Transforming Growth Factor β1 Regulation of Metalloproteinase Production in Cultured Human Cervical Epithelial Cells

Chapla Agarwal, Joan R. Hembree, Ellen A. Rorke, and Richard L. Eckert

Departments of Physiology and Biophysics [C. A., J. R. H., A. E. R., R. L. E.], Biochemistry [J. R. H., R. L. E.], Reproductive Biology [A. E. R., L. E.], Dermatology [R. L. E.], and Environmental Health Sciences [A. E. R.], Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

ABSTRACT

Collagenase levels are regulated in a cell type-specific manner by a variety of growth factors and cytokines, and increased type IV collagenase activity in tumor cells has been linked to metastatic growth. In this study we compare the effects of epidermal growth factor (EGF) and transforming growth factor β1 (TGFβ1) on gelatinase production in cervical epithelial cell lines. EGF is a strong mitogen for cervical epithelial cells and TGFβ1 suppresses growth. Metalloproteinase zymograms of conditioned medium from normal human ectocervical cells reveal two major bands of metalloproteinase activity at 72 and 92 Kd. In contrast, the level of the 92-Kd activity is greatly reduced in the human papillomavirus type 16-positive ECE16-1 and CaSki cells. EGF treatment produces minimal changes in metalloproteinase levels. Treatment of CaSki cells with 20 ng/ml of EGF reduces by 30 to 50% the level of both activities. In ECE16-1 cells, EGF decreases the 72-Kd activity by 50% and the 92-Kd activity slightly. TGFβ1 treatment, in contrast, increases the 72-Kd activity 3- to 10-fold and the 92-Kd activity by ≥25-fold in each cell type. In CaSki and ECE16-1 cells, the changes in metalloproteinase level are mediated by changes in level of the corresponding mRNAs. In each case, the metalloproteinases are secreted as inactive proenzymes which can be activated by in vitro treatment with organomercurials. Tests of a series of additional cervical cell lines reveal that metalloproteinase levels are generally higher in normal cervical cells and in cells immortalized by transfection with HPV16, whereas lower levels are observed in cells derived from human tumors. Moreover, a higher percentage of cell lines derived from human tumors do not respond to TGFβ1 regulation of metalloproteinase levels. Parallel studies indicate that the TGFβ1-stimulated increase in the 72- and 92-Kd activities is correlated with enhanced chemotactic and chemoinvasive behavior in both ECE16-1 and CaSki cells.

INTRODUCTION

Tumor invasion can be defined as the active migration of neoplastic cells out of their tissue of origin and into adjacent tissues (1). During the transition from in situ to invasive carcinoma, tumor cells develop the ability to migrate through the basement membrane and enter the underlying interstitial stroma. It has been postulated that the successful invasive cell must have, among other properties, a high migratory ability and an ability to degrade connective tissue barriers. Evidence in favor of this hypothesis includes the observation that benign growths are surrounded by intact basement membranes, whereas invasive tumors lack an intact basement membrane (1). Type IV collagen is a major component of the basement membrane (2) and one way that invasive tumor cells can penetrate this structure is to acquire the ability to degrade type IV collagen. Collagenase activity in malignant tumor tissue is frequently higher than in the corresponding benign tissue (3-7). Recent studies have provided detailed characterizations of several of these enzymes (8) and their induction by oncogenes and transfected growth factors (8, 9).

EGF2 and TGFβ1 are important regulators of epithelial cell growth and metalloproteinase production and a loss of TGFβ1 responsiveness has been correlated with increased malignancy in a variety of cell types (10, 11). However, little is known of the role of these growth factors in regulating metalloproteinase production and activity in cervical epithelial cells. The majority of cervical cancers contain a high-risk HPV. The HPV integrates into the host genome, which is thought to be an initiating event leading to an immortal, but nontumorigenic phenotype (12). Poorly defined secondary events are required for malignancy to develop, a process to which growth factors may contribute. It is, therefore, important to elucidate the effects of growth factors on matrix metalloproteinase production and invasiveness in cervical epithelial cells.

