Proteinase expression in early mouse embryos is regulated by leukaemia inhibitory factor and epidermal growth factor

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SUMMARY

Several proteinases from different multigene families have been implicated in the uterine invasion required for establishment of pregnancy in some mammals. In this study, the expression of matrix metalloproteinase gelatinase B (MMP-9), urokinase-type plasminogen activator (uPA) and their inhibitors was investigated during early mouse embryo development. Transcripts for tissue inhibitors of metalloproteinases (TIMP-1,-2,-3) and uPA receptor were detected throughout pre- and peri-implantation development whilst MMP-9 and uPA mRNAs were first detected in peri-implantation blastocysts associated with the invasive phase of implantation. Through use of in situ hybridization, it was shown that MMP-9 transcripts were strongly expressed in the network of trophoblast giant cells at the periphery of implanting 7.5 day embryos and TIMP-3 transcripts were strongly expressed in the decidua immediately adjacent to the implanting embryo. uPA transcripts were preferentially expressed in the ectoplacental cone and its derivatives. Because these proteinases are regulated by growth factors and cytokines in other tissues, the effect of leukaemia inhibitory factor (LIF) and epidermal growth factor (EGF) on their activity was investigated. Both LIF

and EGF, like the proteinases, have been implicated in peri-implantation development. Blastocysts collected on day 4 of pregnancy were cultured 2 days in TCM 199 + 10% fetal bovine serum to allow outgrowth followed by 24 hour culture in defined media containing either LIF or EGF. Conditioned media were assayed for uPA activity by a chromogenic assay and MMP activity by gelatin zymography. Both LIF and EGF stimulated uPA and MMP-9 activity in blastocyst outgrowths after 3 days of culture (day 7). Proteinase activity was assayed again at the 5th to 6th day of culture (day 9 to 10). EGF was found to have no effect whereas LIF decreased production of both proteinases. These results demonstrate that proteinase activity in early embryos can be regulated by growth factors and cytokines during the implantation process and, in particular, they demonstrate the possible involvement of LIF in establishment of the correct temporal programme of proteinase expression.

Key words: implantation, mouse embryos, urokinase, matrix metalloproteinases, EGF, LIF, TIMP-3, growth factor, cytokine

INTRODUCTION

Implantation of the embryo into the uterine stroma in some mammals is a highly controlled process of tissue invasion by trophoblast cells from the embryonic trophectoderm that involves localized production and activation of extracellular matrix (ECM)-degrading proteinases. Members from two proteinase families including the plasminogen activators (PAs) and the matrix metalloproteinases (MMPs) have been implicated. PAs, which are serine proteinases, activate plasmin from plasminogen (Danø et al., 1985) and, although plasmin may attack ECM proteins directly, it participates principally as a component of a proteolytic cascade that activates latent forms of the MMPs. The MMPs are a multigene family of zinc-dependent proteinases divided into three subclasses based on substrate specificity: collagenases, stromelysins and gelatinases (Matrisian, 1992; Birkedal-Hansen et al., 1993). They are

implicated as the key, rate-limiting enzymes in ECM remodelling (Werb, 1989). Several MMPs and PAs have been shown to be expressed or produced by developing embryos; in particular, MMP-9 and uPA are expressed and produced by peri-implantation mouse blastocysts (Librach et al., 1991; Behrendtsen et al., 1992; Brenner et al., 1989; Strickland et al., 1976; Sappino et al., 1989; Astedt et al., 1986). However, a complete phenotype for these gene products during early development has not been documented.

The aim of this study was to define the temporal expression of these proteinases and their inhibitors during pre- and peri-implantation development and to determine if these proteinases can be regulated by growth factors and cytokines. The effects of leukaemia inhibitory factor (LIF) and epidermal growth factor (EGF) during the implantation process have been well documented. In the case of LIF, transcripts are localized to uterine endometrial gland cells of the mouse and levels peak

at day 4, the time just prior to implantation (Bhatt et al., 1991). LIF transcript levels are absent or barely detectable in females undergoing delay of implantation but increase to normal levels when delay is broken by hormone administration (Bhatt et al., 1991). Ligand binding studies have demonstrated that the embryonic trophectoderm contains receptors for LIF (Robertson et al., 1990). Most importantly, embryos fail to implant in mice homozygous for a mutated, non-functional LIF gene but decidual responses containing embryos undergoing implantation can be induced in these mice by exogenous supply of LIF (Stewart et al., 1992). Like LIF, EGF transcripts are also detectable in the day 4 mouse uterus and are concentrated in the luminal epithelium (Huet-Hudson et al., 1990). Also, like LIF, immunoreactive EGF levels in the rodent uterus increase as a consequence of estrogen treatment, suggesting that EGF is also regulated hormonally (Huet-Hudson et al., 1990). Finally, EGF receptors are present on preimplantation embryos (Dardik et al., 1992; Wiley et al., 1992).

To assess whether there is a relationship between factors like LIF and EGF and proteinase production by blastocysts we have used a combination of reverse transcription-polymerase chain reaction (RT-PCR) techniques, in situ hybridization and assays for proteinase activity to assess gene expression and enzymatic activity in pre- and post-implantations embryos and blastocyst outgrowths. The results indicate that the expression of both MMP-9 and uPA proteinases can be regulated by LIF and EGF in peri-implantation blastocysts.

MATERIALS AND METHODS

Gene expression

Fertilized eggs, 2-cell embryos, morulae and blastocysts were collected (at 24, 48, 72 and 96 hours after 7.5 I.U. human chorionic gonadotrophin [hCG] administration) from mated, superovulated CD-1 mice (Charles River Breeding Laboratories, Lachine, P.Q., Canada) and RNA obtained using phenol/chloroform extraction and ethanol precipitation as previously described (Arcellana-Panlilio and Schultz, 1993). RNA was also extracted from blastocyst outgrowths after 24, 48 and 72 hours culture (corresponding to days 5, 6, and 7 of development) of day 4 blastocysts cultured in groups of 50 to 100 in 50 µl droplets of TCM 199 medium (Gibco BRL; Burlington, Ontario, Canada) containing 10% fetal calf serum (FCS; Gibco BRL), as described by Glass et al., (1983). RNA was reverse transcribed (RT) by oligo(dT) priming and AMV reverse transcriptase (Gibco BRL); and the cDNA derived from equivalent amounts of total RNA from 6 to 10 embryos was used in polymerase chain reactions (PCR) to specifically amplify cDNAs of interest (as previously described in Arcellana-Panlilio and Schultz, 1993). The PCR products were resolved on 2% agarose gels containing 0.5 µg/ml ethidium bromide. cDNA samples were first tested, and discarded if found to be contaminated with genomic DNA. This was performed by PCR with a primer pair for mouse β-actin, which gives a predicted 243 bp fragment for the cDNA and a 330 bp fragment (due to presence of an intron) if contaminating genomic DNA is present (Telford et al., 1990). Primer pairs used in the PCR reaction were derived from published mouse sequences and the sizes of the expected PCR fragments are shown in Table 1. To confirm identity, PCR products were either sequenced or subjected to cleavage with an appropriate diagnostic restriction enzyme (Table 1).

