The expression and function of cystatin C and cathepsin B and cathepsin L during mouse embryo implantation and placentation

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SUMMARY

The implantation of the mouse embryo requires the controlled invasion of the uterine stroma by the embryonic trophoblast. This event is dependent, in part, on the secretion of matrix metalloproteinases and serine proteinases for the extracellular degradation of the uterine matrix. Proteinase activity is controlled by stromal decidualization and specific proteinase inhibitors. This work adds to our understanding of implantation and placentation by reporting the expression and function of another class of proteinases/inhibitors closely related to invasive cell behavior. We focused on the cysteine proteinases, cathepsins B and L, and their inhibitor cystatin C. Northern blots showed that trophoblast expressed cathepsin B throughout the invasive period (days 5.5-10.5). Both cathepsin B message and cathepsin L protein were localized to the mature, invasive trophoblast giant cells. Substrate gel electrophoresis showed an increase in giant cell cathepsin activity with enzyme profiles changing at the end of the invasive period. Northern and western blotting showed that cystatin C, the main inhibitor of cathepsins, was a major product of the decidualizing stroma. Message levels first increased in peripheral decidualizing cells, with the protein localizing close to the embryo during implantation (days 5.5-8.5). With the regression of the decidua beginning on day 9.5, a coordinated upregulation of both cathepsin B and cystatin C was observed implying a role for controlled cathepsin expression during apoptosis. E-64, a synthetic inhibitor of cathepsins B and L, was injected into pregnant females at the stage of blastocyst attachment (days 4.5-5.5). High doses resulted in the complete failure of implantation while lower doses resulted in stunted embryos and a reduced decidual reaction. These results suggested that cathepsins B and L are necessary for normal embryo development and uterine decidualization, and that decidua contributes to their control by a coordinated expression of cystatin C within the implantation site.

Key words: mouse, implantation, placentation, trophoblast, decidua, cathepsin B, cathepsin L, cystatin C.

INTRODUCTION

Mouse embryo implantation is a highly controlled event requiring invasion of the uterine stroma by the extraembryonic trophoblast (for review see Cross et al., 1994). On day 4.5 of gestation, the blastocyst attaches to the uterine epithelium and initiates implantation. The outer cells of the blastocyst, the mural trophoblast, begin differentiation into invasive primary trophoblast giant cells. These cells breach the uterine epithelium and degrade the underlying extracellular matrix to connect with the maternal blood supply. By day 7.5, the implantation site is established. Concurrently, the cells adjacent to the inner cell mass, the polar trophoblast, continue to proliferate and form the ectoplacental cone (EPC). On day 6.5, the outer cells of the EPC begin differentiation into invasive secondary trophoblast giant cells. Placentation is dependent upon the invasion of the uterine stroma by these cells from days 7.5-10.5.

In ectopic sites, trophoblast invasion is uncontrolled and destructive but is strictly limited within the microenvironment of the uterus (Kirby, 1965). Uterine control of trophoblast invasion is due, in part, to the decidualizing stroma. Decidua is transient tissue, which first develops antimesometrially in the uterine chamber. The antimesometrial zone (AMZ) begins differentiation at the time of blastocyst attachment on day 4.5. Decidualization spreads to the mesometrial zone (MZ), which begins its differentiation 2 days later at the time of secondary trophoblast giant cell invasion. Decidualizing cells increase in size and form extensive junctional complexes (O’Shea et al., 1983; Ono et al., 1989). They remodel the matrix by phagocytosis of collagen type I and produce basement membrane components including laminin and collagen type IV (Zorn et al., 1986; Wewer et al., 1986). These changes allow decidua to serve as a thick tissue barrier to uncontrolled trophoblast invasion. However, beginning on day 6.5, the decidua cells in close proximity to the invasive trophoblast undergo apoptosis and are removed by trophoblast cell phagocytosis, thus providing room for the growing embryo (Bevilacqua and Abrahamson, 1989; Alexander et al., 1996). By the end of the invasive period (day 10.5), the remaining AMZ decidua and portions of the MZ decidua totally regress, leaving the mature placenta and decidua basalis.
Trophoblast invasion is dependent on proteinases for the extracellular degradation and phagocytosis of maternal cells and matrix (Bevilacqua and Abrahamsohn, 1989). Mouse trophoblast has been shown to synthesize and secrete the metalloproteinases, gelatinase and stromelysin (Harvey et al., 1995; Lefebvre et al., 1995; Alexander et al., 1996) the serine proteinase, plasminogen activator (Teesalu et al., 1996; Zhang et al., 1996) and the cysteine proteinase, cathepsin L (Hamilton et al., 1990, 1991). The decidua participates in the control of these proteinases by secreting plasminogen activator inhibitor and tissue inhibitors of metalloproteinases (TIMP1, TIMP2 and TIMP3) in specific temporal and spatial patterns (Waterhouse et al., 1993; Apte et al., 1994; Harvey et al., 1995; Alexander et al., 1996). Inhibitors of cysteine proteinases have not been well characterized in mouse decidua. However, in vitro, cysteine proteinase inhibitors were shown to prevent EPC trophoblast invasion (Babiarz et al., 1992). This suggests that the control of cysteine proteinases is important during mouse implantation.

