Membrane Type I-Matrix Metalloproteinase-Mediated Degradation of Type I Collagen by Oral Squamous Cell Carcinoma Cells

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ABSTRACT

Oral squamous cell carcinomas are highly invasive lesions that destroy adjacent tissues and invade bone and muscle, which is most likely the result of matrix metalloproteinase (MMP) activity. We examined three cell lines derived from squamous cell carcinoma of the tongue for their intrinsic capacities to degrade interstitial collagen with the goal of identifying the matrix-degrading enzymes. SCC-25 and SCC-15 cells degrade reconstituted fibrillar type I collagen in the absence of exogenous growth factors or cytokines when seeded as a colony on dried films. Degradation is confined to the subjacent matrix, is enhanced 2–3-fold by phorbol ester, and is strictly MMP-dependent, as it is blocked by BB-94 and tissue inhibitor of metalloproteinases-2 but not by inhibitors of serine and cysteine proteases. Both cell lines express active (M, 57,000) membrane type I-MMP (MT1-MMP) on their surfaces, as detected by surface biotinylation and immunoprecipitation. Concomitantly, both cell lines activate endogenious MMP-2 when cultured on type I collagen films, as assessed by zymography. Phorbol ester treatment enhances collagen-induced MMP-2 activation, which is accompanied by the appearance of a surface-labeled Mr 43,000 form of MT1-MMP. Treatment of cells with a synthetic furin inhibitor, which inhibits processing of the MT1-MMP zymogen, blocks collagen degradation. In contrast, CAL 27 cells do not degrade collagen under either basal or phorbol 12-myristate 13-acetate-stimulated conditions. Although proMT1-MMP (Mr, 63,000/65,000) is detectable in these cells by immunoblot analysis, they express greatly reduced levels of active MT1-MMP on their surfaces relative to SCC-25 and SCC-15 cells. Correspondingly, CAL 27 cells cultured on collagen express neither latent nor active gelatinases. Immunoblots of lysates and conditioned media revealed the constitutive expression of proMMP-1 and proMMP-13 in all three cell lines. We conclude that in the absence of exogenous growth factors or accessory stromal cells, degradation of interstitial collagen by oral squamous cell carcinoma cells requires a threshold level of active MT1-MMP on cell surfaces.

INTRODUCTION

SCCs are the most common malignant tumors of the oral and maxillofacial region and are characterized by a high degree of local invasiveness and metastasis to cervical lymph nodes (1, 2). The poor prognosis and survival rate of patients presenting with advanced disease underscores the need to better understand the molecular mechanisms of invasion in these tumors. Invasiveness of tumor cells is a complex process that requires the proteolytic degradation of ECM (3) barriers, coupled with migration of the cells through the modified region (3, 4). Degradation of ECM in malignant tumors is accomplished primarily by members of the MMP family of enzymes, which currently includes at least 26 distinct gene products (5, 6). MMPs are zinc-dependent endopeptidases, which collectively are capable of degrading virtually all ECM components (6, 7). This family of matrix-degrading enzymes participates in tissue remodeling processes under both physiological and pathological conditions including morphogenesis, angiogenesis, wound healing, arthritis, and tumor invasion (3).

Studies of MMP expression in oral SCCs have implicated mainly the secreted MMPs in invasion and metastasis. For example, individual studies have correlated the increased expression of MMP-1, -2, -3, -9, and -13 in tissue sections of oral SCC with increased local invasion or incidence of lymph node metastases in the patients from whom they were derived (8–12). In vitro experiments with established cell lines from oral SCCs have implicated the involvement of MMPs -1, -3, -9 in the invasion of collagen gels and reconstituted basement membrane matrix (13–16). In several of these studies, epidermal growth factor and/or hepatocyte growth factor/scatter factor were required to initiate MMP-mediated invasion through three-dimensional matrices (17). However, a role for MT1-MMP in oral cancer progression has received less attention. A limited number of studies have documented its expression in oral tumor specimens (12, 18–20), with one study detecting predominantly stromal expression of the mRNA (18) and another showing strong expression of the protein on tumor cells at the invasive edge (20). Originally described as an activator for membrane-bound proMMP-2 (gelatinase A; 21), MT1-MMP has more recently been demonstrated to have matrix-degrading activity in its own right, including activity against interstitial collagen (22, 23).