In situ hybridization

Restriction fragments of mouse MMP-9 (220 bp *Pst*I to *Bam*HI

fragment; Tanaka et al., 1993), mouse TIMP-3 (300 bp *Eco*RI to *Pst*I fragment; Leco et al., 1994), and mouse uPA (400 bp *Eco*RI to *Hin*dIII fragment; Belin et al., 1985) were subcloned into pBluescript KS⁻ (Stratagene, La Jolla, CA) for riboprobe preparation. Identity and orientation of all plasmids were confirmed by sequence analysis. After linearization of plasmid DNA with the appropriate restriction enzyme, riboprobes were generated incorporating digoxygenin (DIG)-labeled rUTP (Boehringer Mannheim, Laval, Quebec, Canada) following the manufacturer's instructions. Confirmation of sense and antisense riboprobe was confirmed by northern blot analysis. Antisense probes, but not sense probes, detected a single band of the appropriate size for all three genes.

CD-1 mouse embryos in utero were collected on day 7.5 post coitum (p.c.), fixed in paraformaldehyde and embedded in paraffin as previously described (Arcellana-Panlilio and Schultz, 1994). 6 µm sections were mounted on glass slides coated with 2% aminopropyl-triethoxysilane and incubated at 42°C overnight to facilitate adherence of the section to the slide. The following day, sections were dewaxed in two changes of toluene, then rehydrated through a 100%, 95%, 80%, and 70% ethanol series for 2 minutes each and placed in 4× SSC. Sections were incubated with 20 µg/ml proteinase K in 2× SSC for 7.5 minutes at room temperature and acetylated by incubation in a freshly prepared mixture of 0.1 M triethanolamine and 0.56% (v/v) acetic anhydride for 10 minutes at room temperature. Sections were washed in 4× SSC twice for 5 minutes, then prehybridized in a solution of 50% formamide, 4× SSPE, 20 mM DTT, and 1× Denhardt's solution at 50°C for 4 hours. Sections were hybridized with 200 ng probe and 1.5 µg *E. coli* tRNA in 20 µl prehybridization solution under a coverslip sealed with rubber cement for 36 hours at 50°C. After hybridization, rubber cement was removed and the coverslips were allowed to fall off in 2× SSC. The sections were then washed once in 2× SSC at 50°C for 30 minutes, once in 2× SSC with 20 µg/ml RNase A at 37°C for 30 minutes, once in 2× SSC at 37°C for 30 minutes, and once in 0.5× SSC at 50°C for 30 minutes. Sections were then rinsed in buffer 1 (100 mM Tris-HCl pH 7.5, 150 mM NaCl), transferred to blocking buffer (buffer 1 plus 0.3% Triton X-100 and 2% normal sheep's serum) for one hour at room temperature and incubated with 100 µl anti-DIG antibody (Boehringer Mannheim) diluted 1:500 in blocking buffer for 4 hours at room temperature. Sections were washed twice for 15 minutes in buffer 1 and once for 5 minutes in buffer 2 (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl₂). Subsequently, 500 µl color development solution [10 ml buffer 2, 35 µl nitroblue tetrazolium salt (75 mg/ml), 35 µl 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml), and 25 µl 1M livamisol] was added and sections were incubated 20 hours in the dark. The reaction was terminated by placing the sections in buffer 1. Sections were dehydrated though an ethanol series for 2 minutes each, cleared in xylene for one minute, rehydrated though an ethanol series, counterstained with nuclear fast red for 2 minutes, rinsed and coverslipped with water, and photographed on Kodak Royal Gold 35mm film using a Zeiss photomicroscope II under bright-field illumination.

LIF/EGF effect on proteinase activity

Blastocysts were collected on day 4 of pregnancy (96 hours after hCG) and cultured in groups of 50 to 100 in 50 µl droplets of TCM 199 containing 10% FCS for 48 hours to allow the embryos to attach to the culture dish and undergo trophoblast outgrowth. The culture medium was then replaced with fresh TCM 199 without serum but containing 1 mg/ml BSA (Pentex, Miles Inc, Illinois, USA) or the same medium with LIF (1000 U/ml; Amrad Corp., Australia) or EGF (10 ng/ml; UBI, Lake Placid, NY, USA). The conditioned medium was removed 24 hours later on day 7 of development and assayed for proteinase activity. The same outgrowths were then resupplied with fresh serum-containing medium and cultured for 2 to 3 more days. Media samples were again taken on day 9 or 10 of development after transfer of defined medium plus LIF or EGF for 24 hours. PA activity was determined by modification of the chromogenic assay described by a

Coleman and Green (1981). Briefly, 5 μl from the 50 μl samples were first incubated for 45 minutes at 37° with plasminogen. The plasmin substrate (220 μM 5,5′-dithiobis-2-nitrobenzoic acid; Sigma, St Louis, MO, USA) and chromogenic substrate (220 μM Na-CBZ-L-lysine thiobenzyl ester; Sigma, St Louis, MO, USA) were then added to the incubation and further incubated for 30-60 minutes. Absorbance at 410 nm wavelength, indicative of PA activity, was obtained using a Beckman Model 35 Spectrophotomer. uPA activity standards (Calbiochem, San Diego, CA, USA) were included in each assay for estimating the PA activity in the samples. The PA activity in blastocyst outgrowth-conditioned media was shown to be completely due to uPA as addition of 0.1 mM amiloride (a specific uPA inhibitor; Vassalli and Belin, 1987) to the chromogenic reaction completely abolished activity (results not shown).

