Stromelysin-1 Activation Correlates with Invasiveness in Squamous Cell Carcinoma[1]

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The expression of selected metalloproteinases and tissue inhibitors of metalloproteinases (TIMP) was examined in three squamous cell carcinoma (SCC) cell lines (FaDu, SiHa, A431) and a keratinocyte cell line (HaCaT) to determine which metalloproteinases function in SCC invasiveness. A Matrigel invasion assay was used to assess invasiveness of the cell lines. Only the FaDu cell line showed invasiveness in this assay, and invasion of Matrigel by FaDu cells was inhibited by treatment with the metalloproteinase inhibitor, batimastat. No correlation was found between mRNA expression for matrilysin, stromelysins 1–3, TIMP-1, or TIMP-3 and secretion of these proteins, indicating that the extracellular activity of these molecules is regulated post-transcriptionally. The SCC cell lines differed from the HaCaT line in that matrilysin and TIMP-1 proteins were detected in conditioned medium from all SCC cell lines, but not in medium from HaCaT cells. Only the invasive cell line, FaDu, released active stromelysin-1 into the culture medium. These results indicate that while matrilysin contributes to the invasive phenotype, activation of stromelysin-1 is a key regulatory step for invasiveness in SCC cells. ***Key words: batimastat/invasion/Matrigel/metalloproteinase/tumorigenesis. J Invest Dermatol 118:759–766, 2002***

In order to migrate into the stroma, invade other tissues, and metastasize, epithelioid tumor cells must digest and migrate through their basal lamina and then pass through the extracellular matrix of surrounding stromal tissue (Woodhouse *et al*, 1997). Proteolysis of the basal lamina and extracellular matrix results from the action of metalloproteinases produced by both tumor cells and surrounding stromal cells (Kleiner and Stetler-Stevenson, 1999). Gene transfer experiments have consistently shown that increased metalloproteinase activity is associated with increased invasiveness of tumor cells (reviewed in Knox *et al*, 1998), but metalloproteinases have also been shown to affect cell proliferation and tumorigenesis (Meade-Tollin *et al*, 1998; Boulay *et al*, 2001). Consequently, inhibitors of metalloproteinases are being tested for their therapeutic effects on tumor development, growth, invasiveness, and metastasis.

Batimastat is a general matrix-metalloproteinase inhibitor that suppresses extracellular matrix degradation *in vitro* and reduces the growth of solid tumors in mice (Rasmussen and McCann, 1997). Batimastat resembles metalloproteinase substrates and acts by competitive inhibition of metalloproteinase activity, but has no effect on metalloproteinase expression, cell viability, or cell proliferation (Chirivi *et al*, 1994; Sledge *et al*, 1995; Rasmussen and McCann, 1997). Batimastat treatment has been shown to significantly reduce invasiveness of malignant human glioma cells (Tonn *et al*, 1999) and neoplastic human prostate cells (Knox *et al*, 1998) in Matrigel invasion assays. Studies on mouse models have demonstrated that Batimastat also reduces the incidence of intestinal tumors (Heppner-Goss *et al*, 1998), inhibits metastasis of human breast cancer cells (Sledge *et al*, 1995), reduces tumor growth and enhances survival in mice bearing human ovarian tumor grafts (Davies *et al*, 1993), and prevents metastasis of human pancreatic cancer xenografts (Rasmussen and McCann, 1997). Research on prostate tumors and pancreatic xenografts indicates that metalloproteinase inhibition by batimastat allows tumor cells to reform a basal lamina, thereby limiting invasiveness (Rasmussen and McCann, 1997; Knox *et al*, 1998). The effects of batimastat on squamous cell carcinoma (SCC) have not been described.

Expression of the metalloproteinase matrilysin (MMP-7) is not found in epidermal or stromal cells in normal skin, but in skin cancer, matrilysin is expressed by tumor cells at the stromal interface in SCC, and in aggressive and recurrent morpheaform and infiltrative basal cell carcinomas (BCC). Nodulocystic, keratotic, and adenoid BCC, which are not invasive, do not express matrilysin (Karelina *et al*, 1994). Matrilysin is released from the cell as an inactive proenzyme that is cleaved to yield an active protein (Woessner, 1991). In addition, transformation of HaCaT – a nontumorigenic, noninvasive, keratinocyte cell line – with c-*ras*^{Ha} gives rise to tumorigenic cells with increased expression and secretion of promatrilysin (Meade-Tollin *et al*, 1998).

Another family of metalloproteinases, the stromelysins, promote tumor growth through different mechanisms. Stromelysin-1 (MMP-3) digests extracellular matrix proteins and cleaves promatrilysin, releasing active matrilysin (Imai *et al*, 1995; Tsukifuji *et al*, 1999). Like matrilysin, stromelysin-1 is not expressed by keratinocytes in normal skin, but is highly expressed in SCC, and is expressed by keratinocytes after wounding (Muller *et al*, 1991; Saarialho-Kere *et al*, 1994; Kusukawa *et al*, 1996; Tsukifuji *et al*, 1999). Stromelysin-2 (MMP-10) is synthesized by SCC cells, but is associated with inflammation and wound healing rather than invasiveness (Kerkelä *et al*, 2001; Madlener *et al*, 1996).

Manuscript received September 12, 2001; revised December 16, 2001; accepted for publication December 21, 2001.

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Abbreviations: BCC, basal cell carcinoma; SCC, squamous cell carcinoma; TBS, tris-buffered saline; TIMP, tissue inhibitor of metalloproteinase.

[1]The authors have declared not to have a conflict of interest.

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Stromelysin-3 (MMP-11) does not digest extracellular matrix. Instead of stimulating invasiveness through matrix degradation, stromelysin-3 inhibits cell death in malignant cells, thus promoting tumor growth (Noël *et al*, 2000; Boulay *et al*, 2001).

Metalloproteinase activity is inhibited by the tissue inhibitors of metalloproteinases (TIMP) (Kleiner and Stetler-Stevenson, 1999). TIMP proteins bind metalloproteinases with 1:1 stoichiometry and form noncovalent complexes (Umenishi *et al*, 1991; Gomis-Ruth *et al*, 1997). TIMP binding not only inhibits the activity of active metalloproteinases, but also suppresses conversion of pro-metalloproteinase molecules to their active forms (Umenishi *et al*, 1991). In normal skin, TIMP-3 and matrilysin exhibit the same patterns of expression, suggesting that TIMP-3 regulates matrilysin activity in skin (Airola *et al*, 1998).