Cysteine proteinases are controlled by members of the cystatin superfamily, which include both intracellular (cystatin A and cystatin B) and secreted (cystatin C) forms. Cystatin C is a low molecular mass protein (14 kDa) that is expressed in several tissues and is present in high concentrations in a number of biological fluids (Abrahamson et al., 1986; Tavera et al., 1990; Huh et al., 1995). It is known to be an inhibitor of the cysteine proteinases cathepsin B and cathepsin L (Barrett et al., 1984; Abrahamson et al., 1986), which are present in the lysosomal apparatus of phagocytic cells (Everts et al., 1996). The invasive phenotype of many metastatic cell types is associated with the increased production and secretion of cysteine proteinases and aberrant regulation of cystatin C (Lah et al., 1989; Gottesman, 1990; Sloane et al., 1990; Chambers and Tuck, 1993). Cystatin C is also the principle cathepsin inhibitor regulated in chronic inflammatory diseases (Lenarcic et al., 1988; Billing et al., 1992; Lah et al., 1993; Henskens et al., 1994) and tissue remodeling events (Lerner and Grubb, 1992).

In this paper, we analyze the expression and function of cystatin C and cathepsins B and L during mouse implantation and placentation in vivo. The results show that cystatin C message and protein levels are regulated in a specific spatial and temporal pattern that correlates with cathepsin B and L production. We also show that perturbation of cysteine proteinase activity during early implantation causes abnormal embryo development and uterine decidualization.

MATERIALS AND METHODS

Materials

Tissue dissociation enzymes, proteinase inhibitors, SDS-PAGE supplies, laminin antiserum, secondary antibody conjugates and in situ hybridization reagents were from Sigma (St Louis, MO). All culture media, serum and supplements were from Gibco Laboratories (Grand Island, NY). Tri-Reagent was from Molecular Research Center Inc. (Cincinnati, OH) and the Megaprime random priming kit was from Amersham (Arlington Heights, IL). GeneScreen Plus nylon membrane, Renaissance chemiluminescence kit and [γ-32P]ATP were from DuPont NEN (Boston, MA). The Lowry-based DC Protein Assay kit was from BioRad (Hercules, CA) and the western blotting medium was Immobilon-P from Millipore (Bedford, MA). Non-reduced protein standards (Mark 12) were from Novex (San Diego, CA). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). The digoxigenin labeling mix and immunodetection kit were from Boehringer Mannheim (Indianapolis, IN). The DAB (3-3′-diaminobenzidine) substrate kit was from Vector (Burlingame, CA). The cystatin C cDNA probe from American Type Culture Collection (Rockville, MD) was subcloned into Bluescript II KS+ (Stratagene, La Jolla, CA). Mouse cathepsin L probe was purchased from University Technologies International, Inc. (Calgary, Alberta, Canada). Preprocathepsin B cDNA was the generous gift of S. J. Chan, University of Chicago, IL. The IgG fraction of normal rabbit serum and the 18s rRNA probe were kindly provided by D. T. Denhardt, Rutgers University, NJ. Cystatin C antiserum was purchased from DAKO (Carpinteria, CA) and cathepsin L antiserum was the generous gift of R. Hamilton, Iowa State University, IA.

Mice

Naturally mated mice (CF1) were killed on days 4.5-13.5 of development (day 0 = morning of vaginal plug). Stroma was enzymatically isolated from virgin female endometrium and females on day 4.5 of pregnancy as described (Romagnano et al., 1996). Decidual capsules (days 5.5-13.5) were split open and the embryo and trophoblast teased free. Decidual tissue was collected from whole decidual capsules or separated into antimesometrial and mesometrial regions by dissection at the level of the eutocplacental cone (Babiarz et al., 1996). Trophoblast tissue was collected by microdissection of the EPC from day 7.5 embryos (Romagnano and Babiarz, 1990) and, using fine forceps, the embryo was withdrawn from the surrounding sheath of primary trophoblast. Sheets of primary trophoblast were peeled from days 9.5 and 11.5 implantation sites with fine needles. Whole placenta were collected on days 9.5-13.5.