MMP activity in conditioned media was determined by gelatin zymography (Brenner et al., 1989; Behrendtsen et al., 1992). Briefly, 45 μl from the 50 μl samples were lyophilised, reconstituted in SDS sample buffer without 2-mercaptoethanol and electrophoresed on 10% polyacrylamide gels co-polymerised with 1 mg/ml gelatin. Gels were then washed in 2.5% Triton X-100 and incubated for 48 hours at 37°C in 50 mM Tris-HCl, 10 mM CaCl$_2$ (pH 7.8). Gelatinolytic activities were visualized as clear bands after staining the gels with 0.5% Coomassie Blue R250 and de-staining. Confirmation that the gelatinase activities of blastocyst outgrowths were MMPs was shown by a complete inhibition of activity on gels developing in the presence of 10 mM EDTA (Fig 3A,B). The position of the two bands on the gel ($M_r=105\times10^3$ and $M_r=97\times10^3$) identified the activities as MMP-9. Gels were photographed and the band intensities were quantified by densitometry of the negatives on a PDI Protein plus DNA Imageware System (Huntington Station, NY, USA).

RESULTS

Gene expression

To develop an mRNA phenotypic map for the expression of various proteinase and proteinase inhibitor genes in early mouse embryos and blastocyst outgrowths, RT-PCR studies were carried out with primer pairs specific for MMP-9, uPA, uPA receptor, TIMPs-1,-2,-3, plasminogen
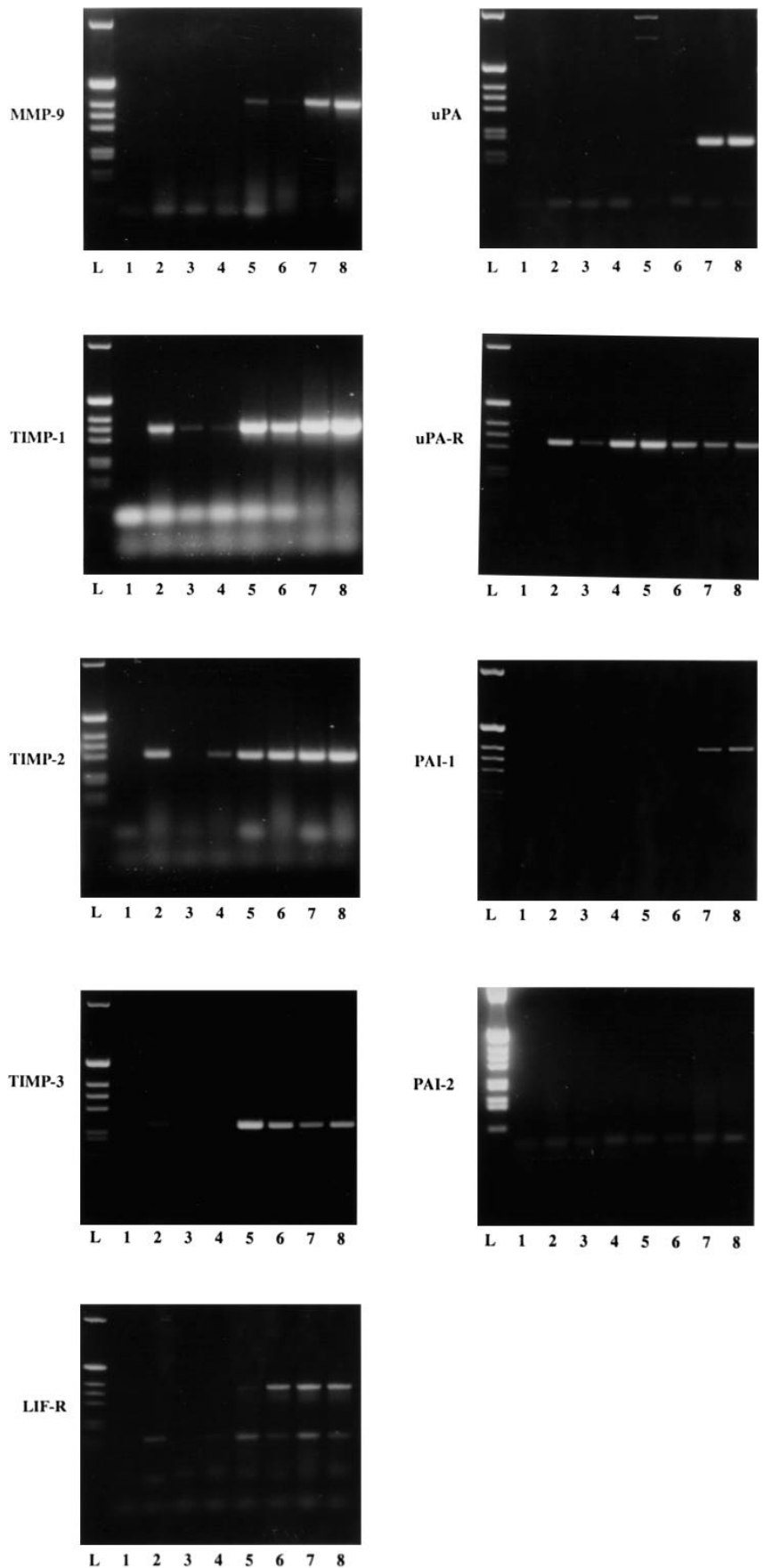

Fig. 1. Gene expression of proteinases and inhibitors during early development. Each lane was produced using a cDNA aliquot derived from RNA from the equivalent of 10 embryos for lanes 2-5 and 6 embryos for lanes 6-8. The RNA preparations were reverse transcribed and amplified by 40 cycles of PCR using oligonucleotides specific for various genes as described in Table 1. Lanes are **L**=DNA ladder (Bands from top to bottom - 1018 bp, 516/506 bp, 394 bp, 344 bp, 298 bp, 220/200 bp, 154/142 bp); **1**=Negative control (no cDNA); **2**=Fertilized eggs; **3**=2-cell embryos; **4**=morulae; **5**=blastocysts; **6**=day 5 blastocyst outgrowth; **7**=day 6 blastocyst outgrowth; **8**=day 7 blastocyst outgrowth.

