Expression of extracellular matrix-degrading metalloproteinases and metalloproteinase inhibitors is developmentally regulated during endoderm differentiation of embryonal carcinoma cells

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Summary
The differentiation of F9 and PSA-1 embryonal carcinoma cells to embryoid bodies composed of a mixture of parietal and visceral endoderm was accompanied by changes in their secretion of metalloproteinases. Differentiation was induced by retinoic acid and dibutyryl cyclic AMP (for F9 cells) or by removing cells from a substrate of feeder cells to alter cell–cell interaction and adhesion (for PSA-1 cells). The embryoid bodies attached to gelatin-coated dishes, and the parietal endoderm cells spread out over the matrix. The differentiated cells secreted specific gelatin- and casein-degrading proteinases, including enzymes that comigrated with proenzyme forms of collagenase and stromelysin. Total proteinase activity as well as specific collagenase activity increased with the time of differentiation. All of the gelatin- and casein-degrading proteinases detectable by substrate gel zymography were inhibited by inhibitors of metalloproteinases but not by inhibitors of serine or cysteine proteinases, indicating that they were metalloproteinases. Both cell lines showed increased collagenolytic activity, which was activated by treatment with plasmin. In addition, both cell lines showed increased secretion of specific metalloproteinase inhibitors, including tissue inhibitor of metalloproteinases, with differentiation. Analysis of mRNA from undifferentiated and differentiated F9 cells by RNA blot analysis or reverse transcription coupled with the polymerase chain reaction showed that increased expression of genes for collagenase, stromelysin and tissue inhibitor of metalloproteinases is associated with differentiation of these cells. These results suggest that the expression of extracellular matrix-degrading metalloproteinases and their inhibitors is developmentally regulated during the differentiation and spreading of the parietal endoderm.

Key words: embryonal carcinoma; endoderm; proteinase; extracellular matrix.

Introduction
Development is characterized by the changing interactions of cells with their extracellular matrix (ECM) and by accompanying changes in cellular phenotype and gene expression. The preimplantation mouse embryo begins to synthesize and secrete components of the ECM during the 4-cell stage. By the time the blastocyst forms, there is ECM between all of the cells and newly differentiated tissue layers, as well as on the surface of the mural trophoderm facing the blastocyst cavity (Adamson, 1982). This suggests that all growth and cell movement after the 4-cell stage requires remodeling of the ECM. For example, at about 5.5 days of gestation the cells on the blastocoel surface of the inner cell mass (ICM) differentiate to form primitive endoderm; this tissue subsequently gives rise to the visceral endoderm, which continues to cover the ICM, and the parietal endoderm, which migrates out of the ICM and spreads over the blastocoelic surface of the mural trophoderm. Because the inner surface of the mural trophoderm is coated with ECM composed of collagen, laminin and fibronectin (Adamson, 1982), the outgrowth of parietal endoderm cells must require interaction with and remodeling of that matrix.

It has been shown that ECM remodeling often involves the activity of specific proteolytic enzymes of the metalloproteinase family, such as collagenase and stromelysin (reviewed in Werb, 1989; Alexander and Werb, 1989). These enzymes are expressed during early mouse development, with a large increase in expression at the blastocyst stage (Brenner et al. 1989). However,
owing to the small size of the embryo, it is often difficult to use whole embryos to determine the specific cell or tissue that is secreting these enzymes or to demonstrate the specific role that the enzymes play in cell migration or differentiation. Nor is it possible to obtain sufficient material from embryos to fully characterize these activities. This problem has been solved with the use of embryonal carcinoma (EC) stem cells, which not only provide an unlimited supply of embryonic material but can also be induced to differentiate in culture. The cell lines PSA-1 and F9 have been studied extensively because of their biochemical and morphological resemblance to the ICM and because of their ability to differentiate and form parietal endoderm in vivo (Martin and Evans, 1975; Strickland et al. 1980; Hogan et al. 1981; Grover and Adamson, 1986; Casanova and Grabel, 1988; Edwards et al. 1988; Pecorino et al. 1988; Dahl and Grabel, 1989). In the present study, these cell lines were used to investigate the expression of ECM-degrading proteinases and their inhibitors during the differentiation of parietal endoderm. The results indicate that these proteins are developmentally regulated during EC cell differentiation.