This study was performed to determine whether Batimastat inhibits invasiveness in SCC cells and to determine which metalloproteinases regulate invasiveness in SCC cells.

MATERIALS AND METHODS

Cell culture Cell lines used in this study were HaCaT, an immortalized, nontumorigenic keratinocyte cell line (Boukamp *et al*, 1988, 1990); SiHa, a SCC from the cervix (ATCC HTB-35, American Type Culture Collection, Rockville, MD); FaDu, a SCC from the pharynx (ATCC HTB-43); and A431, a metastatic SCC from the epidermis (ATCC CRL-1555). Each cell line was maintained in D-MEM/F-12 medium (Gibco-BRL, Grand Island, NY) containing 3% fetal bovine serum (Gibco-BRL), 80 units penicillin per ml, and 80 μg streptomycin per ml (Gibco-BRL). Cultures were grown in a humidified incubator at 37°C, 5% CO_2 in air.

Matrigel invasion assay The invasion assay was adapted from the method described by Kawahara *et al* (1995). Chambers were prepared for invasion assays by coating porous (8 μm pores) tissue culture inserts (Falcon, Franklin Lakes, NJ) with 90 μg per cm^2 of Matrigel (Becton Dickinson Labware, Bedford, MA) suspended in phosphate-buffered saline (PBS; Sigma, St. Louis, MO). Inserts were placed in a 24 well plate containing medium with 10% fetal bovine serum, which stimulates cell migration. Cells were plated on the inserts at 5×10^4 cells per insert in serum-free medium. For control experiments, cells were plated in serum-free medium on inserts that were not coated with Matrigel. Cells were incubated for 24 h, then fixed and stained with Diff-Quick (Dade Behring, Newark, DE). Each assay was performed in triplicate. For each cell line the number of cells passing through uncoated and Matrigel-coated filters were counted. Cells on each insert were viewed with a Zeiss Axioscop microscope, and counted in 30 optical fields (2.5×2.5 mm grid) at 40× magnification. Statistical significance was determined using a one-tailed *t*-test for samples of unequal variance.

To assess the effects of Batimastat (BB-94, British Biotech Pharmaceuticals, Oxford, U.K.) on invasion of Matrigel, cells were plated on inserts in serum-free medium containing 0.1 or 1.0 μM Batismastat and incubated for 24 h before counting as described.

Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) Total RNA was extracted from cells using the Qiagen RNeasy kit (Valencia, CA). RNA 100 ng was amplified by RT-PCR, using the Titan kit (Roche Biochemicals, Indianapolis, IN). The RT-PCR program was as follows: one cycle of 50°C for 30 min, 90°C for 2 min; 15 cycles of 90°C for 1 min, 55°C for 30 min, 68°C for 1 min; 10 cycles of 90°C for 1 min, 58°C for 30 min, 68°C for 1 min; one cycle of 68°C for 4 min. Primers for human matrilysin were 5′-GAACAATTGTCTCTGGACGG and 3′-TTGGCTTCTACTGTT-GGC. Primers for human TIMP-1 were 5′-GACTCTTGCACATCAC-TAC and 3′-ACACTGTGCAGGCTTCAGT. Primers specific for human TIMP-3 were 5′-AATGACCCCTTGGCTCGG and 3′GGT-GTAGACCAGCGTGCC. Primers for human stromelysin-1 were 5′-AAGAGCAACGACGAGTACTT and 3′-AGGTCCTTGCTAGTAA-CTTCA. Human stromelysin-2 primers were 5′-AGCTAGACACT-GACACTCTG and 3′-TGCCTGATGCATCTTCTGTC. Human stromelysin-3 primers were 5′-TCGACTATGATGAGACCTG and 3′-CAGTCTGAGACAGTCGCTG. RT-PCR products were separated on a 2% SeaKem agarose gel (FMC, Rockland, ME), detected with Sybr Green I (FMC) and analyzed with a FluoroImager (Molecular Dynamics, Sunnyvale, CA). Semiquantitative evaluation of expression was performed by comparison with expression of the GADPH gene in the same sample. Primers for human GAPDH were 5′-ACAGTCCA-TGCCATCACTGCC and 3′-GCCTGCTTCACCACCTTCTTG.

Immunoblotting Levels of metalloproteinase and TIMP proteins were analyzed in conditioned medium by immunoblotting. For conditioned medium samples, each cell line was grown in serum-free medium for 48 h. The culture medium was then collected, concentrated 10-fold with Millipore Ultrafree-probind membrane filters (Millipore, Bedford, MA), and stored at –80°C. For each sample, 30 μg of protein was loaded onto a 4%–20% gradient SDS-polyacrylamide gel (FMC) in reducing buffer (Laemmli buffer, Bio-Rad Laboratories, Hercules, CA) and electrophoresed at 70 V. Colored molecular weight markers (Rainbow markers, Amersham-Pharmacia, Piscataway, NJ) were run on each gel. The proteins were transferred to Immobilon-P membrane (Millipore) for immunostaining. Membranes were blocked for 1 h at room temperature with 10% fat free milk in TBS (20 mM Tris, 150 mM NaCl, pH 7.4) containing 0.1% Tween-20, then incubated for 90 min with primary antibody diluted in TBS-0.1% Tween-20. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Amersham-Pharmacia) for 45 min. Antibody binding was detected using enhanced chemiluminescence (Amersham-Pharmacia).

Immunocytochemistry To synchronize the cell cultures for mitosis, all cell lines were treated with 4.0 μg aphidicolin per ml for 8 h. Medium was then replaced to wash out the aphidicolin and cells were fixed for immunocytochemistry at 2, 4, 8, 12, 16, and 24 h after removal of aphidicolin. For antibody labeling, cells were fixed with ice-cold 50% acetone in methanol, washed with PBS, washed twice with 5% bovine serum albumin in PBS, then incubated with primary antibodies at 4°C overnight. Samples were washed three times with 0.5% bovine serum albumin in PBS, then incubated with secondary antibodies for 1 h at room temperature. After washing, DNA was stained with 100 ng DAPI (4,6-diamidino-2-phenylindole) per ml (Sigma). DNA staining was used to identify mitotic cells.

