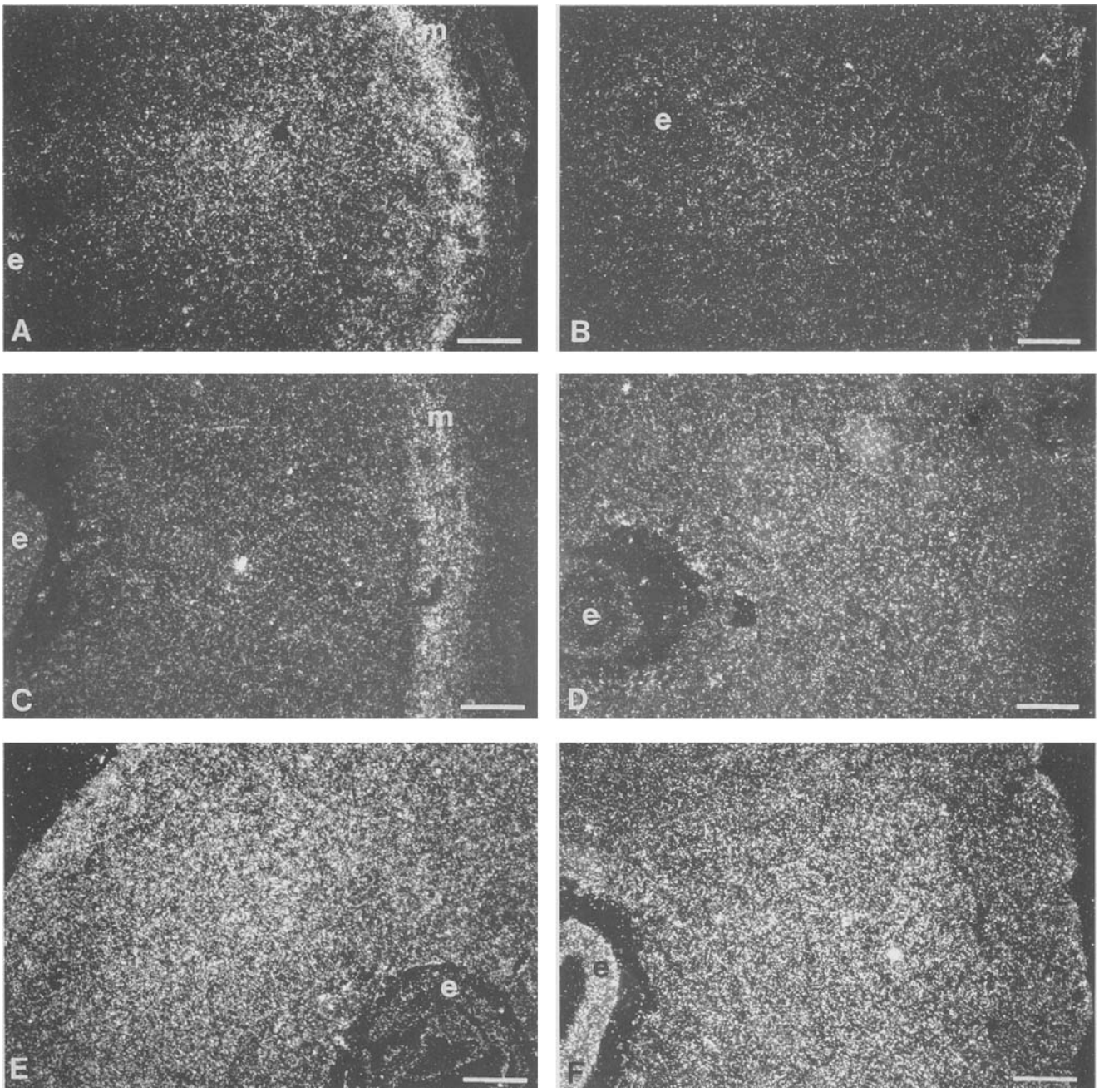

Fig. 4. Expression of 72 kDa type IV collagenase at 6.5 days of gestation and TIMP-1 and TIMP-2 at 7.5 days of gestation by in situ hybridization. **A:** 72 kDa type IV collagenase mRNA is seen in the circular layer of myometrial smooth muscle (m). Also some positive reaction is seen in the uterine mucosal stroma cells outside the decidualized area. The embryo proper (e) is negative. **B:** Negative in situ hybridization control of an adjacent section to A with sense probe. **C:** The expression pattern of the TIMP-1 gene is quite similar to the signals observed for the 72 kDa type IV collagenase. **D:** Negative control with sense probe. **E:** Expression of TIMP-2 cannot be distinguished from background obtained with the sense probe (**F**). Bar: 200 μm.

ine stroma cells distant from the site of implantation and in the circular layer of myometrial smooth muscle, but signals above background for TIMP-2 were not observed. Brenner et al. (1989) have reported the presence of TIMP-1 mRNA of maternal origin in unfertilized eggs and in preimplanting embryos by the use of reverse transcription-polymerase chain reaction (RT-PCR). These contradictory results may be due to differences in sensitivity of techniques, as the high sensitivity of the RT-PCR can reveal the presence of a single transcript and easily detects even so-called illegitimate transcription of no apparent biological significance

(Chelly et al., 1989). Furthermore, Nomura et al. (1989) have detected TIMP-1 message by RNase protection in the whole embryo from E 7.5 to 8.5 or at a slightly later stage than analyzed here. Also Waterhouse et al. (1993) have analyzed TIMP-1 and TIMP-2 transcripts by Northern hybridization. Maximal levels of TIMP-1 mRNA were observed from day 6 to