In the present study we primarily utilize two human cervical cell lines, modeling two stages in malignancy, to study the effect of growth factors in metalloproteinase production. The ECE16-1 cells, which were immortalized by transfecting normal cervical epithelial cells with HPV16 (13), do not form tumors in nude mice or colonies on soft agar. CaSki cells, derived from a patient with cervical cancer, contain integrated HPV16 DNA and are fully transformed (14, 15). Our results reveal that TGFβ1 stimulates an increase in 72- and 92-Kd metalloproteinase in ECE16-1 and CaSki cells at the protein and RNA level. The growth factor-dependent increase in 72- and 92-Kd metalloproteinase activity was correlated with enhanced chemotactic and chemoinvasive behavior of these lines. Screening of a series of additional cervical cell lines revealed that TGFβ1 increased metalloproteinase levels in six of the nine cell types tested and that the absolute level of metalloproteinase production was not correlated with malignant potential.

MATERIALS AND METHODS

Cell Lines. Two cell lines were studied in detail in the present studies. ECE16-1 cells are immortalized by transfection with the molecularly cloned HPV16 genome (13). These cells are immortal as measured by continuous growth in vitro, but are not transformed as evidenced by their inability to form tumors in nude mice or grow on soft agar. CaSki cells are a transformed human cell line derived from a cervical epidermoid carcinoma present in a 40-year-old woman who also had metastatic tumors in the small bowel mesentery (16). Caski cells contain approximately 500 copies of integrated HPV16 DNA and are able to form tumors in nude mice. We also studied ECE16-D1 and ECE16-D2 cells which were immortalized by transfection with HPV16.5 Hela cells which contain HPV18 (18, 19), ME180 cells which contain an unknown HPV type (20), and HT3 cells and C33A cells which are HPV-negative, but contain p53 mutations (21, 22).

Cell Culture Conditions. Cell lines were routinely cultured in growth medium containing a mixture of Dulbecco’s modified Eagle’s medium:F12...
Detection of Collagenase Activity. Cells were seeded in regular growth medium and after a 24-h attachment period were shifted to DM. After incubation for 24 h in DM, treatment was initiated with fresh DM or DM supplemented with growth factors and cells were grown at a standard 3 days (time course) or 3 days (dose response). Cells were harvested with isotonic buffer containing 0.025% trypsin and 1 mM EDTA, fixed in isoton buffer containing 4% formaldehyde and counted using a Coulter counter. Conditioned medium was collected, cleared of debris by centrifugation at 1,500 × g for 8 min and stored at −20°C. Collagenase activity was detected by diluting conditioned medium 1:1 with 2× Laemmli sample buffer (without reducing agent) and incubating for 20 min at room temperature. Equal quantities of culture medium, normalized based on total cell number, were electrophoresed on nonreducing sodium dodecyl sulfate acrylamide gels containing 1% gelatin (23). After electrophoresis, the gels were washed twice with 30 min with 2.5% Triton X-100, rinsed three times for 5 min with 50 mM Tris-HCl (pH 8.0) containing 5 mM CaCl2 and 0.2% sodium azide, and incubated at 37°C in this buffer overnight. The gels were stained with 0.5% coomassie blue stain in acetic acid/isopropanol-water (1:3:6) and destained with water. Collagenase activity, detected as unstained regions in the gel, was quantified by densitometric scanning of the lanes.

Organomercurial activation of prometalloproteinases was achieved by incubating conditioned medium with 1 mM APMA for 30 min at 22°C, prior to adding 1 vol of reducing agent-free 2× Laemmli sample buffer and then processed for zymography (24). To study inhibition of metallocproteinase activity, samples were electrophoresed through gelatin-containing gels, washed twice with 30 min in 2.5% Triton X-100, rinsed three times for 5 min in 50 mM Tris-HCl, pH 8 containing 5 mM calcium chloride and 0.2% sodium azide, and incubated in this mixture in the presence or absence of 10 mM 1,10-phenanthroline (9) or 2 mM phenylmethyl sulfonyl fluoride for 12 h at 37°C. The gels were then stained with coomassie blue as described above.

Chemoinvasion Assay. The chemoinvasion assay was performed as described previously (25). Nucleopore polypyrrolidone-free, 8-μm pore size, polycarbonate filters (Costar, Cambridge, MA, #150046) were coated with 25 μg of matrigel (Collaborative Biomedical Products, Bedford, MA, #40234) in 100 μl of DM/18 mm2 of filter surface, dried, reconstituted, and placed in Boyden Chambers. Fibroblast-conditioned medium, obtained by incubating 3T3 cells for 24 h in DM, was placed in the lower compartment of the Boyden chamber as a chemoattractant. Cervical cell lines were grown in DM ± growth factor(s) as outlined above. They were then harvested with trypsin, washed three times with DM, and 2 × 105 cells in 0.2 ml of DM were added to the top compartment of the Boyden chamber. After an 8- to 9-h incubation at 37°C in a 5% CO2 incubator, the upper surface of the filter was wiped free of cells with a cotton swab (this removes cells that have not migrated through the matrix and polycarbonate filter), the filters were fixed in 10% formalin and stained with hematoxylin and eosin, and the number of cells that had migrated to the lower surface of the membrane was determined. Three to five 1-mm2 fields were counted using a hemocytometer grid on each of two filters per experiment and each experiment was repeated at least three times.