Northern analysis

Tissues were collected as described above and total RNA was isolated using Tri-Reagent. Samples (5 μg) were denatured with glyoxal and dimethylsulfoxide, resolved on a 1% agarose gel and transferred to GeneScreen Plus membrane. Membranes were hybridized with 32p-labeled cystatin C or preprocathepsin B (CB) cDNA probes according to standard protocol and exposed to X-ray film for 24-48 hours. Subsequently, membranes were stripped and rehybridized with the 18s rRNA loading control. Scanning densitometry was used to quantify relative mRNA levels by normalizing band intensity to the 18s rRNA. All experiments were repeated in triplicate and analyzed using Image-Quant software (Molecular Dynamics, Sunnyvale, CA).

Western analysis

Tissue samples were collected as described above and incubated in 0.02% NP-40 with 0.01% phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin and 10 μg/ml soybean trypsin inhibitor at 37°C for 30 minutes, sonicated on ice and spun at high speed to remove unsolubilized material. Total protein from each sample (100 μg) was analyzed by SDS-PAGE on 5-15% gradient gels under reducing conditions and transferred to Immobilon-P membranes. Membranes were blocked with 5% Carnation milk in phosphate-buffered saline (PBS), then incubated with rabbit anti-human cystatin C diluted 1:2,000 in 1% Carnation milk in PBS. After washing, the membrane was incubated with goat anti-rabbit IgG-peroxidase conjugate (1:2,000). All incubations were performed for 1 hour at room temperature. Bands were visualized by chemiluminescence. Experiments were repeated three times and relative intensities of the bands were quantified by scanning densitometry as above. For cathepsin L (CL) detection in EPC-conditioned media (CM), 12-14 EPCs from day 7.5 embryos were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% bovine calf serum in a single well of a 96-well plate. After 48 hours, cultures were rinsed in DMEM to remove serum proteins and incubated a further 8 hours in 40 μl of DMEM supplemented with 0.2% lactalbumin hydrolysate. The medium was then collected and added to 10 μl of 4× nonreducing...
Acidic proteinase activity was detected in CM (20). Zymography secondary antibody alone. CM was collected from day 7.5 EPCs cultured for 48 hours (see paraffin and sectioned at 7 non-reducing treatment buffer. Aliquots of 20 μl were analyzed on 10% SDS-PAGE minigels. The separated proteins were transferred to Immobilon-P membrane, blocked and incubated overnight with rabbit anti-mouse cathepsin L diluted 1:200. After washing, the membrane was incubated for 2 hours with goat anti-rabbit IgG peroxidase conjugate (1:200). Bands were visualized by staining with 4-chloro-napthol.

**In situ hybridization**

The CB cDNA plasmid was linearized with NheI or SalI and the CL cDNA was linearized with BamHI or HindIII to provide template for antisense or sense riboprobes, respectively. The cystatin C cDNA was subcloned into Bluescript II KS+, purified and linearized with either SpeI or SalI to generate antisense or sense cRNA probes, respectively. Linearized CB, CL or cystatin C template (1 μg) was used to synthesize T7 or T3 polymerase-directed digoxigenin-labeled riboprobes with DIG RNA labeling mix. The in situ procedure followed was described in Schaeren-Wiemers and Gerfin-Moser (1993). Briefly, samples were frozen in liquid nitrogen. Cryosections (15 μm) were cut and mounted, fixed with cold phosphate-buffered 4% paraformaldehyde, then acetylated with 0.1 M triethanolamine, pH 8, 0.25% acetic anhydride. The slides were prehybridized at room temperature for 4-6 hours in hybridization buffer (50% formamide, 5× SSC, 2% blocking solution provided by Boehringer Mannheim, 0.1% N-laurylsarcosine, 0.02% SDS, 250 μg/ml RNA and 500 μg/ml sheared salmon sperm DNA). Hybridization mixture was prepared by adding 200 ng of DIG riboprobe per ml of hybridization solution, heating to 85°C, then chilling on ice. Sections were hybridized overnight at 72°C, washed with 2× SSC buffer and incubated with anti-digoxigenin antibody. Labeled probe was detected using a colorimetric immunodetection kit containing NBT (4-nitroblue tetrazolium chloride) and X-phosphate (BCIP, 5-bromo-4-chloro-3-indolyl-phosphate). For negative controls, sections were incubated with sense strand cRNA or without probe.