activator inhibitor (PAI)-1 and -2 as well as LIF receptor (Fig. 1). TIMP-1,-2 and -3 transcripts were detectable in all stages of preimplantation embryo development examined (from 1-cell to blastocyst stages) and yielded strong signals in blastocyst outgrowths (Fig. 1). TIMP-1, TIMP-2 and uPA receptor all showed a pattern typical of many genes that are constitutively expressed during early development; namely, strong expression in the oocyte and 1-cell embryo, decreased abundance at the 2-cell stage due to degradation of maternal mRNA and reaccumulation in morulae and blastocysts due to new transcription from the zygotic genome (Fig. 1). TIMP-3 showed a similar pattern although signals were not as strong. Under conditions utilized in these studies, ethidium-bromide-stained bands for RT-PCR products for MMP-9 and LIF-receptor mRNAs were not detectable until blastocyst stages on day 4 of development. Transcripts for uPA, although first detected in day 5 blastocysts in some preparations, are not visible in the panel shown prior to blastocyst outgrowth (Fig. 1). The intensity of the signals for MMP-9, LIF-receptor and uPA increased through day 7 in blastocyst outgrowths although caution is needed in making quantitative assessments in RT-PCR assays unless internal standards are included in the reaction. PCR products representing PAI-1 transcripts were not detectable in preimplantation embryos recovered from the reproductive tract but signals were detected following RT-PCR of RNA extracted from day 6 and 7 blastocyst outgrowths (Fig. 1). RT-PCR products for PAI-2 were not detectable in any of the samples examined herein (Fig. 1) although positive signals were obtained from RNA extracted from later stages of embryos (egg cylinders) dissected from decidua at day 7.5 from females made pregnant through natural matings (data not shown).

The identity of all PCR products was confirmed either through DNA sequencing for MMP-9 and PAI-2 (data not shown) or restriction digestion for the remainder (Table 1).

Localization of MMP-9, uPA and TIMP-3 expression in implanting mouse embryos

The expression patterns of proteinase and proteinase inhibitor genes were compared between blastocyst outgrowths maintained in vitro and embryos developed in vivo and dissected from the decidua on day 7.5 p.c. By RT-PCR studies, the mRNA phenotypic map obtained for day 7.5 embryos derived in vivo was virtually identical to that shown for day 7 blastocyst outgrowths in Fig. 1. All transcripts examined in blastocyst outgrowth samples were detectable in day 7.5 embryos, including the product for PAI-2 (data not presented). The transcripts for MMP-9, TIMP-3 and uPA were also examined by in situ hybridization to establish their localization (Fig. 2). With the alkaline phosphatase-based detection system used in these studies, positive signals representing hybridization of DIG-labelled antisense RNA probes to target transcript sequences yield a blue precipitate at the site of mRNA localization. To assess background staining, DIG-labelled RNA probes prepared from the sense-strand of the cDNA clone were used for in situ hybridization under identical conditions to that used for antisense probes. Essentially no staining was observed with the sense probes in any of the experiments (Fig. 2).

The staining for MMP-9 transcript is shown for a section of day 7.5 embryo (in deciduum) that includes the ectoplacental cone, embryo and decidual components. Strongest staining occurs in a network of cells at the periphery of the embryo in contact with the adjacent decidual cells (Fig. 2). This network surrounds the entire embryo (not shown) and corresponds to the trophoblast giant cells that become organized around the mural and abembryonal regions of the embryo (Abrahamsohn and Zorn, 1993). Lighter staining can also be observed in other trophoblast cells within the ectoplacental cone as well as in the embryo, but clearly the greatest concentration of transcripts is in the trophoblast giant cell network at the periphery of the embryo.

Because proteinase expression is often counter-balanced by proteinase-inhibitor expression, an examination of the localization of transcripts for various isoforms of TIMPs was included in the experiment. Preliminary experiments with radiolabelled probes had indicated that TIMP-1 and TIMP-2

### Table 1. Proteinase and inhibitor PCR primer sequences

| Gene	Primer sequence	Reference	Fragment size	Restriction diagnosis	
				Enzyme	Size of fragments
MMP-9*	5′ Primer=5′TTGAGTCCGGCAGACAATCC3′ 3′ Primer=5′CCTTATCCACGCGAATGACG3′	Tanaka et al., 1993	433		
TIMP-1	5′ Primer=5′CGCAGATATCCGGTACGCCTA3′ 3′ Primer=5′CACAAGCCTGGGATTCCGTGG3′	Edwards et al., 1986	354	*Pst*1	130, 224
TIMP-2	5′ Primer=5′CTCGCTGGACGTTGGAGGAA3′ 3′ Primer=5′CACGCGCAAGAACCATCACT3′	Leco et al., 1992	309	*Rsa*1	142, 167
TIMP-3	5′ Primer=5′CTTGTCGTGCTCCTGAGCTG3′ 3′ Primer=5′CAGAGGCTTCCGTGTGAATG3′	Leco et al., 1994	244	*Acc*1	81, 163
uPA	5′ Primer=5′GTGCCGCACACTGCTTCATT3′ 3′ Primer=5′CGTGCTGGTACGTATCTTCA3′	Belin et al., 1985	194	*Acc*1	48, 146
uPA-R	5′ Primer=5′TGTGCCTGCAGCGAAAAGACCAACA3′ 3′ Primer=5′GCATCCGCGGGAGACTGCCACA3′	Kristensen et al., 1991	321	*Xho*1	148, 173
PAI-1	5′ Primer=5′CCTTGCTTGCCTCATCCTGG3′ 3′ Primer=5′CTGGAAGAGCTTGAAGAAGTGG3′	Prendergast et al., 1990	406	*Pst*1	188, 218
PAI-2*	5′ Primer=5′AGAGAACTTCAGTGGCTGTG3′ 3′ Primer=5′CACTGCTTCTGGTTCTGTTG3′	Belin et al., 1989	250		
LIF-R	5′ Primer=5′TGGTGCAACTCATCTCGGTCTG3′ 3′ Primer=5′TGTGAGTCACCATGTGGTTGCTG3′	Tomida et al., 1993	360	*Hin*dIII	172, 188

*PCR product verified by sequencing.