Materials and methods

Cell lines, culture, and differentiation

The following cell lines were used: STO, an embryonic fibroblast line derived from a SIM mouse (Martin et al. 1977; obtained from Gail Martin, University of California, San Francisco); PSA-1, an ICM-like cell line (Martin et al. 1977; obtained from Gail Martin); and F9, another ICM-like cell line (Strickland and Mahdavi, 1978; obtained from Eileen Adamson, La Jolla Cancer Institute, La Jolla, CA).

PSA-1 cells were maintained on X-ray-inactivated feeder layers of STO cells cultured in Dulbecco's modified Eagle's medium (DME) containing 10% calf serum. Confluent cultures of PSA-1 cells growing on STO feeder layers were dissociated in trypsin, then suspended in 50 ml DME containing calf serum. These suspensions were incubated 3 times for 15 min each time in T150 culture flasks (Falcon) at 37°C to remove the more rapidly adhering STO cells. The PSA-1 cells were then plated in 100 mm tissue culture dishes (Costar) at a density of 1 x 10⁵ cells/dish and incubated for 3 days, by which time they had formed small clusters of attached cells called embryoid bodies. The embryoid bodies were removed from the culture dishes by gentle pipetting. Embryoid bodies from three dishes were pooled, suspended in 50 ml fresh DME containing calf serum, transferred to 150 mm bacteriological dishes, and grown in suspension for up to an additional 21 days. The culture days are expressed as the total number of days off feeder layers.

F9 cells were maintained in DME containing 10% fetal bovine serum. For differentiation, F9 cells were plated in 100 mm culture dishes at a density of 1 x 10⁵ cells/dish in a 1:1 mixture of Ham's F12 and DME containing 10% fetal bovine serum and 1 mM retinoic acid (Sigma). The cells were fed every 2 days, and after 7 days the cells were removed from the dishes by gentle pipetting, resuspended in 50 ml of the same medium plus 0.5 mM dibutyryl cyclic AMP (db-cAMP, Sigma) and 1.0 mM isobutylmethylxanthine (Sigma), then transferred to 150 mm bacteriological dishes and grown in suspension for up to 4 days.

To isolate parietal endoderm cells for analysis of proteinase expression, outgrowth of parietal endoderm-like cells was stimulated by transferring the suspension cultures of either PSA-1 or F9 onto gelatin-coated tissue culture dishes. Dishes were prepared by briefly coating them with 0.3% gelatin and washing with sterile H₂O. Cells or embryoid bodies from one 150 mm dish were suspended in 20 ml of the appropriate culture medium and distributed equally into two gelatin-coated dishes. Cells and embryoid bodies were incubated for 24 h in medium containing serum to allow them to attach. For analysis of secreted proteinases, cells were transferred to DME containing 0.2% lactalbumin hydrolysate (DME-LH) for 48 h before collection of cell-conditioned medium. Rabbit synovial fibroblasts were cultured and treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) in DME-LH as described previously (Unemori and Werb, 1988).

Detection of proteinases and proteinase inhibitors

Zymography was used to detect the secretion of proteinases and inhibitors by cultured cells as described previously (Herron et al. 1986a,b; Brenner et al. 1989). Protein concentration in all conditioned medium samples was determined by the Bio-Rad protein assay (Bradford, 1976; Grover and Adamson, 1986; Casanova and Grabel, 1989). In the present study, these cell lines were used to investigate the expression of ECM-degrading proteinases and their inhibitors during the differentiation of parietal endoderm. The results indicate that these proteins are developmentally regulated during EC cell differentiation.