**Immunohistochemistry**

To localize cystatin C, 7 μm cryosections of uterine tissue from virgin or pregnant mice were fixed in cold acetone for 10 minutes and rehydrated with PBS. Sections were blocked in 5% Carnation milk in PBS, incubated in anti-human cystatin C antiserum diluted 1:50 in 1% Carnation milk in PBS. After washing, slides were incubated in goat anti-rabbit IgG-FITC conjugate (1:100). All incubations were performed for 1 hour at room temperature and signal was visualized by epifluorescence using a Nikon Diaphot 300 inverted microscope. To localize extracellular laminin, 7 μm cryosections of normal and E-64-treated day 7.5 capsules were fixed in methanol for 10 minutes, rehydrated in PBS, and digested for 10 minutes in 0.5% Triton X-100 (Glasser et al., 1987). Sections were blocked as above, then incubated in laminin antiserum (1:100) followed by goat anti-rabbit IgG-FITC conjugate (1:100). Antibody incubations were performed at 37°C for 1 hour and signal was visualized by epifluorescence as above. To localize CL, decidual capsules were fixed with Bouin’s, embedded in paraffin and sectioned at 7 μm. Tissue sections were incubated with CL antibody (1:600). The signal was visualized with peroxidase-conjugated goat anti-rabbit IgG (1:200) using the DAB Substrate kit for peroxidase (Hamilton et al., 1991). Negative control experiments included incubation with the IgG fraction of normal rabbit serum or secondary antibody alone.

**Zymography**

Acidic proteinase activity was detected in CM (20 μl) or tissue lysates (25 μg total protein) after separation on 10% SDS-polyacrylamide minigels that were copolymerized with 1 mg/ml gelatin. CM was collected from day 7.5 EPCs cultured for 48 hours (see above). Tissue samples including day 7.5 EPCs, primary trophoblast from days 7.5, 9.5 and 11.5, and 48-hour day 7.5 EPC outgrowths were lysed in 2× non-reducing treatment buffer. After electrophoresis, gels were washed 2× 15 minutes in 2.5% Triton X-100 in 0.1 M sodium acetate at pH 5.2 to reactivate enzymes by removal of SDS. Gels were then incubated overnight in 0.1 M sodium acetate substrate buffer at pH 5.2 containing 2 mM diithiothretol, a cysteine proteinase-specific enhancer (Dowd et al., 1994). To demonstrate specific proteinase activities, gels were incubated in substrate buffer with class-specific enzyme inhibitors. These included 1 mM PMSF, 5 mM disodium ethylenediamine tetracetic acid (EDTA), 0.5 mM trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E-64), 20 μg/ml leupeptin and 100 μM peptatin A. To detect areas of protease digestion (indicated by clear bands against a dark background), gels were stained in Coomassie Blue for 45 minutes, destained in 50% methanol/10% acetic acid for 5-10 minutes, followed by rehydration in 5% methanol/7% acetic acid. Zymograms were replicated a minimum of three times to ensure reproducibility of results.

**In vivo cathepsin perturbations**

E-64 was diluted in PBS and injected intraperitoneally at concentrations of either 10, 30 or 50 mg/kg. Pregnant mice were injected once on days 4.5 and 5.5, then killed on day 7.5. Control mice were injected with PBS. Uterine capsules were separated, fixed in Bouin’s and embedded in paraffin by standard protocol. Serial 7 μm sections were stained with haematoxylin and eosin. Measurements of capsule length and width were taken from longitudinal serial sections through the center of implantation sites. Embryo and EPC lengths were calculated from serial cross sections from 6 capsules/treatment. Statistical significance was analyzed using the Student's t-test with P<0.01.

**RESULTS**

**Cystatin C expression in decidualizing tissue**

Northern analysis showed that cystatin C was expressed and upregulated during stromal decidualization (Fig. 1A). A 0.7 kb transcript was observed in total RNA samples isolated from non-pregnant and pregnant uteri on days 5.5-10.5 of gestation. The relative levels of cystatin C mRNA in whole decidual capsules increased by a factor of 5 from days 5.5-10.5 (Fig. 1A). Message levels accumulated slowly during days 5.5-7.5, then increased sharply at day 8.5 (Fig. 1A). Western analysis also identified an increase of cystatin C protein (Fig. 1B). In non-pregnant uteri, cystatin C was only detected in overexposed films (data not shown). By the time of blastocyst attachment on day 4.5, a band of 14 KDa was observed. From days 4.5-10.5, cystatin C levels showed a 20-fold increase with the largest change in protein accumulation occurring at day 7.5. Regional differences in cystatin C protein expression were analyzed in decidual capsules dissected into the two zones of differentiation. The relative levels of cystatin C in the AMZ decidua increased in a linear fashion from days 6.5-10.5 (Fig. 1C). MZ decidua levels remained fairly consistent until late in development, with a dramatic increase at day 10.5 (Fig. 1C). Control western blots using only secondary antibody showed no labeling (data not shown).