Chemotaxis Assay. Chemotaxis assays were performed as described above for the chemoinvasion assay (25) except that instead of matrigel, the filters were coated with 5 μg of type IV collagen (C-0543, Sigma Chemical Company, St. Louis, MO) in 100 μl of DM/18 mm2 (26, 27). The collagen was then dried, reconstituted, and washed in DM. Fibroblast-conditioned medium was included in the lower compartment of the Boyden chamber as a chemottractant. These experiments were performed in parallel with the chemoinvasion assays.

Detection of Metallocproteinase RNA. The isolation, electrophoresis, and blotting of RNA was performed as previously described (13, 28). RNA encoding the 92- and 72-Kd metalloproteinases was detected by hybridization with cDNAs, kindly provided by Dr. Stetler-Stevenson, encoding the 72- and 92-Kd metalloproteinases.

RESULTS
Identification of Metallocproteinases Produced by Cervical Epithelial Cells. To identify positively the collagenase activities present in normal cervical cells and cervical tumor cell lines, conditioned medium obtained from 3T3 mouse fibroblasts (A), human epidermal squamous cell carcinoma cell line SCC-13 (B), normal human epidermal keratinocytes (C), SV40 large T-antigen-immortalized human epidermal cell line KER-1 (28) (D), and normal human ectocervical epithelial cells (E) were compared to ECE16-1 cells (F) when grown in medium lacking EGF and TGFβ1 (Fig. 1). Normal human keratinocytes are known to express (29) the previously described 72- and 92-Kd metalloproteinase activities (8, 30). The results shown in Fig. 1 indicate that a variety of cells, including normal cervical epithelial cells (E), produce the classical 72- and 92-Kd metalloproteinases. However, an interesting feature is the reduction in the quantity of 92-Kd metalloproteinase observed in the HPV16-immortalized cervical cells (F).

Time-dependent Changes in Metallocproteinase Activity. The 72-Kd metalloproteinase is abundant in ECE16-1 and CaSk cells grown in DM without added growth factor (Fig. 2, Lanes 4). EGF addition (Lanes 1) results in slight decreases in the level of the 72-Kd activity by 72 h in both cell types. In contrast, treatment of ECE16-1 or CaSk cells with TGFβ1 alone (Lanes 2) or TGFβ1 + EGF (Lanes 3) produced a time-dependent increase (3- to 10-fold) in the level of the 72-Kd activity. The increase was optimal at 48 h in ECE16-1 and at 72 h in CaSk cells. Changes in level were observed as early as 24 h after addition of TGFβ1.

The 92-Kd activity was present at low levels in each cell type in the absence of growth factor stimulation (Lanes 4). Addition of EGF reduced this activity slightly in CaSk cells, with maximal suppression at 72 h (CaSk, Lanes 1). In ECE16-1 cells, EGF increased the 92-Kd activity at 24 and 48 h and slightly suppressed it at 72 h. TGFβ1 dramatically increased (10- to 25-fold) the 92-Kd activity at 48 h in

Fig. 1. Comparison of metallocproteinases secreted by ECE16-1 cells and other epithelial and fibroblast cell lines. Cells were grown in normal growth medium until 80% confluent, washed twice with MC-193 medium containing 2 mM L-glutamine, nonessential amino acids, 5 μg/ml of insulin, 1.8 × 10−4 M adenine, 10 mM hydrocortisone, 5 μg/ml of transferrin, 2 mM I−, 1.5 mM CaCl2, 100 μg/ml of penicillin, 100 μg/ml of streptomycin, and 50 μg/ml of gentamicin and then equilibrated overnight in this medium. Fresh medium was added and 24 h later the conditioned medium was harvested and assayed for collagenase activity. Conditioned medium, normalized based on cell number, from the last 24 h period was electrophoresed on a gelatin-imregnated nonreducing gel and processed for zymography. The 92- and 72-Kd gelatinases are indicated. Conditioned medium was obtained from 3T3 mouse fibroblasts (A), human epidermal squamous cell carcinoma cell line SCC-13 (B), normal human epidermal keratinocytes (C), SV40 large T-antigen-immortalized human epidermal cell line KER-1 (28, D), normal human ectocervical epithelial cells (E), and ECE16-1 cells (F). Similar results to those shown in Lane 1 were observed for normal cervical cells derived from four different subjects.
proteinase at 3 ng/ml of EGF. No further decrease was observed at 24, 48, and 72 h of treatment with or 20 ng/ml of EGF ~ 3 ng/ml of TGF/31 processed for zymography. The 92- and 72-Kd metalloproteinase activities are indicated.