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Differentiation induces proteinases

The cDNA probes used in this study were obtained from the following sources: rabbit collagenase, pCLl (Frisch et al. 1987); rabbit stromelysin, pSL2 (Frisch et al. 1987); mouse tissue-type plasminogen activator (tPA), gift of R. Rickles, SUNY-Stony Brook (Rickles et al. 1987); chicken actin, PA1, gift of M. Kirschner, UCSF (Cleveland et al. 1980); rat transin-1, pTR1, gift of R. Breathnach, University of Strasbourg (Matrisian et al. 1985); human tissue-type plasminogen activator (tPA), gift of R. Rickles, SUNY-Stony Brook (Rickles et al. 1987); mouse tissue-type plasminogen activator (tPA), gift of R. Rickles, SUNY-Stony Brook (Rickles et al. 1987); human 68 × 10^3 MT gelatinase/type IV collagenase, gift of G. Goldberg, Washington University, St Louis (Wilhelm et al. 1987); human 68 × 10^3 MT gelatinase/type IV collagenase, gift of G. Goldberg, Washington University, St Louis (Wilhelm et al. 1987); mouse TIMP, gift of B. Williams, University of Toronto (Gewert et al. 1987), a2 (IV) collagen, gift of M. Kurkinen, UMDNJ/Robert Wood Johnson Medical Center (Saus et al. 1989; Kurkinen et al. 1983). A mouse stromelysin cDNA (Ostrowski et al. 1988) identical to embryonic mouse stromelysin (EMS-2) (Brenner et al. 1989) was a gift of Dr L. Matrisian, Vanderbilt University, Nashville. For mouse collagenase cDNA, amplified fragments generated by reverse transcription followed by the polymerase chain reaction (RT-PCR) from F9 or PSA-1 RNA were purified by agarose gel electrophoresis as described previously (Brenner et al. 1989).

RNA analysis
RNA isolated from F9 and PSA-1 cells cultured as described for proteinase assays was separated and run on denaturing agarose gels, transferred to nylon membranes by capillary action (Thomas, 1980; Reed and Mann, 1985), and then UV cross-linked (Lehrach et al. 1977; Church and Gilbert, 1984). The RNA was then hybridized with inserts from cDNA clones or PCR-amplified cDNA (Brenner et al. 1989) radiolabeled with 32P-dCTP by nick translation (Maniatis et al. 1982). RT-PCR analysis of mRNA was performed as described previously (Rappolee et al. 1988a,b, 1989), with stromelysin oligonucleotide primers (Brenner et al. 1989) and glyceraldehyde-3-phosphate dehydrogenase primers (5' primer: 5'TGATGAACATCAAGAGGTGGTGAAG-3'; 3' primer: 5'TCCTTGGAGGCCATGTAGGCCAT-3') designed from the rat sequence (Fort et al. 1985) to give an amplified cDNA fragment of 240 bp.

Results

EC cell differentiation is associated with secretion of gelatin- and casein-degrading proteinases
PSA-1 cells remain undifferentiated as long as they are cultured on STO feeder cells. Once removed from the feeder layers they form small aggregates of ICM-like cells with some primitive endoderm-like cells on the outside (Martin et al. 1977). When these colonies are then grown in suspension culture, they form hollow balls of cells, known as embryoid bodies, that are composed of a core of undifferentiated stem cells surrounded by a mixture of differentiated visceral and parietal endoderm (Martin and Evans, 1975). When grown on gelatin-coated surfaces, the embryoid bodies attach, and the parietal endoderm cells grow out onto the matrix (Grabel and Watts, 1987).

PSA-1 cells were differentiated for up to 24 days and the embryoid bodies were grown on gelatin, then cultured in DME-LH for 48 h. The resulting conditioned medium was then assayed for proteinase activity. Zymograms from gelatin-SDS gels showed that both undifferentiated and differentiated cells secreted three major gelatinases migrating at 68, 85, and 96 × 10^3 Mf (Fig. 1A). The STO feeder cells also produced copious amounts of the 68, 85, and 96 × 10^3 Mf gelatinases, and contamination from these cells may

![Fig. 1. (A) Gelatin- and (B) casein-degrading proteinases secreted by PSA-1 cells during differentiation. Undifferentiated PSA-1 cells and embryoid bodies from cells cultured for 0–24 days off feeder layers (days in culture) were incubated for 48 h on gelatin-coated dishes in DME-LH. Samples of conditioned medium containing 9.0 μg protein were then separated on substrate gels. Molecular weight markers (×10^-3) are shown on the left in A and on the right in B. Bands comigrating with procollagenase (A) and prostromelysin (B) are marked by arrows. The zymograms are shown as negative images.](image-url)
A Gelatin