**Cystatin C and cathepsin B expression in trophoblast**

To assess whether invasion was associated with the production of trophoblast-derived cystatin C, we examined its expression in day 7.5 EPC lysates and placenta from days 9.5, 10.5 and 13.5. Trophoblast-derived cystatin C mRNA was barely detectable on days 7.5-10.5 but, on day 13.5, the noninvasive placental trophoblast showed increased cystatin C expression (Fig. 2A). During this time, the invasive cells were found to
express mRNA for CB. A 2.2 kb transcript was expressed in EPCs from days 7.5 and 8.5 and placenta from days 9.5-11.5 (Fig. 2B).

Localization of cystatin C and cathepsin B
In situ hybridization was used to elucidate the uterine cell-type-specific expression patterns of cystatin C and its target enzyme, CB. In non-pregnant uteri, cystatin C message was restricted to the uterine glands and epithelium (Fig. 3A). On day 4.5, cystatin C was present in small “islands” of expression that were randomly distributed in the surrounding stroma (Fig. 3B). No localized increase in synthesis was invoked at the site of blastocyst attachment. Sections through the center of the day 5.5 implantation site showed cystatin C localized to the peripheral decidualizing cells of the AMZ and MZ (Fig. 3C). The primary decidual zone (PDZ), which is the avascular region adjacent to the implanting embryo and consists of the most differentiated stromal cells, showed less intense labeling at this stage (Fig. 3C). The undifferentiated basal stem cell layer, adjacent to the inner myometrium, did not express cystatin C (Fig. 3C). CB was expressed in the uterine glands and epithelium in the non-pregnant uterus (Fig. 3D). This was similar to the pattern seen for cystatin C (Fig. 3A). Sections through the implantation site on day 5.5 showed that CB expression was restricted to the uterine epithelium and glands, and trophoblast giant cells (Fig. 3E). Control sections hybridized with sense strand riboprobes of either cystatin C or CB showed no labeling (Fig. 3F,G, respectively).

In situ localization of cystatin C message in day 7.5 decidual capsules showed higher levels in the AMZ cells relative to the MZ cells (Fig. 4A). Lower levels of expression were seen in the visceral endoderm (Fig. 4A). The invasive trophoblast giant cells remained negative while the adjacent decidua showed the highest cystatin C expression (Fig. 4B). Concomitantly, CB message was localized to the differentiated trophoblast giant cells and not to the immature cells of the developing EPC (Fig. 4C,D). High levels of CB were also expressed in the visceral endoderm and uterine glands but not in the decidua (Fig. 4C,D).

As development continued, decidual cell regression along with extracellular matrix degradation occurred in order to make room for the growing conceptus. From days 9.5-11.5, high levels of cystatin C mRNA were observed throughout the decidual capsule with increased expression in the apoptotic decidua adjacent to the invading trophoblast (Fig. 5A). As the trophoblast reached the end of the invasive period, giant cells began upregulating the synthesis of cystatin C message (Fig. 5B). These cells continued to express high levels of CB (Fig. 5C,D). In addition, high levels of CB were also seen in the visceral yolk sac and were observed for the first time in the regressing AMZ decidua (Fig. 5C,D).

Immunofluorescence using cystatin C antiserum revealed that, on day 4.5, cystatin C protein was restricted to uterine epithelium and glands and scattered cells within the uterine stroma (Fig. 6A). This was similar to the distribution of cystatin C mRNA (Fig. 3B). Blastocyst attachment did not appear to induce an increase in cystatin C around the attachment site. However, on day 5.5, higher concentrations of
cystatin C protein accumulated in the PDZ (Fig. 6B), whereas mRNA was localized to the uterine epithelium and glands (arrowheads). By day 4.5, at the site of blastocyst attachment, low levels of cystatin C were observed randomly distributed within the surrounding stroma cells (arrows). (C) Within the center of the day 5.5 implantation site, cystatin C was localized in the peripheral decidualizing stroma while the primary decidual zone and basal stem cell layer (arrowheads) remained negative. (D) CB expression in the non-pregnant uterus showed colocalized expression with cystatin C in the uterine epithelium and glands (arrowheads). (E) By day 5.5, CB expression was confined to the primary trophoblast giant cells, the uterine epithelium and glands (arrowheads). Inset: high magnification of embryo, surrounding giant cells and uterine epithelium. (F) Negative control sections probed with sense strand RNA of cystatin C or (G) CB. Bars indicate 100 μm in A,C,D,G, 20 μm in B,E inset, F and 50 μm in E, ul, uterine lumen; b, blastocyst; pdz, primary decidual zone; e, embryo; amz, antimesometrial zone; mz, mesometrial zone; gc, giant cells.