In this study, we examined the interstitial collagen-degrading capacities of three established oral SCC cell lines derived from malignant lesions of the tongue, with the goal of identifying the relevant MMP(s). Collagen degradation was assessed with an assay developed to measure dissolution of reconstituted type I collagen fibrils by keratinocytes and fibroblasts (24–27), which is sensitive to low levels of cell-associated collagenolytic activity. Two of these cell lines, the SCC-25 and SCC-15 cells (28), were capable of subjacent collagen degradation in the absence of exogenous growth factors, cytokines, or proteases such as trypsin or plasmin. Degradation was enhanced by the phorbol ester PMA and was strictly MMP-mediated as determined by its protease inhibitor profile. The third cell line, CAL 27 (29),...
incapable of collagen degradation under either basal or PMA-stimulated conditions. Limited MMP profiling by zymography and Western blotting was performed along with studies of MMP regulation by collagen culture and PMA. We present evidence that cell surface expression of active MT1-MMP is a requirement for pericellular collagen degradation in this system.

MATERIALS AND METHODS

Reagents. Cell culture reagents (DMEM, trypsin, and PBS) were from Life Technologies, Inc. (Grand Island, NY), BSA, PMA, benzamidine, EACA, and monolastic Ab to β-actin were from Sigma Chemical Co. (St. Louis, MO). Precast 10% Tris-glycine polyacrylamide gels, gelatin zymograms, and polyvinylidene difluoride membranes were from Novex/Invitrogen (Carlsbad, CA). The synthetic furin inhibitor, decanoyl-Arg-Val-Lys-Arg-CMK (30) was from Alexis Biochemicals (San Diego, CA). Aprotinin, leupeptin, 4-(2-aminoethyl)-benzenesulfonyl fluoride, peptatin A, and E-64 were from Boehringer Mannheim/Roche Diagnostics (Indianapolis, IN). Batimastat (BB-94; Ref. 31) was from R&D Systems (Minneapolis, MN). The synthetic furin inhibitor, decanoyl-Arg-Val-Lys-Arg-CMK (30) was from Alexis Biochemicals (San Diego, CA). 4-(2-aminoethyl)-benzenesulfonyl fluoride, peptatin A, and E-64 were from Boehringer Mannheim/Roche Diagnostics (Indianapolis, IN). Batimastat (BB-94; Ref. 31) was from R&D Systems (Minneapolis, MN).

Cell Culture. SCC-25 (CRL-1628), SCC-15 (CRL-1623), CAL-27 (CRL-9053), and HT-1080 (CCL-121) cell lines were purchased from American Type Culture Collection, The SCC-25, SCC-15, and CAL-27 cell lines were derived from SCCs of the tongue (28, 29). Cell lines were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (HyClone, Logan, UT). Cells were derived from SCCs of the tongue (28, 29). Cell lines were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (HyClone, Logan, UT), penicillin (10 units/ml)-streptomycin (10 μg/ml), and gentamicin (10 μg/ml; Sigma Chemical Co.) at 37°C in 10% CO2 in air. Each cell line was used within 15–20 passages after initiation of cultures from American Type Culture Collection.

Isolation of Rat-Tail Tendon Type I Collagen. Type I collagen was isolated from rat tail tendons of Wistar rats 4–6 weeks of age as previously described in detail (33, 34). Lyophilized collagen was stored at −80°C, and stock solutions were prepared as needed. Acid-soluble type I collagen forms fibrils at neutral pH which (at 37°C) are resistant to degradation by proteinases other than collagenases, similar to native collagen type I in vivo.