F9-RA + db-cAMP

1 2 3 4

days

B Casein

Fig. 2. (A) Gelatin- and (B) casein-degrading proteinases secreted by F9 cells during differentiation. Undifferentiated F9 cells, embryoid bodies from F9 cells treated with retinoic acid (F9-RA), and embryoid bodies from F9 cells treated with retinoic acid followed by 1–4 days with db-cAMP were incubated for 48 h on gelatin-coated dishes in DME-LH. Samples of conditioned medium containing 7.5 μg protein were then separated on substrate gels. Molecular weight markers (×10^3) are shown on the left in A and on the right in B. Bands comigrating with procollagenase (A) and prostromelysin (B) are marked by arrows. The zymograms are shown as negative images.

have accounted, in part, for the enzymes seen on day 0 and day 3 of cells off feeders. However, in mixing experiments that included contaminating STO cells, proteinases from these cells did not contribute substantially at days 0 and 3 and had no detectable contribution after day 3. The undifferentiated cells also secreted a 37 × 10^3 Mr gelatinase that was initially lost on differentiation and then reexpressed by day 12. After 12 days of differentiation, new gelatin-degrading proteinases appeared at 51, 53, and 100 × 10^3 Mr. The activity of most of the gelatinases increased with differentiation through 24 days (Fig. 1A). Casein gels also showed constitutive and differentiation-induced proteinase activity (Fig. 1B). The undifferentiated cells showed several caseinase bands above 75 × 10^3 Mr. At 3 days of differentiation new bands appeared at 51, 53, 72 and 100 × 10^3 Mr. With further differentiation all of the new bands showed increased activity. In general, the casein gels and gelatin gels showed distinct enzymes.

F9 cells differentiate to form primitive endoderm on the surface of embryoid bodies when they are treated with retinoic acid. When the embryoid bodies were treated with 0.5 mM db-cAMP, they further differentiated to form a mixture of parietal and visceral endoderm (Strickland and Mahdavi, 1978; Strickland et al. 1980; Hogan et al. 1981; Grover and Adamson, 1986). Culture of these embryoid bodies on gelatin allowed the parietal endoderm cells to spread out onto the matrix. The embryoid bodies from differentiated and undifferentiated F9 cells were incubated for 48 h in DME-LH, and the conditioned medium was analyzed by zymography. With differentiation there was a large increase in the secretion of specific gelatinases, particularly at 51, 92, 96, and 150 × 10^3 Mr, and of a caseinase at 52 × 10^3 Mr (Fig. 2A,B).

ECM-degrading proteinases secreted by EC cells are metalloproteinases

To characterize these proteinases, the proteins secreted by the undifferentiated and differentiated cells were separated on polyacrylamide gels containing gelatin or casein, and the gels were incubated for 24 or 48 h with various enzyme inhibitors. As positive controls, the metalloproteinases secreted by TPA-treated rabbit synovial fibroblasts were also separated on these gels. PMSF and leupeptin, inhibitors of serine proteinases, had no effect on the gelatinase or caseinase activity in any of the conditioned medium analyzed; however, TIMP, 1,10-phenanthroline and EDTA, which are potent inhibitors of metalloproteinases, blocked all of the proteolytic activity seen by zymography (Fig. 3, Table 1), as shown previously (Brenner et al. 1989). At the concentration used, all the enzymes were inhibited in parallel by the metalloproteinase inhibitors. The
Differentiation induces proteinases

Latent and active collagenase activity increases with EC cell differentiation

Because most major metalloproteinases are secreted as zymogens (Werb, 1989), the effect of proteolytic activation on the proteinases secreted by PSA-1 and F9 cells was studied by zymography. There was a significant increase in both gelatin- and casein-degrading activity in samples treated with plasmin. In addition, the intensity of the 51 and 53 x 10^3 Mr gelatinase bands that migrated with rabbit procollagenase decreased after plasmin treatment, and two bands of greater gelatinase activity appeared at 41 and 43 x 10^3 Mr. Similarly, the 52 x 10^3 Mr proteinase comigrating with rabbit stromelysin seen on the casein gels disappeared with plasmin treatment, and a new band appeared at 42 x 10^3 Mr (data not shown).