were expressed at low levels throughout the day 7.5 conceptus but that TIMP-3 transcripts were enhanced in decidual tissue adjacent to the implanting embryo. On re-examination using DIG-labelled TIMP-3 probes, very strong staining was indeed observed in the area of the deciduum adjacent to the network of MMP-9 positive trophoblast giant cells (Fig. 2). The section
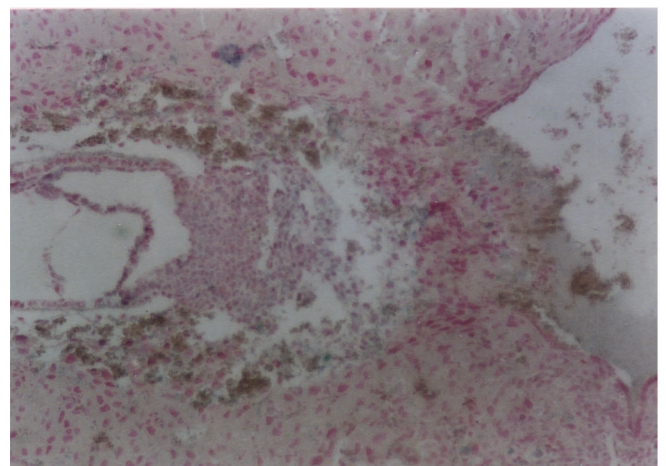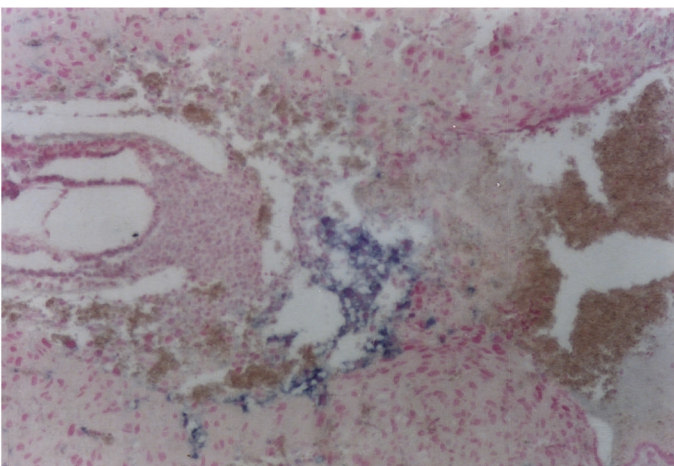
MMP-9
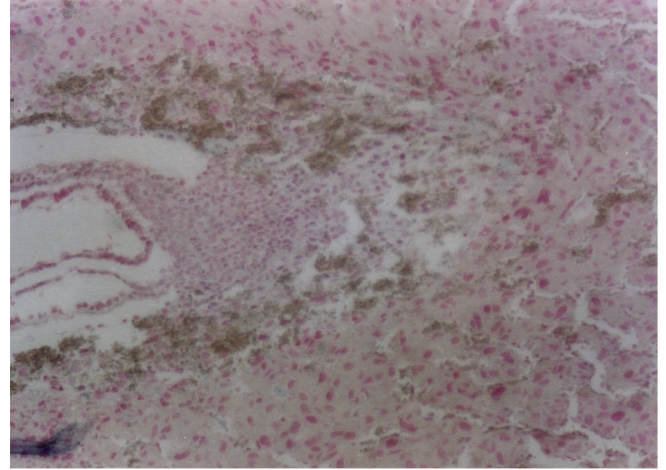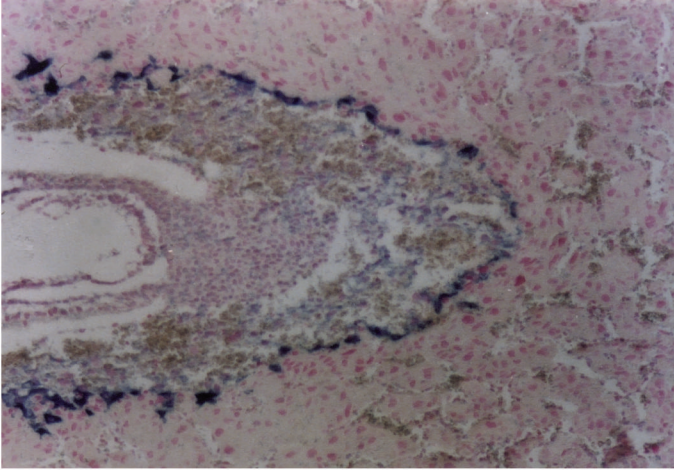
TIMP-3
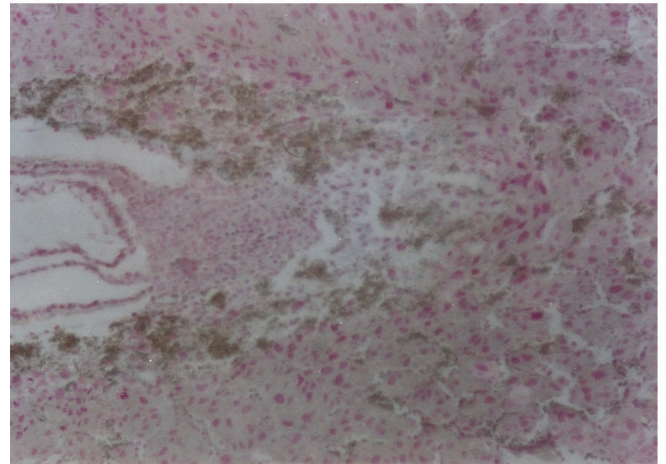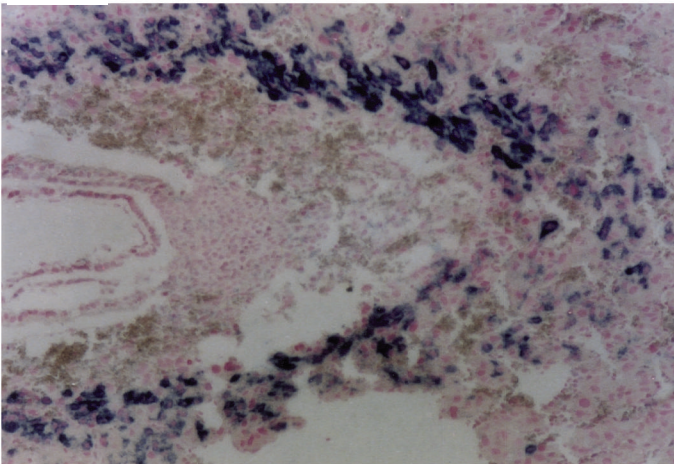
uPA

Fig. 2. In situ hybridization of 7.5 day p.c. mouse embryos and maternal decidua with MMP-9, TIMP-3 and uPA riboprobes. Sections were hybridized to antisense (left panels) and sense (right panels) riboprobes. MMP-9 signal (blue staining) was localized primarily over trophoblast giant cells at the periphery of the invading embryo, while TIMP-3 signal was localized predominantly to cells within the maternal deciduum adjacent to the embryo. uPA localized to trophoblast cells of the ectoplacental cone. Magnification ×100.

shown in the photomicrograph for TIMP-3 localization in Fig. 2 is derived from the same embryo shown for MMP-9 localization in Fig. 2 although it is not immediately adjacent.