Preparation of Collagen-Coated Plates. Six-well culture plates (35 mm diameter wells; Corning Glass Works, Corning, NY) were coated with a film of reconstituted type I collagen by a modification of techniques described previously (24–27). Briefly, a stock solution of rat tail tendon type I collagen in 13 mM HCl was diluted and mixed with neutralizing phosphate buffer to a final concentration of 300 μg/ml. Aliquots of 1.5 ml/well (50 μg/cm2) were dispensed, and collagen fibrils were formed by heat gelation at 37°C for 2–4 h. Collagen gels were then air-dried to a film and washed extensively with sterile distilled water to remove salt precipitates. Tissue culture plates (100 and 150 mm; Falcon) were coated similarly, maintaining a coating concentration of 50 μg/cm2.

Cell-Mediated Collagen Fibril Dissolution. Cells were detached from subconfluent cultures in 0.25% trypsin, washed, and finally resuspended in serum-free DMEM supplemented with 0.1% BSA (DMEM/BSA). Cells were then seeded in collagen-coated, six-well plates as a droplet (60,000 cells in 50 μl) in the central part of each well. After 3 h in a humidified 37°C incubator, 2 ml of DMEM/BSA were added to each well, with or without PMA (160 nM; final DMEM concentration in media, 0.016%) plus other reagents/inhibitors as indicated. Culture was continued for up to 3 days, during which cells formed a compact monolayer and did not migrate outward from the colony. Collagen degradation by the cells was examined after removing cells with 0.25% trypsin/1 mM EDTA for 10 min before the addition of Triton X-100 to a final concentration of 0.3%. Wells were then washed and residual collagen stained with a solution of Coomassie Blue (0.2%) in 20% methanol/7.5% acetic acid. The fibrillar collagen film is resistant to trypsin at 37°C, therefore cellular collagen degradation was visualized as clear areas against a blue background. Samples done in duplicate were indistinguishable from one another visually. In selected experiments, the degree of clearing in equivalent areas under the cell button was quantitated with the Bio-Rad Gel Doc1000 using Molecular Analyst software (Bio-Rad, Richmond, CA).

Preparation of Cell Lysates. Cells were plated at 70–80% confluence on substrate-coated or -uncoated dishes in serum-containing DMEM (DMEM/+ and incubated for 16 h. Adherent cells were rinsed and then incubated in DMEM/BSA, with or without PMA for the duration indicated. Alternatively, in some experiments (Fig. 5A), cells were resuspended and plated directly into DMEM/BSA. Lysates were harvested by scraping the cells into a small volume of ice-cold PBS/0.1% Triton (pH 7.4), 150 mM NaCl, 1.1 mM MgCl2, 0.5% NP-40, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 1 μg/ml pepstatin A. Extracts were incubated on ice for 45 min, then clarified by microfuge at 14,000 × g for 15 min at 4°C. Protein concentrations of lysates and membranes (below) were determined using the bicinchoninic acid Protein Assay Kit (Pierce, Rockford, IL).

Preparation of Cell Membranes. Cells were plated in DMEM/+ and incubated for 16 h. Medium was then replaced with DMEM/BSA and culture continued for 2–3 days until confluent. When PMA (160 nM) was included, it was added during the final 24 h of culture. To harvest membranes, cells were rinsed in icecold PBS; scraped into 20-ml HEPES (pH 7.4), 0.25% sucrose buffer with 2 mM benzamidine, 2 mM EACA, and protease inhibitors; and homogenized with 100 strokes of a glass Dounce homogenizer ( Kontes Glass, Vineland, NJ) and then passed 10 times through a 26-gauge needle. Homogenate was centrifuged at 100,000 × g for 10 min to pellet nuclei and unbroken cells, then the postnuclear supernatant was ultracentrifuged at 100,000 × g for 1 h at 4°C. The crude membrane pellet was finally resuspended in 20 mM HEPES (pH 7.4) with 10 mM CaCl2, 1 mM MgCl2, 1 μM ZnCl2, and protease inhibitors to a concentration of 2–4 mg/ml. For Western blots or zymograms, membrane suspensions were mixed with SDS sample buffer before to electrophoresis. Alternatively, for immunoprecipitation, rinsed membrane pellet was directly resuspended in NP40 lysis buffer with protease inhibitors, incubated on ice for 2 h at 4°C, and then clarified by microfuge at 14,000 × g for 20 min at 4°C.