All primary antibodies for immunoblotting and immunocytochemistry were mouse monoclonal antibodies obtained from Oncogene Research Products (Boston, MA). Specifically, these were IM40T, MMP-7 (Ab-1) specific for promatrilysin; IM47T, MMP-7 (Ab-2) specific for active matrilysin; IM69T, MMP-3 (Ab-4) specific for pro- and active stromelysin-1; IM75T, MMP-10 (Ab-2) specific for pro- and active stromelysin-2; IM86T, MMP-11 (Ab-2) specific for pro- and active MMP-11; IM68T, MMP-2 (Ab-8) specific for pro- and active gelatinase A; IM37T, MMP-9 (Ab-3) specific for pro- and active gelatinase B; IM63T, TIMP-1 (Ab-4); IM82T, TIMP-2 (Ab-4); IM43T, TIMP-3 (Ab-1). Secondary antibodies for immunocytochemistry were conjugated with Texas Red or FITC and obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

RESULTS

Invasion assays were performed to determine whether cell invasiveness correlates with the production of matrilysin or other metalloproteinases. Invasiveness was measured as the ability of cells to digest Matrigel. The four cell lines differed in ability to pass through uncoated filters, indicating inherent differences in cell mobility (**Fig 1*a***). The number of FaDu cells passing through uncoated filters was significantly ($p < 0.05$) greater than the number of HaCaT cells crossing uncoated filters. HaCaT and SiHa cell lines exhibited both poor cell mobility and Matrigel invasion. A431 cells demonstrated greater mobility and Matrigel invasion than HaCaT and SiHa cells, although assay results did not reach statistical significance.

Treatment with the inhibitor Batimastat at 0.1 μM reduced by half the mean number of FaDu or HaCaT cells passing through uncoated filters (**Fig 1*a***). Treatment with 1.0 μM Batimastat reduced by at least half the number of HaCaT, FaDu, or A431 cells traversing uncoated filters, but mobility of treated cells was significantly different from untreated cells only in the HaCaT cell line (**Fig 1*a***). Batimastat treatment had no effect on the mobility of SiHa cells (**Fig 1*a***). On Matrigel-coated filters, both FaDu and A431 cells were active in digesting Matrigel; however, FaDu cells were four times more active than A431 in invading the Matrigel (**Fig 1*b***). The number of FaDu cells traversing Matrigel-coated filters was significantly ($p < 0.05$) greater than the number of HaCaT cells passing through the Matrigel. HaCaT and SiHa

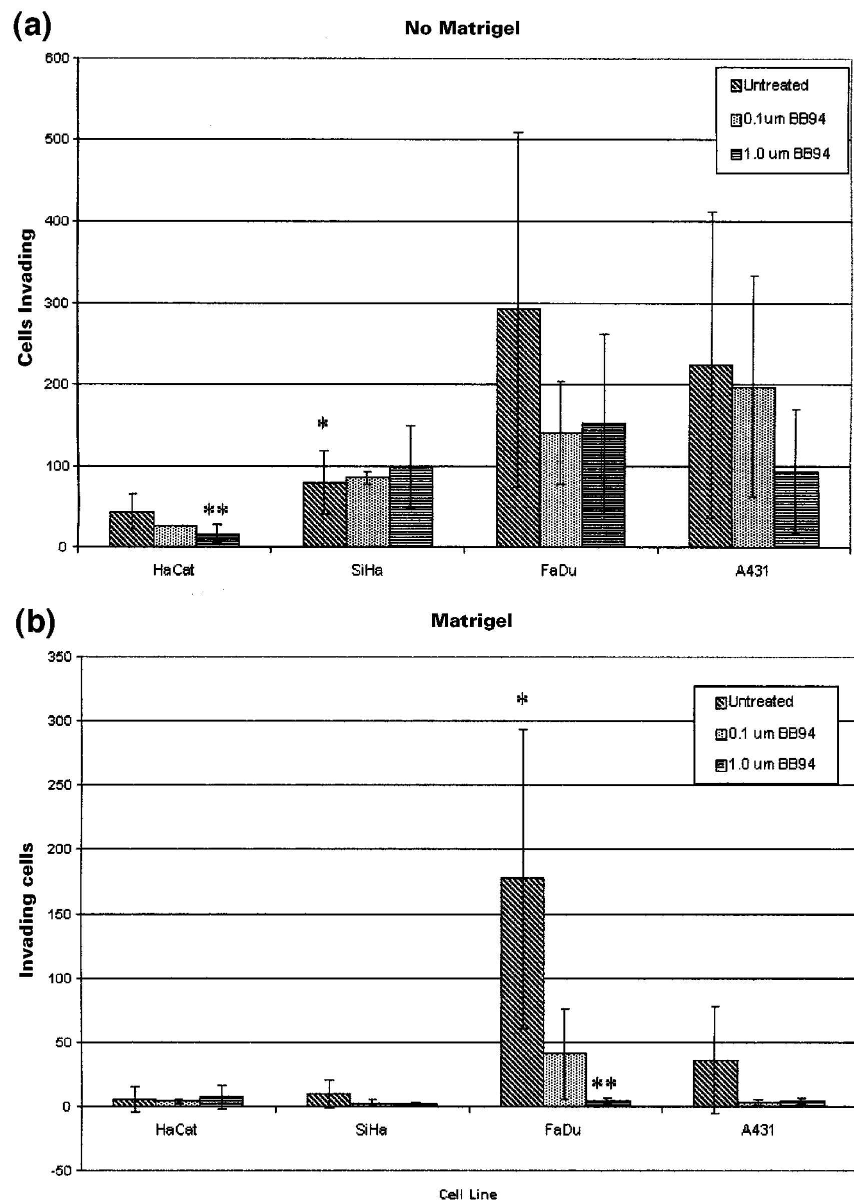

Figure 1. Effect of batimastat on cell invasiveness and motility. Cells were plated on uncoated or Matrigel-coated porous filters. After a 24 h incubation, the cells were stained and the number of cells passing through the filters was determined. Each assay was performed in triplicate. Mean and SD for five assays of each cell line for untreated cells, two assays for 0.1 μM batimastat (BB-94), and four assays at 1.0 μM batimastat. (*a*) Number of cells passing through uncoated filters. For untreated SCC cells compared with untreated HaCaT, $p = 0.07$ for SiHa, 0.04 for FaDu*, 0.06 for A431. For treatment with 1.0 μM batimastat compared with untreated cells, $p = 0.03$ for HaCaT**, 0.29 for SiHa, 0.15 for FaDu, 0.13 for A431. *Statistically significant ($p < 0.05$) difference compared with untreated HaCaT; **statistically significant difference for batimastat-treated compared with untreated cells of the same cell line. (*b*) Invasion of Matrigel-coated filters. Untreated SCC *versus* untreated HaCat, $p = 0.20$ for SiHa, 0.02 for FaDu*, 0.10 for A431. Batimastat at 1.0 μM compared with untreated cells, $p = 0.40$ for HaCaT, 0.09 for SiHa, 0.02 for FaDu**, 0.09 for A431.