TGFI31 for 3 days in DM in the presence or absence of 20 ng/ml of EGF and TGF/31 decreased the TGF/31 response in ECE16-1 cells and CaSki cells at 72 h.

Levels. The EGF effect can be better visualized in the context of a dose-response curve. Treatment of ECE16-1 or CaSki cells with 0-20 ng/ml of EGF for 3 days resulted in a reduction in metalloproteinase secretion as measured using gelatin zymography (Fig. 3). Densitometric scanning of the gels (not shown) revealed a 30% (CaSki) and 50% (ECE16-1) reduction in the level of the major 72-Kd metalloproteinase at 3 ng/ml of EGF. No further decrease was observed at higher EGF concentrations.

TGFB1 Regulation of ECE16-1 and CaSki Metalloproteinase Levels. The EGF effect can be better visualized in the context of a dose-response curve. Treatment of ECE16-1 or CaSki cells with 0-20 ng/ml of EGF for 3 days resulted in a reduction in metalloproteinase secretion as measured using gelatin zymography (Fig. 3). Densitometric scanning of the gels (not shown) revealed a 30% (CaSki) and 50% (ECE16-1) reduction in the level of the major 72-Kd metalloproteinase at 3 ng/ml of EGF. No further decrease was observed at higher EGF concentrations.

Relative Levels of Metalloproteinase Activity in Other Cervical Cell Lines. As described above, TGFB1 increases metalloproteinase levels and, in most cases, EGF reduces levels. To determine whether these responses are typical of human cervical cell types, we evaluated the effects of TGFB1 and EGF on additional cervical epithelial cell lines. Cells were seeded in regular growth medium and after a 24-h attachment period were shifted to DM. After a 24-h equilibration in DM, treatment was initiated with fresh DM or DM supplemented with growth factors. After 3 days, the culture medium from the last 24-h of treatment was harvested and assayed for metalloproteinase activity by zymography. In general, cell lines derived from human cervical tumors (CaSki, Hela, ME180, HT3, C33A) produce less 72- and 92-Kd metalloproteinases than immortalized, nontumorigenic cells (ECE16-1, ECE16-D1, ECE16-D2), or normal cells (Table 1). EGF suppressed and TGFB1 increased the 72- and 92-Kd activities.
Fig. 4. TGFβ1 increases metalloproteinase activity in a dose-dependent manner. ECE16-1 (A and B) or CaSki cells (C and D) were treated with zero (c) to 5 ng/ml of TGFβ1 in the absence (A and C) or presence (B and D) of 20 ng/ml of EGF (+EGF) for 72 h with daily media changes. In B and D, c indicates no growth factor treatment and 0 indicates treatment with 20 ng/ml of EGF but no TGFβ1. Conditioned medium, layered based on cell number, from the last 24-h period was collected, electrophoresed on gelatin-containing gels, and processed for zymography. The 92- and 72-kD gelatinase activities are indicated.

92-kD metalloproteinase levels in five of the cell types tested. All of these, with the exception of CaSki, were nontumorigenic. Cells derived from human tumors were less responsive to these agents (see “Discussion”).

EGF and TGFβ1 Enhance ECE16-1 and CaSki Cell Chemotaxis and Chemoinvasion. An estimate of the chemotactic and chemoinvasive properties of a particular cell type can be obtained by measuring the ability of the cells to migrate through collagen or matrigel, respectively (25). We therefore measured the ability of ECE16-1 and CaSki cells to migrate through films of collagen or matrigel and the effects of growth factors on the migration rate. As shown in Table 2, although EGF and TGFβ1 alone modestly enhanced ECE16-1 cell migration and invasion, the maximal increase (15.5-fold) was observed in cells simultaneously incubated with EGF and TGFβ1 (Table 2). For CaSki cells, maximal migration and invasion were observed when cells were treated with TGFβ1 alone, although EGF - TGFβ1 also heightened the responses compared to control (Table 1).