Detection of cathepsin activity
To determine if trophoblast cells synthesized or secreted acidic cysteine proteinases, substrate gel electrophoresis was performed. Lysates of day 7.5 EPCs and primary trophoblast from days 7.5, 9.5 and 11.5 all possessed bands of gelatinase.
However, the parietal yolk sac remained negative. Bars indicate 100 μm. (A) By day 11.5, cystatin C mRNA was significantly upregulated in the decidua as well as the giant cells, and lower expression was detected in the parietal yolk sac and visceral yolk sac on day 11.5. (arrowhead), trophoblast giant cells and visceral yolk sac. (D) CB were detected in the antimesometrial zone, uterine glands parietal yolk sac and visceral yolk sac. (C) High expression levels of CL were detected in the antimesometrial zone, uterine glands parietal yolk sac and visceral yolk sac; e, embryo.

**Localization and detection of cathepsin L**

In situ analysis localized CL to the trophoblast giant cells and visceral and parietal endoderm on day 7.5 of development (Fig. 7D). CL message was also observed within the decidualizing stroma with the most intense expression in the lateral sinuses of the mesometrial decidua (Fig. 7D). Since a suitable antibody to CB was not available, we analyzed CL protein expression. CL protein was immunolocalized to trophoblast giant cells and visceral and parietal endoderm in the day 7.5 implantation site (Fig. 7E). Within the decidua, CL expression was highest in the lateral decidualizing zone (Fig. 7E). Higher magnification showed intense staining in small cytoplasmic vesicles of invasive trophoblast giant cells (Fig. 7F). This suggested that increased lysosomal activity occurred in the most differentiated cells compared to the immature cells of the EPC core. Further confirming the secretion of trophoblast cathepsins, western blotting detected CL bands at 35 and 36 kDa in EPC-conditioned media (Fig. 7C).

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ferences was the lack of lateral sinus development in the MZ region of E-64-treated capsules (Fig. 8A,B). Light microscopic analysis of treated capsules showed that all expected layers were present, but reduced in size when compared to controls (Fig. 8C,D). No evidence of abnormal cell death or invasion of lymphatic cells was observed in non-resorbing capsules. When animals were treated with 50 mg/kg of E-64, total resorption of all implantation sites was observed (Table 1).

To further characterize the E-64-induced decidual defect, we used immunofluorescence to examine the pattern of extracellular laminin deposition. No qualitative differences were observed in laminin deposition between control and treated day 7.5 capsules (Fig. 9). However, laminin organization within the MZ clearly delineated the elongated sinuses in normal capsules (Fig. 9A). In treated capsules, the lack of these structures was reflected by the presence of only small round sinuses (Fig. 9B).

**DISCUSSION**

We have shown that the cathepsin proteinases B and L are important regulators of normal development in the mouse and observed in laminin deposition between control and treated day 7.5 capsules (Fig. 9). However, laminin organization within the MZ clearly delineated the elongated sinuses in normal capsules (Fig. 9A). In treated capsules, the lack of these structures was reflected by the presence of only small round sinuses (Fig. 9B).