showed only weak invasion of Matrigel (**Fig 1*b***). Batimastat at 1.0 μM significantly inhibited Matrigel invasion by FaDu cells (**Fig 1*b***). These results show that the FaDu cell line is the only SCC line tested that readily invades Matrigel, and that inhibition of metalloproteinase activity inhibits Matrigel invasion by FaDu cells.

To determine if invasiveness and expression of metalloproteinase or TIMP mRNA are correlated, steady-state levels of mRNA for matrilysin, stromelysins-1, 2, and 3, and TIMP-1 and -3 in each cell line were measured by semiquantitative RT-PCR. These results are shown in **Fig 2**. Only mRNA for stromelysin-2 was higher in the FaDu cell line than in the other cell types, and the difference was not substantial. The four cell lines expressed similar levels of mRNA for matrilysin, stromelysin-3, and TIMP-1 (**Figs 2*a*, *b***). Stromelysin-2 mRNA was the most abundantly expressed stromelysin RNA in all four cell lines (**Fig 2*a***). TIMP-3 mRNA levels were nearly 2-fold greater in HaCaT and A431 cells than in SiHa or FaDu cells (**Fig 2*c***). These results do not reveal a clear correlation between invasiveness in Matrigel and steady-state RNA levels for matrilysin, stromelysins-1–3, or TIMP-1 or -3.

Immunoblotting was performed to examine the levels of metalloproteinases and TIMP released into the culture medium by each cell line. These results are shown in **Fig 3**. Conditioned medium from FaDu and A431, the cell lines with greatest mobility, contained the highest amounts of both promatrilysin and active matrilysin. Both forms were present in lesser amounts in medium from SiHa cells, but neither form was detected in conditioned medium from HaCaT cells. A similar pattern was seen for TIMP-1: FaDu and A431 cells produced the greatest amounts of TIMP-1, SiHa cells produced a lesser amount, and no TIMP-1 was detected in medium from HaCaT cells. Neither TIMP-2 nor TIMP-3 could be detected in conditioned medium from any of the cell lines. These results suggest that TIMP-1 regulates the activity of secreted matrilysin in these cell lines.

FaDu and A431 medium also contained the highest amounts of prostromelysin-1, but only medium from FaDu cells contained a substantial level of active stromelysin-1. FaDu cells also produced greater amounts of active stromelysin-2 and active stromelysin-3 than the other three cell lines. Stromelysin 2 was easily detected in medium from HaCaT and A431 cells, but only weakly detectable in medium from SiHa cells. Stromelysin 3 was weak in HaCaT, SiHa, and A431 cell medium. Medium from FaDu and A431 cells contained the highest amounts of active gelatinase A, but strong labeling for gelatinase A was also seen in SiHa and HaCaT cells. In contrast, active gelatinase B (MMP-9) was clearly present in

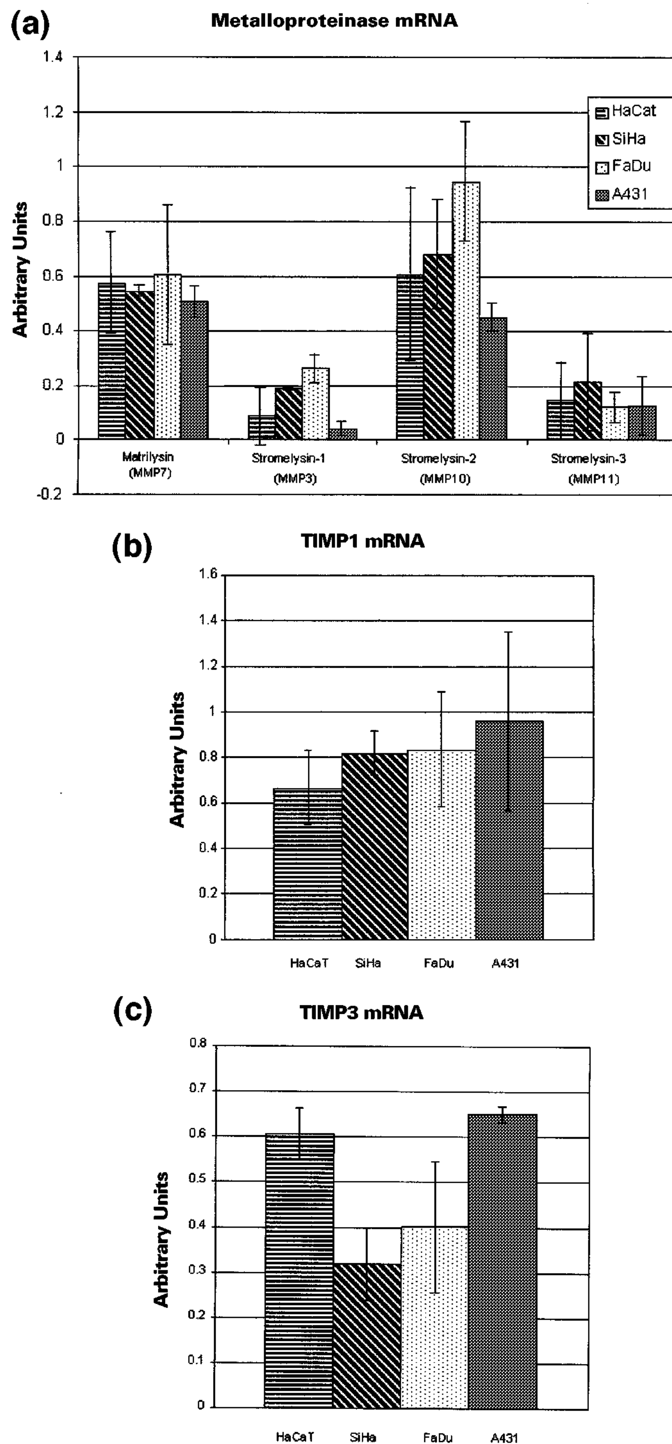

Figure 2. Steady-state levels of mRNA for metalloproteinases and TIMPs. Mean and SD for two RT-PCR reactions for each mRNA. (*a*) Matrilysin and stromelysins 1, 2, and 3; (*b*) TIMP-1; (*c*) TIMP-3.