day 10 in the uterus, decidua, and placenta while the level of TIMP-2 mRNA increased throughout gestation. Therefore, although expression of TIMPs 1 and 2 at the site of implantation may be biologically significant, it appears to be considerably less than that of TIMP-3 which was intense in stromal cells surrounding the implanting embryo.

The present results demonstrated that the onset of TIMP-3 mRNA expression coincides with the early deciduous reaction to embryonic implantation. It remains restricted to the immediate vicinity of the embryo, which is negative, and does not extend to the entire decidualized area. At day 7.5 the number of positive cells as well as the intensity of signals appeared to have decreased. Instead, studies of the placenta from days 12.5 to 16.5 have shown high levels of expression in the trophoblasts with low to background levels in the uterus (Apte et al., 1994b). This suggests that TIMP-3 may have multiple roles during embryogenesis: one being in regulation of implantation, another being in placental development. The TIMP-3 gene is also prominently expressed in connective tissues such as those of cartilage and skeletal muscle, in myocardium, in the choroid plexus, and in epithelia of a variety of tissues. Since TIMP-3 has been shown to have inhibitory activity for at least one metalloproteinase, the 72 kDa type IV collagenase (Staskus et al., 1991), it is possible that TIMP-3 also inhibits the 92 kDa enzyme and, thus, plays a role in the control of metalloproteinase action in the region of decidual reaction to the invasion of embryonic trophoblasts. TIMP-3 may also have growth-promoting activity during implantation as such an additional role has been proposed for all TIMPs (Gasson et al., 1985; Hayakawa et al., 1992, Stetler-Stevenson et al., 1992).