**Table 1. Analysis of day 7.5 decidual capsules from mice treated with the cysteine proteinase inhibitor, E-64**

<table>
<thead>
<tr>
<th>Treatment (No. of mice)</th>
<th>No. of capsules</th>
<th>Embryonic stage</th>
<th>Decidua</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Prim. streak*</td>
<td>Stunted EC†</td>
</tr>
<tr>
<td>Control (4)</td>
<td>45</td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>E-64 (10 mg/kg) (2)</td>
<td>11</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>E-64 (30 mg/kg) (4)</td>
<td>38</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>E-64 (50 mg/kg) (4)</td>
<td>34</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Width (mm)</td>
<td>Length§ (mm)</td>
<td>Area (mm²)</td>
</tr>
<tr>
<td>Control (4)</td>
<td>2.48±0.11</td>
<td>3.32±0.02</td>
<td>6.46±0.92</td>
</tr>
<tr>
<td>E-64 (10 mg/kg) (2)</td>
<td>2.46±0.005</td>
<td>3.13±0.01</td>
<td>6.07±0.14</td>
</tr>
<tr>
<td>E-64 (30 mg/kg) (4)</td>
<td>2.18±0.06</td>
<td>2.57±0.11</td>
<td>4.46±0.98</td>
</tr>
<tr>
<td>E-64 (50 mg/kg) (4)</td>
<td>2.18±0.06</td>
<td>2.57±0.11</td>
<td>4.46±0.98</td>
</tr>
</tbody>
</table>
| *Primitive streak staged embryos with mesoderm formation.  
†Stunted egg cylinder staged embryos lacking mesoderm.  
‡Mean length in millimeters (mm) ± the standard deviation calculated from serial longitudinal sections measured through the center of 6-9 capsules/treatment.  
§Shape determined by the ratio of capsule width to length (w/l) measured from longitudinal sections through the center of 6-9 capsules/treatment.  
¶Significance was determined using the Student’s t-test where P<0.01.
that their expression is coordinated spatially and temporally with their inhibitor, cystatin C, during implantation and placentation. Cystatin C message and protein increased continually in decidualizing stroma. The increase of protein on day 7.5 preceded the increase in message at day 8.5 suggesting post-transcriptional regulation of cystatin C. On day 5.5, high levels of cystatin C message were localized to the peripheral decidua cells within the implantation chamber and CB message was first detectable in trophoblast giant cells. No increases in cystatin C or cathepsin B were observed in the basal fibroblast layer. Although cystatin C message was highest in the peripheral decidual cells within the implantation chamber and CB message was first detectable in trophoblast giant cells. No increases in cystatin C or cathepsin B were observed in the basal fibroblast layer. Although cystatin C message was highest in the peripheral decidual cells, the protein appeared localized in the PDZ. This localization suggested a role for the PDZ in regulating trophoblast cathepsins. From days 7.5 to 9.5, when both AMZ and MZ regions are actively invaded by giant cells, cystatin C message levels increased dramatically and protein continued to be localized close to the invading cells. After the completion of placental trophoblast invasion (day 11.5), the trophoblast cells expressed high levels of cystatin C. This suggested that they are programmed to regulate their invasive capacity as they switch from an invasive cell type to one that provides nourishment and protection to the growing embryo.

Throughout the invasive period, trophoblast giant cells were found to express CB. A single 2.2 kb CB transcript was detected which is typical of non-malignant cellular phenotypes. Aggressive malignant cell types express additional transcripts of 4-5 kb (Qian et al., 1989). Like other proteinases such as gelatinase B (Alexander et al., 1996), we have shown that CB and CL are detected in the outer, more mature trophoblast cells. Both CB message and CL message and protein were localized to these cells, with CL protein accumulating in small cytoplasmic vesicles suggesting increased lysosomal activity. Zymography identified cathepsin activity at 34 and 37/38 kDa. Others have reported active or mature CB and CL species in the same molecular mass range (Hamilton et al., 1991; Gong et al., 1993; Mach et al., 1993; Ryan et al., 1995). Cathepsin activity was greater in day 9.5 giant cells versus the undifferentiated day 7.5 EPCs. The increased production of cathepsins coincided with the period of trophoblast phagocytosis of epithelial cells, maternal blood cells and decidual cells (Bevilacqua and Abrahamsohn, 1989). Non-invasive giant cells showed activity primarily associated with high molecular mass zymogens, most likely representing unprocessed or aggregate forms (Chan et al., 1986). This suggested that the more aggressive trophoblast giant cells process cathepsin precursors into the active or mature forms of enzymes to facilitate invasion.
Both CB and CL are capable of digesting matrix molecules (Burleigh et al., 1974; Mason et al., 1986), including laminin (Lah et al., 1989), collagen IV and fibronectin (Guinec et al., 1993). Increases in cathepsin synthesis and secretion are associated with numerous invasive carcinomas and ras-transformed fibroblasts (Sloane et al., 1990; Chambers et al., 1992). Lower molecular mass forms of cysteine proteinase zymogen activity were observed in EPC-conditioned media compared to EPC lysates suggesting that further processing occurs when these enzymes are secreted. Detection of CL in trophoblast-conditioned media confirmed the secretion of this enzyme by mouse trophoblast (Hamilton et al., 1991) and supported a possible role in extracellular matrix degradation. CL and CB also activate other proteinases involved in matrix degradation. CL activates pro-uPA (Goretzki et al., 1992) and CB activates the metalloproteinase, stromelysin (Murphy et al., 1992). Guinec et al. (1993) reported that soluble cysteine proteinase digests liberated three proteolytically active fibronectin fragments, suggesting that these enzymes can activate latent proteinase activity from the basement membrane and thus initiate a novel proteolytic cascade. In summary, cysteine proteinases may contribute to invasion by the digestion of matrix molecules, the extracellular activation of other pro-enzymes and the intracellular breakdown of molecules phagocytosed by cells.