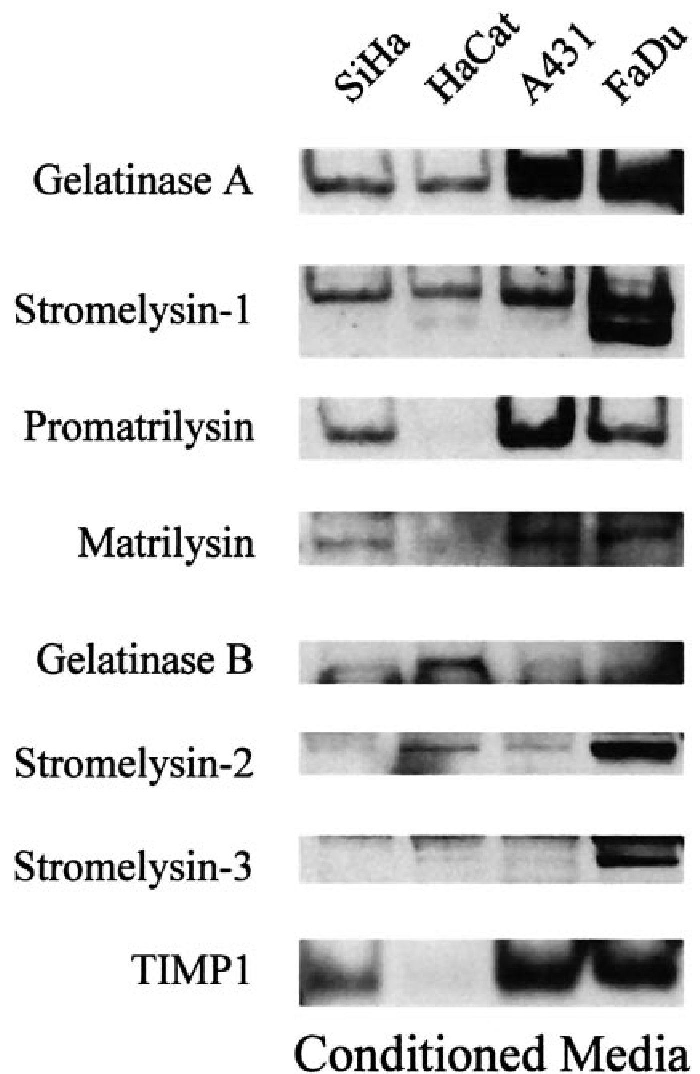

Figure 3. Immunoblots of metalloproteinases and TIMP in conditioned medium. Culture medium from each cell line was collected after 48 h incubation. Expression of specific proteins was analyzed by gel electrophoresis and immunoblotting. Approximate molecular weights listed were determined by comparison with molecular weight standards. MMP2 = gelatinase A, 66 kDa; MMP3 = stromelysin-1, 59 kDa and 48 kDa; MMP7p = promatrilysin, 28 kDa; MMP7a = active matrilysin, 38 kDa aggregate; MMP9 = gelatinase B, 83 kDa; MMP10 = stromelysin-2, 50 kDa; MMP11 = stromelysin-3, 47 kDa. TIMP1 = 28.5 kDa.

medium from HaCaT and SiHa cells, the cell lines with poor mobility, but was barely detectable in medium from FaDu and A431 cells. Thus, only secretion of active stromelysin-1 and stromelysin-3 correlates with the invasive phenotype exhibited by the FaDu cell line, whereas gelatinase A and matrilysin secretion is associated with cell mobility and the SCC phenotype.

Cellular expression of active matrilysin reflected the release of matrilysin into culture medium. Antibody labeling revealed little or no active matrilysin in HaCaT cells, except in mitotic cells (**Fig 4*a***). In contrast, SiHa, FaDu, and A431 cells were strongly labeled for active matrilysin in nondividing and mitotic cells (**Figs 4*b–d***). In all cell lines, labeling for active matrilysin was localized around the perimeter of the nucleus and may be associated with the endoplasmic reticulum. Stromelysin-1 labeling was similarly greater in the SCC lines than in HaCaT cells, and exhibited the same cellular localization as matrilysin (**Fig 5**). Although TIMP-1 was not found in medium from HaCaT cells, cell-associated TIMP-1 was detected by antibody labeling in HaCaT cells, as well as in the three SCC cell lines (**Fig 6**). TIMP-1 labeling was greatest in A431 cells (**Fig 6*d***). In the three SCC cell lines, TIMP-1 labeling was concentrated around the nuclear perimeter (**Figs 6*b–d***); however, in HaCaT cells, nuclei were brightly labeled (**Fig 6*a***). Labelling for matrilysin, stromelysin-1, and TIMP-1 in all four cell lines appeared more intense in mitotic cells.

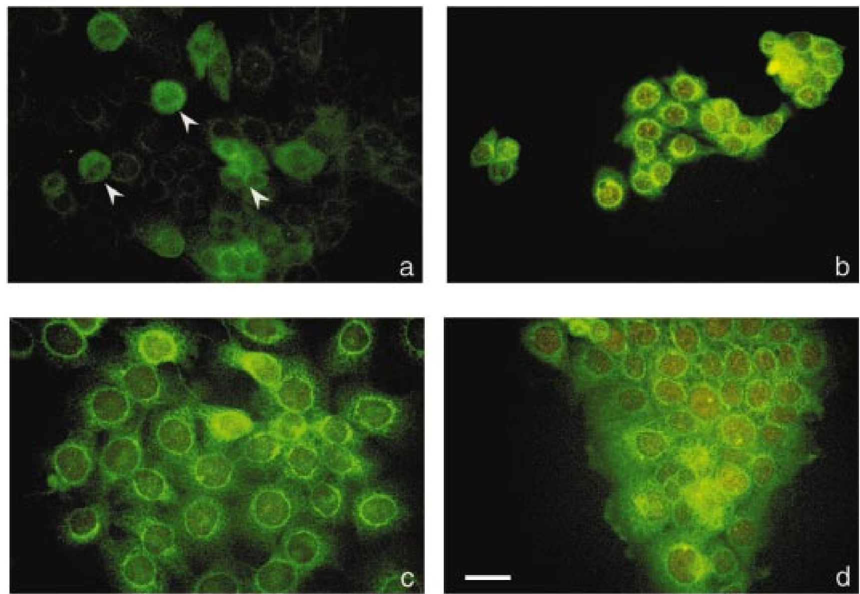

Figure 4. Immunocytochemistry for active matrilysin. All cells were fixed 24 h after removal of aphidicolin. *Arrowheads* designate mitotic cells. (*a*) HaCaT; (*b*) SiHa; (*c*) FaDu; (*d*)A431. *Scale bar*: 100 μm.