Using antisense probes for uPA, expression was localized to trophoblast cells as reported previously by Sappino et al. (1989). In the section shown, staining is strongest in a set of cells at the tip of the ectoplacental cone although positive staining is also seen within cells (or processes of cells) that extend into the deciduum (Fig. 2).

LIF/EGF effect on proteinase activity

Both LIF (1000 U/ml) and EGF (10 ng/ml) significantly increased uPA activity in day 7 blastocyst outgrowths (Fig. 3A,B; $P<0.05$ by paired t-test of 4 experiments). However, 48 hours later at day 9, LIF decreased uPA activity (Fig. 3A; $P<0.05$ by paired t-test of 4 experiments) whilst EGF had no effect (Fig. 3B).

Zymographic analyses of gelatin-degrading activities in media from blastocyst outgrowths are shown in Fig. 4. The major activity was identified as gelatinase B (MMP-9) by virtue of its size (the major band at $M_r=105\times10^3$) and its inhibition by divalent metal ion chelation using 10 mM EDTA (Fig. 4A,B). Slower migrating species may correspond to aggregates of MMP-9 or higher-order complexes of this enzyme with TIMP-1 or interstitial collagenase (Wilhelm et al., 1989; Goldberg et al., 1992). As was the case for uPA, both LIF (0.82 ± 0.47 OD units versus 0.57 ± 0.41 OD units; $P<0.05$ by paired t-test of 3 experiments) and EGF (1.15 ± 0.53 OD units versus 0.52 ± 0.29 OD units; $P<0.05$ by paired t-test of 4 experiments) increased MMP-9 levels (the $M_r=105\times10^3$ form) by 44% and 121% respectively in day 7 embryo outgrowths (Fig. 4C). LIF also induced gelatinase activities at $M_r=95\times10^3$ and $M_r=75\times10^3$ (Fig. 4C) which are likely to represent the activated forms of murine MMP-9 by analogy with previous observations of the human enzyme (latent form is $M_r=92\times10^3$ and activated species migrate at $M_r=83\times10^3$ and $M_r=75\times10^3$, Moll et al., 1990). Alternatively, the band migrating at $M_r=75\times10^3$, which was not present in all experiments, may represent MMP-2 activity (Fig. 4C). At day 10 of development (72 hours later), treatment of outgrowths with LIF led to a 15% reduction in the level of MMP-9 activity relative to controls (2.17 ± 0.71 OD units versus 2.50 ± 0.67 OD units; $P<0.05$ by paired t-test of 4 experiments), which at this time produced high levels of the enzyme (Fig. 4D). In contrast, EGF had no effect (2.59 ± 0.74 OD units versus 2.42 ± 0.76 OD units) on MMP-production compared to control treated blastocyst outgrowths at day 10 (Fig. 4D).

DISCUSSION

Two species of PAs are currently known and are present during early development. Tissue-type PA (tPA) is expressed in rat and mouse oocytes (Huarte, et al. 1985). Subsequent to ovulation and fertilization, the tPA maternal transcripts are degraded and are not detectable beyond the 2-cell stage (Zhang et al., 1994). Urokinase (uPA) genes are first expressed at the 2-cell stage in rat embryos (Zhang et al., 1994) and at the blastocyst stage in mice (Fig. 1). uPA enzymatic activity is detected at the blastocyst stage in both mouse and rat embryos (Strickland et al., 1980; Zhang et al., 1994) and is restricted to

trophoblast cells as no activity is detected in the inner cell mass. During implantation, detailed in situ hybridization experiments (Sappino et al., 1989) have shown that, in day 5.5 to 8.5 embryos, uPA transcripts are localized to trophoblast cells, ectoplacental cone cells and their derivatives. These findings are supported by our studies on day 7.5 embryos (Fig. 2). Receptors for uPA have been shown to occur on human trophoblast cells (Zini et al., 1992) and bind active uPA to localize
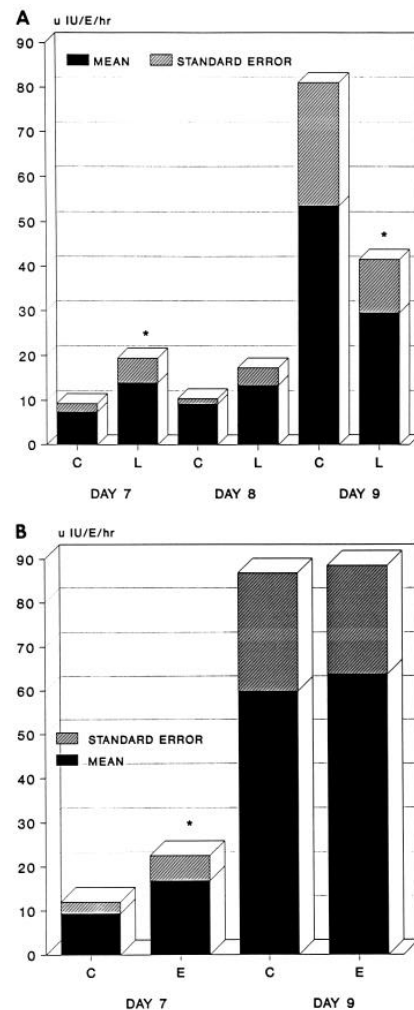

Fig. 3. Effect of LIF and EGF on embryo uPA activity. (A) Blastocyst outgrowths established by 48 hour culture of day 4 blastocysts with 10% FCS were cultured a further 24 hours (corresponding to day 7 of development) in the presence of TCM 199 + 1mg/ml BSA (control; C) or the same media supplemented with 10^3 U/ml LIF (L). Conditioned media was assayed for PA activity by a chromogenic assay using plasminogen as a substrate. Media samples were again taken from the same outgrowths after 24 hours further culture in serum followed by 24 hours culture in defined media ± LIF (corresponding to day 9 of development). Because of the biphasic effect that was observed with LIF treatment at day 7 and 9 of development, PA activity on day 8 of development was determined by 72 hour culture of day 4 blastocysts in the presence of 10% FCS followed by 24 hour culture in defined media ± LIF. (B) The effect of 10 ng/ml EGF (E) at day 7 and 9 of development was similarly determined as described in the LIF experiments. Units are µI.U./embryo/hr. *, significantly different from control ($P<0.05$) by paired t-test of 4 experiments.