DISCUSSION

Normal Ectocervical Epithelial Cells Produce the Classical 92- and 72-kD Metalloproteinases. An aspect of cervical cancer progression that requires further study is regulation of metalloproteinase and metalloproteinase inhibitor production by growth factors (32–34). Metalloproteinases are a family of enzymes able to degrade extracel-

Fig. 5. Regulation of metalloproteinase RNA levels by EGF and TGFβ1. A and B. ECE16-1 or CaSki cells were grown for 48 h in DM (Lanes 1) or DM supplemented with 20 ng/ml of EGF (Lanes 2), 1 ng/ml of TGFβ1 (Lanes 3) or 20 ng/ml of EGF + 1 ng/ml of TGFβ1 (Lanes 4). Individual RNA blots were hybridized with cDNAs encoding the 72- or 92-kD metalloproteinases or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)(31). The level of GAPDH RNA is not regulated by the growth factors included in the present study, therefore, GAPDH RNA level was utilized as a control to ensure uniform loading of lanes. C shows the relative migration of the RNAs encoding the 72- and 92-kD metalloproteinases and GAPDH prepared from CaSki cells treated with 1 ng/ml of TGFβ1 for 48 h. The arrows indicate the position of the 72- and 92-kD metalloproteinase transcripts and the GAPDH transcript. The message sizes (72 kD, 3.4 kilobases; 92 kD, 2.8 kilobases; GAPDH, 1.4 kilobases) are consistent with those previously reported (8, 30, 31). Levels of expression cannot be compared between blots due to differences in film exposure time.
lular matrix proteins (1, 3, 6, 7, 35). As such, specific metalloproteinases have been implicated as having a role in enhancing the metastatic potential of cells, presumably because they provide the cell with the ability to degrade and penetrate the basement membrane (1, 3, 6-8, 36).

Two major collagenase activities have been identified in a variety of cell types. A 92-Kd type IV collagenase is expressed in SV40-transformed human lung fibroblasts (30), normal human macrophages (37, 38), and normal human keratinocytes (29) and a 72-Kd collagenase is expressed in Ha-ras-transformed human bronchial epithelial cells (8) and normal human epidermal keratinocytes (29). Our present studies indicate that normal cervical epithelial cells constitutively express the 72- and 92-Kd metalloproteinases. Similar results were observed for CaSki cells (not shown). The activities were completely inhibited by incubation of the zymograms with 10 ms 1,10-phenanthroline, but not by incubation with 2 ms phenylmethyl sulfonl fluoride (not shown).

Regulation of Metalloproteinase Levels by TGFß1 and EGF. Our initial studies indicated that EGF and TGFß1 modulate the level of metalloproteinases produced and secreted by ECE16–1 and CaSki cells. In general, EGF treatment reduced and TGFß1 treatment increased the level of metalloproteinase secreted by these cell types. However, the effect of EGF on ECE16–1 92-Kd metalloproteinase level was somewhat variable. The altered level of metalloproteinase produced could result from changes at any of several steps, including effects on RNA synthesis and/or degradation, RNA processing, protein translation, and/or protein secretion. To study the mechanism of EGF and TGFß1 modulation of metalloproteinase levels, we compared the level of mRNA encoding the 72- and 92-Kd activities with the level of secreted protease. Our results suggest a direct correlation between RNA level and the level of secreted protease. We have not yet determined whether the effect is at the level of mRNA synthesis and/or mRNA degradation.

Regulation of Metalloproteinase Levels in Other Cervical Cell Lines. To determine whether EGF and TGFß1 produce similar effects in other cervical epithelial cell lines, we monitored the level of metalloproteinase secreted by ECE16–1, ECE16-D1, ECE16-D2, CaSki, Hela, ME180, HT3, and C33A cells in response to treatment with each agent. In the nontumorigenic cell lines and in normal cells, EGF decreased and TGFß1 increased 72- and 92-Kd metalloproteinase levels. In the cell lines derived from human tumors, however, this regulation was not consistently observed, suggesting that responsiveness to these agents is diminished in the more malignant cells.