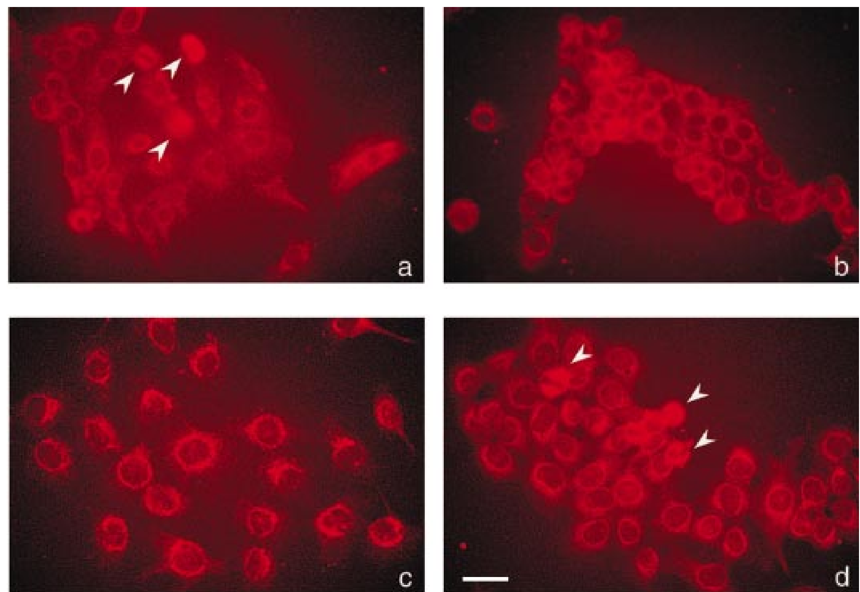

Figure 5. Immunocytochemistry for stromelysin-1. All cells were fixed 24 h after removal of aphidicolin. *Arrowheads* designate mitotic cells. (*a*) HaCaT; (*b*) SiHa; (*c*) FaDu; (*d*) A431. *Scale bar*: 100 μm.

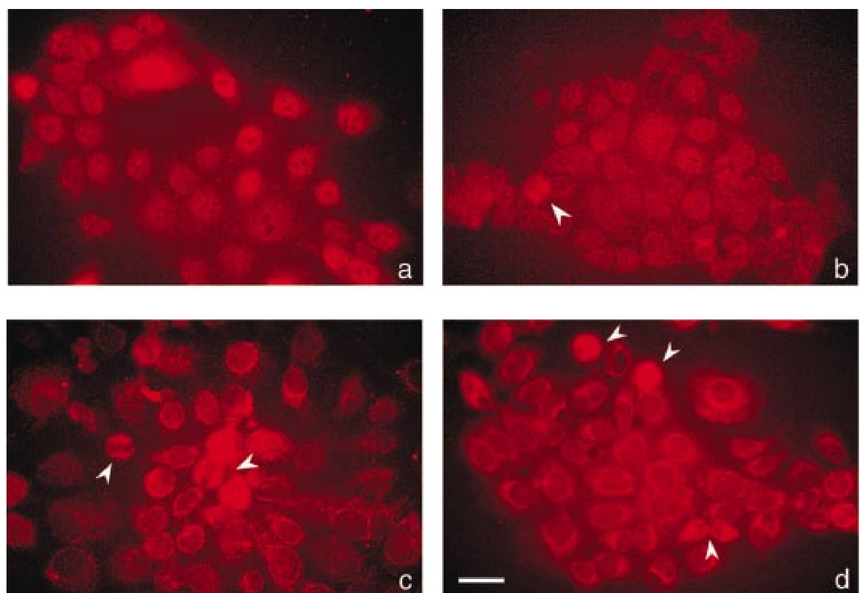

Figure 6. Immunocytochemistry for TIMP1. All cells were fixed 24 h after removal of aphidicolin. *Arrowheads* designate mitotic cells. (*a*) HaCaT, large cell at top of figure is binucleate; (*b*) SiHa; (*c*) FaDu; (*d*) A431. *Arrowhead* at bottom of figure designates daughter cells in cytokinesis. *Scale bar*: 100 μm.

DISCUSSION

Metalloproteinases have been shown to promote tumor progression through several mechanisms (reviewed in Kleiner and Stetler-Stevenson, 1999; Sternlicht *et al*, 2000). Increased metalloproteinase activity increases tumor cell invasiveness through extracellular matrix (Knox *et al*, 1998), and treatment with TIMP inhibits both metalloproteinase activity and invasiveness (Umenishi *et al*, 1991; DeClerck *et al*, 1992; Liu *et al*, 1997); however, at least one metalloproteinase, stromelysin-3, does not digest matrix, but promotes tumor growth by blocking cell death in malignant cells (Boulay *et al*, 2001). Matrilysin and gelatinase A are thought to play a role in tumor initiation rather than invasiveness (Meade-Tollin *et al*, 1998). Metalloproteinase degradation of extracellular matrix may release matrix-associated growth factors that stimulate tumor cell proliferation (Vlodavsky *et al*, 1990). Conversely, contact with particular matrix proteins can suppress tumor cell proliferation, which is enhanced when these matrix components are degraded (Henriet *et al*, 1999). Additionally, matrilysin and stromelysin-1 can cleave E-cadherin, thereby increasing tumor cell invasiveness by blocking cell adhesion (Noë *et al*, 2000).