proteolysis to the leading surface of the invading cell (Roldan et al., 1990). This proteinase has been shown to have a major influence on the ability of an embryo to implant as its inhibition decreases the extent of trophoblast attachment and outgrowth in vitro (Kubo et al., 1981). Embryos homozygous for the mutation t^{w73} have reduced levels of PA and do not implant in vivo (Axelrod, 1985). Other studies investigating the presence of PA inhibitors give further evidence for the importance of PAs during implantation. Uterine fluid from pregnant sows has high levels of a PA inhibitor that is associated with a non-invasive type of implantation in pigs. When pig embryos are transferred to an ectopic site where inhibitors
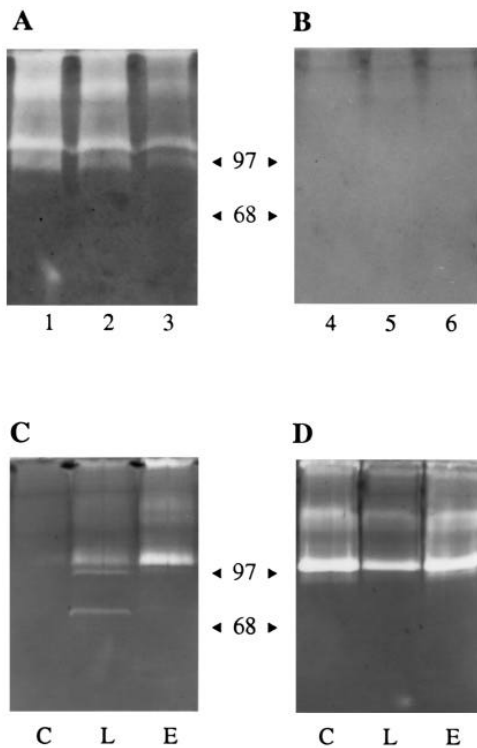

Fig. 4. Effects of LIF & EGF on blastocyst-derived MMP activities. (A, B) Gelatin zymography of conditioned media derived from blastocyst cultures detects a major activity of the size and characteristics of MMP-9. Media samples from three different experiments with varying numbers of day 10 blastocyst outgrowths were analysed in duplicate on the same zymography gel. One set (panel A, lanes 1-3) were treated as usual by washing in Triton X-100 and development for 48 hours as described in Materials and Methods. The second part of the gel (B, lanes 4-6) was incubated throughout in the presence of 10 mM EDTA. Inhibition of the gelatinase activities by EDTA confirmed that the proteins were MMPs, and the migration of a major form at $M_r=105\times10^3$ in A by reference to size markers run in parallel indicates that it is MMP-9. (C, D) A representative experiment of the effect of LIF and EGF on MMP-9 activity at day 7 and 10 of development respectively. Blastocyst outgrowths established by 48 hour culture of day 4 blastocysts with 10% FCS were cultured a further 24 hours (corresponding to day 7 of development) in the presence of TCM 199 + 1 mg/ml BSA (control; C) or the same media supplemented with 10^3 U/ml LIF (L) or 10 ng/ml EGF (E). This conditioned media was electrophoresed as described in Materials and Methods (C). Media samples were again taken from the same outgrowths after 48 hours further culture in serum followed by 24 hours culture (corresponding to day 10) in defined media ± LIF or EGF (D).

are not present, there is vigorous tissue invasion (Mullins et al., 1980). However, targeted mutation studies of the genes for uPA and LDL receptor-related protein (LRP) that internalizes and degrades uPA–PAI-1 complexes yield somewhat conflicting evidence regarding the role of these products during implantation events. In the case of disruption of the LRP gene, development is arrested by day 13.5 and although some, but not all, embryos homozygous for the LRP deficiency are arrested at the early postimplantation stages, some LRP-deficient blastocysts can implant into the uterus (Herz et al., 1992, 1993). Similarly, disruption of the uPA gene apparently has no major consequences for implantation although mice with combined tPA and uPA deficiencies are significantly less fertile (Carmeliet et al., 1994). Perhaps the lack of effect in single uPA deficiencies is due to redundancies (overlap) of function of other gene products (proteinases) that have not yet been defined. In such a situation, the null mutation would exhibit an effect only if other proteinases were not available to compensate or substitute for the uPA deficiency. Thus, a role for PAs in normal implantation events cannot be excluded. Further studies on the roles of PAs during implantation in the mouse were undertaken herein as a step toward elucidation of the role of these factors in this process.

Genes for collagenases, stromelysins and gelatinases are also expressed throughout preimplantation development. In particular, gelatinase-B/MMP-9 has been shown to be released from blastocyst outgrowths (Brenner et al., 1989; Behrendtsen et al., 1992). The importance of this latter MMP in the implantation process has been demonstrated by the observation that treatment of human cytotrophoblasts and mouse blastocyst outgrowths with TIMPs or neutralizing antibodies against MMP-9 blocks invasion and degradation of basement membranes (Librach et al., 1991; Behrendtsen et al., 1992). We show here that transcripts for MMP-9 are strongly expressed in the peripheral network of trophoblast giant cells that are in contact with the deciduum (Fig. 2). This localization is consistent with a role for MMP-9 during the invasive phase of the implantation process.

One aim of this study was to investigate, collectively, the expression of genes encoding uPA, MMP-9 and their inhibitors during preimplantation development. Because of the limited amount of biological material available for study, RT-PCR was chosen for mRNA analyses because of its extreme sensitivity. As blastocyst outgrowths provide a useful model to study the early phase of the implantation process (Glass et al., 1983), they were similarly analysed. Our results show that, while uPA receptor RNAs were detected throughout early development, uPA and MMP-9 transcripts were present in embryos from day 4 and 5 respectively of pregnancy onwards, coinciding with the commencement of trophoblast invasion (Fig. 1). We have also demonstrated localization of uPA and MMP-9 transcripts to trophoblast and ectoplacental cone cells of 7.5 day embryos by in situ hybridization techniques (Fig. 2) lending support to the notion that these proteinases play important roles during implantation. Further, since trophoblast cells elaborate both uPA and gelatinase B/MMP-9 during the implantation phase of development, the question of how cellular invasiveness is controlled is germane. Invasion may be limited by increased expression of TIMPs or PAI in the deciduum or placenta. TIMPs-1, -2 and -3 were found to be expressed throughout pre- and peri-implantation development (Fig. 1).