The specific collagenase activity present in these samples was then analyzed by the collagen fibril assay. The secreted proteins from PSA-1 and F9 cells that had differentiated for various times were assayed directly to measure active collagenase, or after activation of the proenzyme with plasmin to determine total active and latent enzyme. A small amount of active collagenase (10 mU mg^-1 protein) was secreted by undifferentiated PSA-1 cells, and the collagenase activity increased dramatically with differentiation after 9 days off feeder layers, reaching a maximum of 190 mU mg^-1 at 18 days (Fig. 4A). Activation with plasmin increased the measurable collagenase activity in conditioned medium from undifferentiated PSA-1 cells 6-fold to 61 mU mg^-1, and from 18-day differentiated PSA-1 cells 2-fold to 360 mU mg^-1 (Fig. 4A). The appearance of collagenolytic activity paralleled the appearance of the 51 and 53 x 10^3 Mr gelatinase bands on the zymograms.

The F9 cells had a similar pattern of collagenase expression. The unactivated secreted proteins from undifferentiated F9 cells had 16 mU of collagenase per mg, which increased to 57 mU mg^-1 with activation. With differentiation the collagenolytic activity increased rapidly, reaching 180 mU mg^-1 without activation and 270 mU mg^-1 with activation (Fig. 4B). These data suggest that collagenase secretion increases with differentiation of EC cells. Although the majority of the collagenase activity was found in an activatable proenzyme form, a significant amount of active enzyme was present in the serum-free conditioned medium.

Differentiating EC cells secrete inhibitors of metalloproteinases

Many cells and tissues that secrete metalloproteinases also secrete inhibitors of these enzymes, such as TIMP or other inhibitors of metalloproteinases (such as TIMP-2), which are members of the same gene family (Herron et al. 1986a; Werb, 1989; Stetler-Stevenson et al. 1989; Apodaca et al. 1990). To determine if EC cells also secrete these inhibitors, secreted proteins from undifferentiated and differentiated PSA-1 and F9 cells were analyzed for metalloproteinase inhibitor activity by an adaptation of the substrate gel procedure (Herron et al. 1986a). The PSA-1 cells showed a band at 29 x 10^3 Mr, comigrating with human TIMP, that ethanol and dimethyl sulfoxide solvents of the inhibitors had no appreciable effect on the proteolytic activity.

**Table 1. Effect of inhibitors on gelatin- and casein-degrading proteinases**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>RSF</th>
<th>PSA-1 (relative expression)*</th>
<th>F9</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>4+</td>
<td>3+</td>
<td>3+</td>
</tr>
<tr>
<td>DMSO</td>
<td>1%</td>
<td>3+</td>
<td>2+</td>
<td>2+</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1%</td>
<td>4+</td>
<td>2+</td>
<td>2+</td>
</tr>
<tr>
<td>1,10-phenanthroline</td>
<td>4 mM</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EDTA</td>
<td>4 mM</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PMSF</td>
<td>5 mM</td>
<td>4+</td>
<td>2+</td>
<td>2+</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>10 µg ml^-1</td>
<td>4+</td>
<td>3+</td>
<td>3+</td>
</tr>
<tr>
<td>TIMP</td>
<td>10 µg ml^-1</td>
<td>-</td>
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</tbody>
</table>

*Expression refers to appearance of gelatin- and casein-degrading proteinases on SDS-substrate gels. All of the bands seen in Figs 1 and 3 were inhibited by 1,10-phenanthroline, EDTA and TIMP. -, no activity; 2+, moderate activity; 3+, high activity; 4+, very high activity. RSF, rabbit synovial fibroblasts; DMSO, dimethyl sulfoxide.
Metalloproteinase and TIMP gene expression is regulated during EC cell differentiation