Interplay of Proteinases and Inhibitors at the Embryo Implantation Site

Our observations suggest that the trophoblasts of the implanting embryo use the 92 kDa type IV collagenase as part of the machinery needed for the breakdown of the extracellular matrix, presumably first for the degradation of basement membrane collagen and then for the removal of denatured gelatinous fragments of stromal fibrillar collagen, in order to pave the way for migration into the uterine decidual tissue. Furthermore, our results suggest that the metalloproteinase inhibitor TIMP-3 produced by maternal stromal cells at the site of implantation is utilized in the initial phase to neutralize excessive action of this enzyme, and other metalloproteinases that may be secreted by the trophoblasts. It is likely that TIMP-3 can inhibit the activity of 92 kDa type IV collagenase and other metalloproteinases as well as TIMP-1 and TIMP-2 both can act as general metalloproteinase inhibitors. At present, it is not known with certainty if other metalloproteinases are expressed by cells at the implantation site in vivo, but this seems likely because, as discussed above, the 92 kDa type IV collagenase is probably not sufficient for the breakdown of the fibrillar collagen network of the decidual stroma. Based on in vitro studies with isolated metalloproteinases and extracellular matrix proteins as substrates, expression of interstitial collagenase, stromelysins, and possibly also matrilysin would be sufficient to degrade all collagenous and noncollagenous components of the extracellular matrix (see Woessner, 1991; Matrisian, 1992; Birkedal-Hansen et al., 1993). Support for the presence of some of these enzymes in the peri-implantation region comes from studies by Brenner et al. (1989), who showed that fibroblast interstitial collagenase and stromelysin-1 are expressed in mouse implantation embryos using RT-PCR carried out with primers containing rabbit sequences. It would be important to carry out careful in situ hybridization studies with homologous probes to sort out the pattern of metalloproteinase expression in the implantation region.

The absence of TIMP-1 and TIMP-2 expression indicates a role for TIMP-3 in specific physiological situations where metalloproteinases are active. It is clear, however, that although co-expressed at the embryo implantation site, TIMP-3 can not be a specific inhibitor of 92 kDa type IV collagenase in vivo, as they are not generally co-expressed in tissues. For example, during development 92 kDa type IV collagenase is primarily expressed in bone in osteoclasts (Reponen et al., 1994) and sometimes in macrophages, while TIMP-3 is expressed in a large variety of tissues and cells, which do not include osteoclasts. In the developing bone it is expressed in cartilage (Apte et al., 1994b). Another possibility is that TIMP-3 has a function for promoting cell growth as has been shown for TIMPs 1 and 2 (Hayakawa et al., 1992; Stetler-Stevenson et al., 1992).

In addition to metalloproteinases, plasminogen activators and plasmin have been implicated in the trophoblast invasion process based on studies with cultured trophoblastic cells and blastocysts (Sappino et al., 1989; Strickland and Richards, 1992). Sappino et al. (1989) reported the presence of u-PA mRNA in the invasive and migrating trophoblasts of 5.5 to 8.5 day embryos by in situ hybridization analysis. Plasmin has been suggested to have a role in the activation of latent metalloproteinases as well as in the degradation of several noncollagenous matrix components which are known to be susceptible to degradation by plasmin in vitro. However, recent data question the role of plasmin as a pro-metalloproteinase activator, as plasmin does not activate the latent forms of 72 kDa or 92 kDa type IV collagenases (Okada et al., 1990; Morodomi et al., 1992; Goldberg et al., 1992). In fact, these enzymes may not need specific activators as they are autoacti-

vated in vitro (Bergmann et al., 1995). Also, ϵ-aminocaproic acid, an inhibitor of plasminogen activators, does not inhibit rabbit blastocyst attachment or degradation of matrix by mouse trophoblasts in vitro (Denker, 1977; Glass et al., 1983) while inhibitors of metalloproteinases do so (Behrendtsen et al., 1992). It is therefore possible that the plasminogen activator/plasmin system has an insignificant function in degradation of matrix components but could, alternatively, regulate fibrin formation in the area where maternal blood vessels are breached. Further evidence for minor role of the PA/plasminogen activator system for embryonic development comes from experiments with transgenic mice (Carmeliet et al., 1994). Mice containing inactivated u-PA or t-PA genes were shown to develop normally. They were fertile and had normal life span. In contrast, mice with combined deficiency of u-PA and t-PA survived embryonic development, but they suffered from reduced fertility, possibly because of the poor general health of those animals. In conclusion, current data have demonstrated the expression of metalloproteinases and, in particular, strong association of 92 kDa type IV collagenase and TIMP-3 expression with the implantation of the early embryo. Other metalloproteinases and possibly some unknown inhibitors may also be involved and it would be important to be able to probe for them in experimental animal systems to obtain deeper understanding of the entire implantation process.

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