In previous work, Waterhouse et al. (1993) demonstrated that levels of TIMP-1 mRNA peak in mouse uterus, decidua and placenta in the period covering days 6-10 of development. This is the most invasive period of implantation. In this time interval TIMP-2 is expressed at a relatively low and invariant rate, but levels of TIMP-2 RNA increase steadily after day 10, with dramatic increases in the placenta after day 14. Our own in situ hybridization studies indicate high levels of expression of mouse TIMP-3 at day 7.5 in decidual cells that are the most proximal to the embryo, whereas the ectoplacental cone is devoid of signal (Fig. 2). The importance of decidua-derived TIMPs was recognized earlier in studies that examined the effects of conditioned media from decidual cultures in vitro on the invasive characteristics of trophoblast cells. Neutralization by antibodies of either TGFβ or TIMP-1 in such media led to increased ECM invasion (Lala and Graham, 1990; Graham and Lala, 1992). Elevated TIMP-3 expression may be particularly important in this regard because TIMP-3 is an ECM-associated protein (Pavloff et al., 1992; Leco et al, 1994) and its presence in the decidual ECM would provide a protective shield for the uterus that is spatially restricted to the implantation site. Expression of mouse TIMP-3 is apparent in the placenta at later stages of gestation (Apte et al., 1994), and it will be interesting to determine whether this coincides temporally with PAI-2 expression, which is localized to the outermost layer of the syncytiotrophoblast (Astedt et al., 1986).

PAI-2 transcripts were not detected at any stage throughout early development whilst PAI-1 was shown to be expressed in day 6 and 7 outgrowths (Fig. 1). The function of this proteinase inhibitor is yet to be elucidated but its temporal expression corresponding to the most invasive phase of placentation suggests that it may play a role in regulating trophoblast invasiveness. Alternately, expression in blastocyst outgrowths may be confined to the ICM, thus providing a mechanism of protecting the embryo from proteinases used by the trophoblast lineage to invade the maternal decidua. Further studies aimed at localization of PAI-1 expression will be required to help elucidate its potential role in the implantation process.

Because of the potential role that the ECM-degrading proteinases, uPA and MMP-9, may have during establishment of pregnancy, we were interested in investigating the relationship between the production of these two proteinases by mouse blastocyst outgrowths and the actions of growth factors/cytokines. Two prominent candidates are EGF and LIF, which are expressed by the uterus at the time of implantation and are absent in mice in which implantation has been delayed by ovariectomy (Huet-Hudson et al., 1990; Bhatt et al., 1991). EGF receptors have been well characterized on mouse blastocysts at both the protein and RNA level (Dardik et al., 1992; Wiley et al., 1992). However, as there is only one report that describes specific receptors that bind LIF on trophectoderm cells (Robertson et al., 1990), pre- and peri-implantation embryos were screened for the expression of the LIF receptor. Our studies revealed that transcripts for the LIF receptor were present in embryos from day 4 of pregnancy onwards (Fig. 1) which is similar to the temporal pattern of expression found for uPA and MMP-9 (Fig. 1). Because blastocysts have specific receptors that bind EGF and LIF, a mechanism exists whereby uterine-derived EGF and LIF may possibly influence blastocyst implantation.

To investigate this, blastocyst cultures were established in the presence of serum to facilitate attachment and trophoblast outgrowth (migration). After 2 days of culture, EGF or LIF was added in defined medium and the conditioned medium assayed 24 hours later for the presence of proteinases. Our data demonstrate clearly that, at this time in culture (i.e. day 7 of development), both LIF and EGF stimulated uPA and MMP-9 activity relative to control blastocysts (Figs 3, 4C). In the case of LIF treatment, there was increased representation of gelatinase activities at $M_r=95\times10^3$ and $M_r=75\times10^3$ that likely represent the activated forms of murine MMP-9. Co-regulation of uPA and MMP-9 suggests that the functions of these enzymes may be linked, possibly by participation as components of a proteolytic cascade whereby uPA leads to generation of plasmin from plasminogen, which in turn can activate, albeit weakly, the latent form of MMP-9 (Birkedal-Hansen et al., 1993; Kleiner and Stetler-Stevenson, 1993). However, as EGF also induces both uPA and MMP-9 but without leading to the appearance of activated forms of the latter, we infer that additional and specific effects of LIF on the activation mechanism of MMP-9 must occur.

Upon visual inspection, the sizes of blastocyst outgrowths were similar in both LIF- and EGF-treated and untreated samples. This does not entirely exclude the possibility that increased expression of proteinase activity could be due to increased cell numbers (proliferation) in response to LIF or EGF, but the effects of the growth factors/cytokines on uPA activity on day 7 blastocyst outgrowths were already detectable after only 6 hours of treatment (M. Harvey, unpublished observation). A mitogenic response to LIF or EGF would not be expected to occur in this short interval. Furthermore, we observed a decrease in enzymatic activity in the presence of LIF when day 10 outgrowths were examined (see below). Taken together, these observations suggest specific regulation of proteinase activity in blastocyst outgrowths that is independent of mitogenic effects of LIF or EGF.

Exposure of blastocyst outgrowths to LIF or EGF at day 9 of development gave a different result to that seen at day 7. LIF reduced both uPA and MMP-9 activity compared to control cultures whereas EGF had no effect (Figs 3, 4D). This ability of LIF to down-modulate uPA and MMP-9 activity is most interesting. It could reflect either altered responsiveness to LIF through developmental control of elements within its signal transduction pathway, or it may be due to an indirect effect involving induction by LIF of the expression of additional cytokines that negatively regulate proteinase gene expression, such as members of the transforming growth factor-β family (Edwards et al., 1987; Overall et al., 1989).

In conclusion, several members of the PA and MMP families are present during early murine development with uPA and MMP-9 first expressed at a stage that coincides with the onset of trophoblast invasion into the uterus. The production of these proteinases is regulated by LIF and EGF, which are also temporally expressed by the uterus at this time under the influence of maternal estrogen. Our data support a direct mechanism whereby uterine-derived LIF and EGF stimulate production of two important ECM-degrading proteinases during the early, highly invasive phase of implantation. This may explain at least in part why embryos in pregnant mice in which the LIF gene has been mutated do not implant (Stewart et al., 1992). It may be that uterine-derived LIF is essential to stimulate both embryonic uPA and MMP-9 activity to allow

implantation. Our results further suggest that LIF may act either directly or indirectly at later times to down-modulate invasiveness. Thus, LIF in particular may be essential for the correct temporal orchestration of invasiveness during implantation. These studies re-emphasize the important role of these proteinases, proteinase inhibitors and their regulatory factors during embryo implantation.

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