RNA from undifferentiated and differentiated F9 cells was then analyzed by RNA blot analysis (Fig. 6A). The 2 kb collagenase mRNA band seen by hybridization with mouse collagenase cDNA at high stringency was developmentally regulated. In several experiments this transcript appeared highest at 2 days of endoderm differentiation, decreasing thereafter. This mRNA had a different time course of induction than the 51 and 53 x 10⁵ Mᵣ gelatinases. Thus, it is possible that the enzymes seen by zymography are additional enzymes. A 1 kb TIMP mRNA band also increased with differentiation. Differentiation was shown by the induction of mRNA for tPA and type IV collagen, as described previously (Kurkinen et al. 1983; Rickles et al. 1988). In contrast, there was no increase with differentiation in actin mRNA (Fig. 6A) or in the mRNA for the 68 x 10⁵ Mᵣ gelatinase/type IV collagenase, which appeared to be secreted constitutively (data not shown). The expression of mRNA for major excreted protein (cathepsin L), a cysteine proteinase, increased only after treatment of cells with retinoic acid, suggesting that its increased expression may be selective for visceral endoderm. Similar evidence for regulation of these mRNAs with differentiation was obtained with PSA-1 cells (data not shown).

Although an mRNA band hybridizing with a human stromelysin cDNA probe at low stringency recognized a 2 kb mRNA in F9 and PSA-1 cells (Brenner et al. 1989), this washed off at high stringency. cDNA probes for rat and mouse stromelysin hybridized poorly to 10 μg of total RNA from PSA-1 or F9 cells, indicating that stromelysin mRNA was not abundant. Therefore, RTPCR was used to analyze differentiating F9 cells for stromelysin mRNA. As shown in Fig. 6B, stromelysin mRNA increased with differentiation of F9 cells. The appearance of this mRNA paralleled the appearance of the 52 x 10⁵ Mᵣ caseinolytic proteinase by zymography.

Discussion

In previous studies, it was shown that metalloproteinases and their inhibitors are expressed during mouse embryogenesis and by certain EC cell types (Brenner et al. 1989). In the present study, PSA-1 and F9 cells were used to determine if the expression of ECM-degrading metalloproteinases and their inhibitors during parietal endoderm differentiation and spreading on gelatin-coated surfaces is developmentally regulated. The results showed that differentiation of parietal endoderm is associated with an increase in the secretion of specific gelatin- and casein-degrading proteins, which can be detected by zymography. Two of these proteinases comigrated with the metalloproteinases collagenase and stromelysin. Collagenase activity also increased with differentiation, particularly after day 15, as well as a 20 x 10⁵ Mᵣ band (Fig. 5A). A weak inhibitor at 18 x 10³ Mᵣ was also seen, suggesting that EC cells produce additional metalloproteinase inhibitors, as has been found for other cells (Apodaca et al. 1990). The F9 cells also secreted metalloproteinase inhibitors that increased with differentiation. The largest increase occurred after day 1 in db-cAMP, with smaller increments to day 4. However, the major inhibitor in F9 cells was a band at 21 x 10³ Mᵣ, comigrating with human TIMP-2 (Fig. 5B). In some experiments a weak 29 x 10³ Mᵣ band was also seen. These data suggest that TIMP is the major inhibitor produced by PSA-1 cells, whereas TIMP-2 is the major inhibitor of F9 cells. The dark bands migrating above the inhibitors on both of these gels were also visible on conventional SDS-
significantly with differentiation. Expression of these proteins was mirrored by expression of mRNA: Both RNA blot analysis and RT-PCR showed the presence of collagenase and stromelysin mRNA in F9 and PSA-1 cells (Brenner et al. 1989), with an increase in the expression of these mRNA species during differentiation. Thus, the expression of these metalloproteinases is developmentally regulated during parietal endoderm differentiation. Inhibitor studies showed that all of the proteinases detected by zymography were metalloproteinases. Together, these metalloproteinases can degrade the basement membrane collagens, glycoproteins and proteoglycans, and the interstitial ECM, producing the remodeling necessary for changes in ECM-cell adhesion, migration and embryonic expansion as the fetal membranes containing parietal endoderm differentiate and grow. The correlation between collagenase activity, bands comigrating with authentic collagenase on zymograms, and collagenase mRNA levels was not strong. Because TIMP and other metalloproteinase inhibitors were also expressed by the EC cells, this would have effects on the measurable activity in biochemical assays (Herron et al. 1986a, b). Additionally, collagenase activity is also increased about 10-fold by treatment with stromelysin (Alexander and Werb, 1989). Thus, the relative expression of these two proteinases could modify the catalytic activity of collagenase. Similarly, the other gelatinases may facilitate collagen peptide solubilization in the quantitative assay. A third possibility includes post-transcriptional regulation of metalloproteinase expression. This mechanism has been observed for collagenase (Werb, unpublished results). Finally, the comigrating bands seen on zymograms may be due to uncharacterized metalloproteinases. The appearance of a major caseinolytic band at \(52 \times 10^3\) Mr did not correlate strongly with very low amounts of stromelysin mRNA. The strong hybridization of F9 RNA with a rabbit stromelysin probe at low stringency further points to this possibility.