In normal cells and tissues, metalloproteinases also carry out routine tasks such as matrix repair and remodeling, but few metalloproteinases are associated with unique functions, and most participate in similar tasks. Certain metalloproteinases can also exhibit an inhibitory effect on tumorigenesis through an effect on angiogenesis. Matrilysin, stromelysin-1, and murine macrophage elastase (MME, MMP-12) are capable of cleaving plasminogen *in vitro* to yield angiostatin, which blocks endothelial cell proliferation. In tumor tissue, conversion of plasminogen to angiostatin is carried out by macrophage metalloproteinases. MME has been shown to be critical for the production of angiostatin, as MME-null macrophages are incapable of converting plasminogen to angiostatin (Cornelius *et al*, 1998).

This study focuses on the effects of metalloproteinases on invasiveness. By comparing the types of active metalloproteinases released by invasive tumor cells to those produced by normal cells or noninvasive tumor cells, the particular metalloproteinases that contribute to tumor cell invasiveness in different types of cancer can be identified. Matrigel invasion was used to assess invasiveness. The Matrigel assay revealed at least two components to invasiveness: inherent cell mobility, determined by the ability of cells to pass through uncoated filters; and ability to invade Matrigel. Of the three SCC cell lines, both FaDu and A431 showed greater mobility than HaCaT, the noninvasive, nontumorigenic keratinocyte line; however, only FaDu cells demonstrated significantly greater invasion of Matrigel than HaCaT cells, indicating that cell mobility alone does not regulate invasiveness in SCC cells. Differences in cell mobility may reflect the different origins of the cell lines. HaCaT is a keratinocyte line and the A431 line is derived from an epidermal SCC, whereas FaDu and SiHa cells are derived from mucosal SCC. Batimastat-treated HaCaT, FaDu, and A431 cells were less active in passing through uncoated filters, suggesting that metalloproteinase activity contributes to cell mobility. Batimastat treatment, however, significantly decreased Matrigel invasion only in FaDu cells, confirming that invasiveness in this cell line requires metalloproteinase activity.

Because Batimastat does not act specifically on any particular metalloproteinase, expression of specific metalloproteinases was examined and compared among the four cell lines. No correlation was found between steady-state levels of mRNA for any particular metalloproteinase and invasiveness, indicating that regulation of invasiveness occurs at a post-transcriptional step, such as metalloproteinase synthesis or activation. Matrilysin and stromelysin-3 mRNA levels were similar in all four cell lines, but neither promatrilysin nor active matrilysin was detected in conditioned medium from HaCaT cells, whereas varying amounts of matrilysin and stromelysin-3 protein were found in medium from each of the four cell lines. Meade-Tollin *et al* found that matrilysin mRNA levels increased in noninvasive transformed HaCaT cells, but not in the invasive transformants. They further observed that secretion of matrilysin nonetheless increases in invasive transformants, and also concluded that post-transcriptional regulation of matrilysin expression contributes to the invasive phenotype (Meade-Tollin *et al*, 1998).

A431 cells had the lowest steady-state levels of stromelysin-1 mRNA, but higher levels of stromelysin-1 protein in conditioned medium than HaCaT or SiHa cells. No difference was seen in levels of mRNA for TIMP-1 among the four cell lines, yet no TIMP-1 protein was found in conditioned medium from HaCaT cells. These results provide further support for post-transcriptional regulation of metalloproteinase and TIMP activity. TIMP-3 mRNA was present in all four cell lines, but no TIMP-3 protein was detected in conditioned medium from any cell line. This result was unexpected because the localization of matrilysin and TIMP-3 in skin is similar *in vivo* (Airola *et al*, 1998). TIMP-3 binds strongly to extracellular matrix, however, and so may not have been extracted in conditioned medium (Woessner, 2001). Alternatively, TIMP-3 may be not synthesized or secreted by these cell lines *in vitro*, or is produced in amounts that could not be detected by immunoblotting.

Matrilysin has been implicated in promoting tumorigenesis as well as invasiveness. In colon carcinoma cell lines, specific inhibition of matrilysin activity reduces both tumorigenicity and tumor invasiveness; however, transfection of matrilysin into cells that do not express the enzyme increased tumorigenicity but not invasiveness (Witty *et al*, 1994). Thus, whereas matrilysin does not drive invasiveness in these cell types, it nonetheless contributes to invasion and metastasis in colon carcinoma. In our study, media from FaDu and A431 cells, the cell lines with greatest cell mobility, contained the highest amounts of gelatinase A, promatrilysin, and active matrilysin. No matrilysin was detected by immunoblotting in medium from HaCaT cells. Similar results were observed in transformed HaCaT cells: little or no matrilysin or gelatinase A was released into the medium by nontransformed cells, and the greatest amounts of these two enzymes were found in medium from transformed cells with an invasive, SCC phenotype (Bachmeier *et al*, 2000). Therefore, matrilysin and gelatinase A appear to function in tumorigenesis and cell mobility, and contribute to, but do not trigger invasiveness in SCC cells.

Gelatinase B mRNA is expressed by tumor cells at the interface between tumor and stromal cells in SCC, but is not expressed by tumor cells in BCC or by normal skin cells (Pyke *et al*, 1992; Tsukifuji *et al*, 1999). Immunoblotting experiments revealed that HaCaT cells secrete the greatest amount of active gelatinase B, whereas the enzyme is barely detectable in medium from the SCC cell lines. Others have used zymography to show that HaCaT and transformed HaCaT cells secrete gelatinase B, but they did not demonstrate a correlation between invasiveness and gelatinase B secretion. (Meade-Tollin *et al*, 1998; Bachmeier *et al*, 2000). Within our experimental system, active gelatinase B secretion does not correlate with SCC invasiveness, as medium from the invasive cell line, FaDu, contained the least amount of active gelatinase B.