In addition to its role in invasion, cysteine proteinases may contribute to the apoptosis that is necessary for normal development. At attachment and early implantation, the trophoblast and uterine epithelium are in direct contact until the epithelial cells undergo apoptosis (Parr et al., 1987; Abrahamsohn and Zorn, 1993). We have shown that high levels of CB were expressed in the uterine epithelium on day 5.5 suggesting that it was one of the genes upregulated during uterine epithelial cell death. Increased production of CB has been associated with secretory epithelial cell death during mammary gland involution (Tenniswood et al., 1994; Guenette et al., 1994). Decidual cell regression also occurs by apoptosis. This programmed cell death begins on day 6 in the PDZ with apoptotic decidual cells cleared by giant cell phagocytosis to allow for expansion of the implantation site through day 8.5 (Alexander et al., 1996). Shortly after, AMZ decidua begins a program of total regression on day 9.5 spreading in a wave-like fashion to the MZ on day 10.5 (Akcali et al., 1996; Gu et al., 1994). Electron microscopic analysis of regressing decidua showed an accumulation of autophagosomes and lysosomes (Abrahamsohn, 1983). Our data showed that CB expression significantly increased in the AMZ and MZ decidua after day 9.5 of gestation, suggesting that this enzyme may play a role in their apoptotic program. Cystatin C levels also increased in these regions and cystatin C expression was co-localized with CB expression to regressing decidual cells of the AMZ. This led us to hypothesize that cystatin C contributes to decidual apoptosis in these areas by protecting the extracellular matrix.
from aberrant cysteine proteinase activity and/or preventing untimely cysteine proteinase activation of other pro-enzymes that are involved in tissue destruction.

The importance of cysteine proteinases and their inhibitors to normal development was demonstrated by treatment of pregnant females with the cathepsin inhibitor, E-64. At the highest concentration, complete failure of development occurred with resorption of both the embryo and decidualized stroma. At lower concentrations, E-64 caused defects similar to those observed with metalloproteinase inhibitors (Alexander et al., 1996). In both cases, embryos were stunted and the decidual reaction was diminished. CB and CL were localized to the visceral endoderm starting at day 5.5. This tissue, which develops into the visceral yolk sac, performs an absorptive function beginning as early as day 6.5 of development (Palis and Kingsley, 1995). In vivo perturbation of yolk sacs with E-64 or leupeptin resulted in decreased protein processing and embryo growth retardation (Daston et al., 1991), suggesting that enzyme activity is required for normal breakdown of proteins during embryogenesis (Grubb et al., 1991). A similar effect may underlie the decreased embryonic growth observed in animals treated with E-64. It is possible that the accumulation of cystatin C in the PDZ may also play a role in normal control of cathepsins produced by the extraembryonic endoderm. On day 11.5 of development, cystatin C begins to increase becoming a major visceral yolk sac product by day 13.5. The continued presence of cystatin C as a component of amniotic fluid (Abrahamson et al., 1986) supports its regulation of yolk sac cathepsin activity throughout development. Perturbation of cathepsin activity also retarded the decidualization process producing small, abnormally shaped capsules. One of the hallmarks of normal stromal differentiation to decidua is the remodeling of the extracellular matrix from an environment composed of primarily fibronectin and collagen type I to a pericellular basement membrane-like matrix, with increases in laminin (Farrar and Carson, 1992) and collagen type IV (Wewer et al., 1986). This process is mediated in part by the phagocytosis of collagen type I by stromal cells (Zorn et al., 1986). The reported expression of CL in normal day 7.5 decidua is most likely critical to this phagocytic process. Disruptions of the matrix remodeling process by inhibiting cysteine proteinase activity could prevent normal expansion and architectural changes of the decidual tissue. This possibility was supported by the observed decrease in thickness of the PDZ and lateral decidualizing zone in treated capsules, as well as the lack of lateral sinus development.

It is clear that the events surrounding implantation and development are dependent on the regulation of metalloproteinases and their inhibitors (Apte et al., 1994; Alexander et al., 1996). The work presented here provides strong evidence that the cysteine proteinases CB and CL, and their inhibitor cystatin C, also play an important role in these processes. Future directions of study include determining the regulation of the proteolytic cascade, including the interactions between multiple families of enzymes and inhibitors, to provide an understanding of the process of embryo implantation.

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REFERENCES


Cystatin and cathepsins in mouse development