| Table 1 Effects of EGF and TGFß1 on metalloproteinase levels in a variety of cervical epithelial cell linesa |
|-----------------|-----------|-----------|-----------|-----------|
| Cell Line       | Phenotype | Basal Level | 72 Kd     | 92 Kd     |
|                 |           | EGF        | TGFß1     | EGF + TGFß1 |
| Normal          |           | Basal Level | 72 Kd     | 92 Kd     |
| ECE16–1         | HPV16     | +++ ++++++  | D         | D         |
| ECE16-D1        | HPV16     | +++ ++++++  | D         | D         |
| ECE16-D2        | HPV16     | +++ ++++++  | D         | D         |
| CaSki           | HPV16     | ++          | D         | D         |
| Hela            | HPV18     | +           | nc        | nc        |
| ME180           | HPV+      | +           | nc        | nc        |
| HT3             | p53m      | -           | +         | nc        |
| C33A            | p53m      | -           | -         | nc        |
| DM 1            |           | 30.4 ± 8.7 (1) | 4.1 ± 1.0 (1) |
| DM 2            |           | 11.3 ± 4.0 (0.37) | 8.9 ± 3.4 (2.2) |
| DM 3            |           | 158.5 ± 10.3 (5.52) | 77.2 ± 4.3 (19) |
| DM 4            |           | 59.8 ± 11.7 (2.0) | 31.2 ± 1.6 (7.7) |
| Treatment Collagen Matrigel |
| CaSki DM       | 30.4 ± 8.7 (1) | 4.1 ± 1.0 (1) |
| CaSki EGF      | 11.3 ± 4.0 (0.37) | 8.9 ± 3.4 (2.2) |
| CaSki TGFß1    | 158.5 ± 10.3 (5.52) | 77.2 ± 4.3 (19) |
| CaSki EGF + TGFß1 | 59.8 ± 11.7 (2.0) | 31.2 ± 1.6 (7.7) |

*All cell lines are derived from the human cervical tissue. ECE16–1, ECE16-D1, and ECE16-D2 were derived by transfection of normal human cervical epithelial cells with cloned HPV16 DNA as previously described (13). The other cell lines were derived from human tumors (14, 15, 16–22). Normal cells are normal cervical epithelial cell cultures. Phenotype lists the available information regarding the HPV subtype expressed in the cells. ME180 cells contain an unknown HPV type. HT3 and C33A lines lack HPV, but contain mutated p53 (21, 22). Basal expression is defined as the activity observed when the cells are maintained in DM (+, absence of activity; +, low level activity; + +, + ++, + +++ indicate increasing levels of activity). EGF and TGFß1 effects on activity are listed for the 72- and 92-Kd activities (D, decrease; I, increase; nc, no change).

Table 2 Migration of ECE16–1 and CaSki cells through collagen or matrigila

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Collagen</th>
<th>Matrigel</th>
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<tr>
<td>CaSki DM</td>
<td>30.4 ± 8.7 (1)</td>
<td>4.1 ± 1.0 (1)</td>
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<tr>
<td>CaSki EGF</td>
<td>11.3 ± 4.0 (0.37)</td>
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*ECE16–1 or CaSki cells were treated with DM or DM containing 20 ng/ml of EGF, 3 ng/ml of TGFß1, or 30 ng/ml of EGF + 3 ng/ml of TGFß1 for 72 h. The cells were then harvested and 2 X 10^6 CaSki cells or 1 X 10^6 ECE16–1 cells were plated per square centimeter onto 5-μm pore size polycarbonate filters coated with either type IV collagen (μg/filter) or matrigel (μg/filter). Migration studies were performed using 3T3-conditioned medium as the chemotactant. After 8 to 9 h, the filters were processed and cell number per square millimeter was determined and expressed as the mean ± SE of four separate experiments. The fold change, expressed relative to the DM control, is shown in parentheses. Fewer cells migrate through the matrigel than the collagen in the course of the assay. This is presumably due to fact that the cells can migrate through collagen, but must invade matrigel.

Fig. 6. Comparison of the level of metalloproteinase activity in ECE16–1 and CaSki cells. ECE16–1 (lanes 1) or CaSki (lanes 2) cells were grown for 72 h in DM or DM supplemented with 20 ng/ml of EGF, 3 ng/ml of TGFß1, or 20 ng/ml of EGF + 3 ng/ml of TGFß1. Fresh medium and growth factor was added daily and conditioned medium from the last 24 h was collected and assayed for collagenase activity. Conditioned medium, normalized based on cell number, from the last 24 h of treatment, were electrophoresed on a gelatin-containing gel and processed for zymography. The 92- and 72-Kd gelatinases are indicated.