The most notable difference among the four cell lines was the release of active stromelysin-1 only by FaDu cells. Active stromelysin-1 could not be detected in conditioned medium from HaCaT, SiHa, or A431 cells. Bachmeier *et al* also detected weak production of prostromelysin-1 by HaCaT cells. In the transformed HaCaT cells, invasive cells produced much greater amounts of active stromelysin-1 than benign cells (Bachmeier *et al*, 2000). *In vivo*, keratinocytes in normal skin do not express stromelysin-1, but expression is stimulated by wounding (Windsor *et al*, 1993; Saarialho-Kere *et al*, 1994). In SCC, stromelysin-1 expression is associated with advanced pathology and metastasis (Muller *et al*, 1991; Kusukawa *et al*, 1996; Tsukifuji *et al*, 1999). Stromelysin-1 also affects both tumorigenesis and invasiveness in some cancer cells. In stromelysin-1-transfected mammary epithelial cells, stromelysin-1 induction leads to phenotypic changes characteristic of tumor cells (Lochter *et al*, 1997; Sternlicht *et al*, 2000). In DMBA-TPA-induced SCC in

mouse skin, stromelysin-1 expression in tumor cells is coincident with the epithelial-to-mesenchymal transition that yields invasive, metastatic tumor cells (Wright *et al*, 1994). Stromelysin-1 can activate matrilysin and cleave E-cadherin, releasing a soluble E-cadherin fragment that blocks E-cadherin function (Imai *et al*, 1995; Noë *et al*, 2000). Thus, in addition to digesting extracellular matrix, active stromelysin-1 can enhance basal lamina degradation by matrilysin and facilitate invasion and metastasis by disrupting cell–cell and cell–matrix attachment. Active stromelysin-1 accordingly plays a key role in invasiveness, and our results suggest that activation of stromelysin-1 is an important regulatory step for invasiveness in SCC cells.

FaDu cells also released substantially greater amounts of stromelysins 2 and 3 into conditioned medium than the other cell lines. Both enzymes were barely detectable in SiHa medium, and stromelysin-3 was weakly detected in HaCaT and A431 medium. Because stromelysin-2 secretion was greatest in medium from HaCaT and FaDu cells, production of this metalloproteinase does not correlate with invasiveness. SCC tissue also expresses stromelysin-2 mRNA, which is transcribed *in vivo* by epithelial and tumor cells, but expression of this metalloproteinase is associated with tissue inflammation rather than tumor cell invasiveness in skin cancer (Kerkelä *et al*, 2001). Stromelysin-2 can activate matrilysin and gelatinase B, however, and may contribute to tumor invasiveness through these enzymes (Nakamura *et al*, 1998). Although stromelysin-3 secretion was greatest in the invasive cell line, FaDu, this metalloproteinase does not digest matrix proteins and is unlikely to play a direct role in invasiveness (Murphy *et al*, 1993; Pei and Weiss, 1995). Instead, stromelysin-3 appears to foster the growth of tumors by blocking cell death and promoting implantation of malignant epithelial cells (Masson *et al*, 1998; Boulay *et al*, 2001).

Stromelysin-3 mRNA is expressed in head and neck SCC, but not in normal tissues from the head and neck. In SCC tissue, stromelysin-3 mRNA and protein was found only in fibroblasts surrounding invasive cancer cells and not in tumor cells, suggesting a paracrine effect on tumor cells (Muller *et al*, 1993; Thewes *et al*, 1998). We have demonstrated stromelysin-3 production in HaCaT and SCC cell lines, however, and Bachmeier *et al* have shown that this metalloproteinase is expressed by HaCaT and transformed HaCaT cell lines (Bachmeier *et al*, 2000). Similarly, in skin cancer tissue, gelatinase A mRNA is expressed by stromal cells adjacent to tumor nodules in SCC and BCC, but is not expressed by tumor cells (Pyke *et al*, 1992; Tsukifuji *et al*, 1999), yet SCC cell lines produce gelatinase A *in vitro*. Normal keratinocytes do not synthesize stromelysin-1 *in vivo*, but HaCaT cells *in vitro* do produce stromelsyin-1 (Windsor *et al*, 1993; Saarialho-Kere *et al*, 1994). Paracrine regulatory factors present in tissue are likely to explain the difference between stromelysin-3 and gelatinase A expression by cell lines *in vitro* and cells *in vivo*.

Cell-associated labeling for matrilysin, stromelysin-1, and TIMP-1 appeared to increase in mitotic cells, particularly in metaphase. The apparent increase may result from a change in shape as cells round up for cell division. Alternatively, these results may indicate a correlation between cell cycling and metalloproteinase or TIMP expression. Henriet *et al* have shown that plating melanoma cells on collagen I elevates expression of $p27^{KIP1}$, and arrests the cells in G1 of the cell cycle (Henriet *et al*, 1999). TIMP-1 localization to the nucleus has also been shown to be cell-cycle dependent in gingival fibroblasts (Zhao *et al*, 1998). Therefore it is not unreasonable to hypothesize an association between metalloproteinase activity and the cell cycle. Nuclear localization of TIMP-1 was also observed in HaCaT cells in this study. Whereas precise phases of the cell cycle were not distinguished in these assays, nuclear localization of TIMP-1 was seen in HaCaT cells at all time points after removal of aphidicolin and was pronounced in aphidicolin-blocked cells that arrest in G1 (data not shown). Less dramatic nuclear expression of TIMP-1 was also observed in SiHa cells. Because neither of these two cell lines was able to invade Matrigel, it can be speculated that TIMP-1 has a nuclear function that affects cell mobility and invasiveness.

This study is not intended to be a comprehensive examination of the role of all known metalloproteinases in invasiveness. It is also limited to an *in vitro* model system. Other metalloproteinases may also play important roles in various aspects of tumorigenesis *in vivo*, including invasiveness. For example, the recently described collagenase-3 (MMP-13) can also degrade basement membrane components and may function in invasiveness. In head and neck SCC *in vivo*, collagenase-3 mRNA is expressed by tumor cells found on the leading edge of the tumor that are in contact with stromal fibroblasts (Johansson *et al*, 1997). In laryngeal SCC, collagenase-3 expression correlates with overexpression of gelatinase A and MT-1-MMP, suggesting a cooperative effect of these enzymes on tumor progression (Cazorla *et al*, 1998). We hope that our results on the expression of stromelysins and matrilysin in invasive SCC cell lines will stimulate further research on the functions of stromelysin-1 and other metalloproteinases in tumor cell invasiveness, and on the mechanisms through which these enzymes facilitate tumor cell invasion and metastasis *in vivo*.

The authors thank British Biotechnology, Ltd for providing Batimastat, Dr. Ulrich Rodeck for kindly sharing the HaCaT cells, and The Skin Cancer Foundation who supported this research through the Theodore S. Tromovitch Memorial Award. We are also grateful to Mr Ray Hörsch for his assistance with the figures, and Dr. Sue Menko for critical comments on the manuscript.

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