In addition to proteolytic activity, PSA-1 and F9 cells also showed increased secretion of specific metalloproteinase inhibitors with differentiation. However, PSA-1 cells secreted inhibitor activity comigrating with human TIMP, whereas the major inhibitor activity of F9 cells comigrated with human TIMP-2 (Stetler-Stevenson et al. 1989). In both F9 and PSA-1 cells, mRNA for TIMP increased with differentiation, although the amounts were low. In recent studies using nuclease protection assays, Nomura et al. (1989) found TIMP mRNA in mouse fetal membranes, including parietal endoderm. These inhibitors may regulate the amount of degradation of Reichert's membrane so that net accumu-
Fig. 6. Analysis of specific mRNA transcripts expressed by F9 cells during differentiation. Cells were cultured as described in the legend to Fig. 2 and RNA was isolated and (A) 10 μg analyzed by RNA blot analysis or (B) 0.1 ng analyzed by RT-PCR analysis. GL, 68 × 10^3 M, gelatinase; CL, collagenase; MEP, major excreted protein (cathepsin L); type IV, type IV collagen; SL, stromelysin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. The RT-PCR amplification products in B are shown as negative images.

...lation takes place while the ECM is remodeled to allow growth.

Previous studies have shown that the differentiation of parietal endoderm from either F9 or PSA-1 cells requires specific cell–cell and cell–ECM interactions and may be mediated by cell shape (Hogan et al. 1981; Grabel and Watts, 1987; Casanova and Grabel, 1988; Dahl and Grabel, 1989). F9 differentiation is also accompanied by the development of a fibrillar fibronectin matrix (Dahl and Grabel, 1989). All of these types of interactions have been implicated in the stimulation of metalloproteinase gene expression by fibroblasts and endothelial cells (Aggeler et al. 1984b; Unemori and Werb, 1986; Werb, 1989; Werb et al. 1989). Furthermore, F9 cell differentiation is associated with the expression of tPA (Pecorino et al. 1988; Sabbag et al. 1989) and c-fos (Edwards et al. 1988), both of which have been shown to be associated with metalloproteinase expression in other systems (Mignatti et al. 1986; Kerr et al. 1988).

The present study has demonstrated that mouse EC cell metalloproteinases are developmentally regulated and produced, in part, in enzymatically active form, and that the latent zymogens can be activated by plasmin. It is possible that tPA expression by these cells may be directly related to the plasminogen-dependent activation of the observed metalloproteinases. The abundant expression of cathepsin L by these cells also provides a potential mechanism for plasminogen-independent activation of the metalloproteinases (Werb, 1989).

Finally, it is encouraging that similar results were observed for parietal endoderm differentiation in both F9 and PSA-1 cells. Because PSA-1 cell differentiation is stimulated by a change in cell–cell interactions and cell shape, whereas F9 differentiation requires stimulation by retinoic acid and db-cAMP, the results indicate that the metalloproteinase and inhibitor gene expression and secretion observed were characteristic of the parietal endoderm phenotype and not the type of stimulation. These data suggest that the signals required for differentiation regulate expression of the metalloproteinase genes in development. These signals, including retinoid–receptor binding and modulation of cAMP-mediated second messenger pathways, as well as pathways induced by changes in cell–cell and cell–ECM interaction, have all been elucidated in fibroblast cells in vitro (Unemori and Werb, 1986, 1988; Kerr et al. 1988; Werb et al. 1989; Werb, 1989). Our results may be applicable to an understanding of normal development in vivo.
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References


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