Fig. 7. Metalloproteinases are released from ECE16–1 cells in an inactive form. ECE16–1 cells were plated into 9.6-cm² wells in growth medium and allowed to attach overnight. They were then washed, shifted to DM for 24 h, and grown in the presence of DM or DM containing 3 ng/ml of TGFß1 for 3 days. Fresh medium was added daily. Conditioned medium from the last 24 h of treatment was collected and incubated in the presence (+) or absence (−) of 1 ms APMA for 30 min at 22°C, prior to addition of 1 vol of reducing agent-free 2X Laemmli sample buffer (24). The samples were then electrophoresed on a gelatin-impregnated gel and processed for zymography. The arrow indicates the nonactivated 72-Kd metalloproteinase. Similar results were observed for CaSki cells (not shown). The activities were completely inhibited by incubation of the zymograms with 10 ms 1,10-phenanthroline, but not by incubation with 2 ms phenylmethyl sulfonl fluoride (not shown).
It is important to note that the various cervical lines display differing levels of the 72- and 92-Kd metalloproteinases and that the level of expression is not directly correlated with extent of malignancy or presence or absence of HPV. It is interesting that the two cell lines that possess p53 mutations and lack HPV, HT3, and C33A, produce low metalloproteinase levels. In general, it appears that cell lines developed by in vitro transfection with HPV16 (ECE16--1, ECE16-D1, ECE16-D2) express higher levels of each metalloproteinase than cell lines derived from human tumors (C33A, HT3, ME180, Hela, CaSkI) (Fig. 6; Table 1). Based on these results, we can hypothesize (a) that high level metalloproteinase expression is a hallmark of differentiated function of normal cervical cells that is progressively lost during malignant progression or (b) that selective pressure has acted on the cells derived from human tumors to diminish metalloproteinase expression. Assuming that the former hypothesis is correct, these results suggest that although type IV collagenases may contribute to metastatic potential in some cervical cell lines, other factors are likely to contribute to the net metastatic potential. Further studies need to be performed to clarify these possibilities, however, alterations in the pattern of expression are consistent with previously published results indicating that immortalization or malignant transformation alters metalloproteinase production in other systems (1, 3, 8, 30, 39–44).

**Metalloproteinases and Invasiveness.** We tested a limited number of cell lines in an in vitro assay of metastatic potential. As noted in Fig. 6 and Table 1, under all growth conditions tested, ECE16--1 cells produce higher levels of 92- and 92-Kd collagenase than CaSkI cells. Taken alone, this finding predicts that ECE16--1 cells should display a more invasive phenotype than CaSkI cells. However, when ECE16--1 and CaSkI cells are grown in DM supplemented with TGFβ1 (Table 2), CaSkI cells are much more efficient at penetrating matrix than ECE16--1 cells, suggesting that other factors, in addition to the 72- and 92-Kd metalloproteinase levels, are likely to be important in determining the invasiveness of cervical tumor cells. Indeed, a lack of correlation between type IV collagenase levels and metastatic potential has been reported in human lung cancer cells (42) and in oncogene-transformed rat cell lines in which stromelysin was found to be a better predictor of metastatic potential (45). Increased stromelysin expression has also been correlated with progression toward malignancy in chemically induced tumors in mouse skin (46, 47).

Metalloproteinases are secreted as proenzymes that require activation for activity (35, 44). The 72- and 92-Kd metalloproteinases released from ECE16--1, CaSkI, and normal cells are released as inactive proenzymes that can be converted to active forms in vitro using organonemurcular reagents (Fig. 7). Several endogenous activators and inhibitors of metalloproteinases have been identified (35, 48–52). It is possible that the net higher levels of 72- and 92-Kd metalloproteinase produced by ECE16--1 cells are countered by a lower production of activator(s) and/or a higher production of inhibitor(s) of type IV collagenolytic activity. As noted by Liotta and Stetler-Stevenson (44), potent levels of collagenolytic activity are produced by ECE16--1 cells in the presence of TGFβ1 and CaSkI cells are determined by the level of the corresponding mRNA; (d) EGF and TGFβ1 can work together in some cell lines to facilitate invasion; and (e) tumorigenic cervical cell lines produce less 72- and 92-Kd metalloproteinases than normal or nontumorigenic cell lines.

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