Gelatin Zymography. Samples were mixed with SDS sample buffer without heating or reduction and applied to 10% polyacrylamide gels copolymerized with 1 mg/ml gelatin. After electrophoresis, gels were washed for 1 h at room temperature in buffer containing 2.5% (v/v) Triton X-100 in 50 mM Tris-CI (pH 7.5). Gels were then incubated at 37°C in 50 mM Tris-CI (pH 7.5) with 5 mM CaCl2 and 1 μM ZnCl2 for 24 h. After staining with Coomassie Blue (0.2%), zones of gelatinolytic activity were detected as clear bands against a blue background.

SDS-PAGE and Western Blotting. Samples (cell lysates, membranes, or conditioned medium) were mixed with reducing SDS sample buffer, heated, and electrophoresed on 10% polyacrylamide gels according to the method of Laemmli (35). Gels were then electroblotted onto polyvinylidene difluoride membrane for immuno blot analysis. After blocking for 1 h with 5% nonfat dry milk in PBS/0.1% Tween 20 (PBST; Bio-Rad), blots were probed with primary antibody diluted in 0.5% milk in PBST (for 1.5 h at 25°C) and then HRP-conjugated goat antimouse or antirabbit IgG (Pierce), diluted 1:5,000. Signals were detected by chemiluminescence using the ECL Western blotting detection reagents from Amersham Pharmacia. Where indicated, blots were stripped of antibodies after signal detection by incubation for 30 min at 50°C in 2% SDS in Tris-CI (pH 6.7) with 2 mM β-ME and then reblocked and reprobed with a different antibody.

Surface Biotinylation and Immunoprecipitation. Adherent cells were rinsed with ice-cold PBS, then incubated with 0.5 mg/ml of water-soluble, cell-impermeable EZ-Link Sulfo-N-hydroxysuccinimide-long chain-biotin (Pierce, Rockford, IL) in PBS for 40 min at 4°C. After rinsing dishes with cold Tris-buffered saline [50 mM Tris-CI (pH 7.5) and 150 mM NaCl], the reaction was quenched with 0.1 M glycine for 10 min. Cells were finally lysed with NP40 lysis buffer as described above.

For immunoprecipitation, a known quantity of lysate (300–1000 μg) was precleared with rabbit IgG immobilized onto Protein A-Sepharose CL-4B (Amersham Pharmacia). Precleared lysate was incubated for 2 h at 4°C with 5 μg anti-MT1-MMP (AB8153) immobilized onto protein A-Sepharose beads.

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Immune complexes on beads were washed 5 times with cold 0.1% Triton X-100 in 50 mM Tris-Cl (pH 7.4), 300 mM NaCl. Complexes were eluted for 5 min at 95°C in reducing 2X SDS sample buffer, and eluates were electrophoresed and transblotted as described above. Biotinylated proteins were detected with ExtrAvidin Peroxidase Conjugate (Sigma Chemical Co.) and ECL. For negative controls, 5 µg of rabbit IgG was substituted for anti-MT1-MMP. Alternatively, samples were incubated with protein A-Sepharose beads in the absence of antibody; a similar pattern of nonspecific bands was seen in either case.

**Immunofluorescence.** Cells were seeded on glass coverslips in DMEM/+ and grown to 60% confluency. The last 15 h of culture was in serum-free DMEM supplemented with 160 nM PMA. Cells were washed and fixed with phosphate-buffered 3% formaldehyde for 30 min at 25°C. After fixation, cells were washed with PBS and blocked with 10% normal goat serum (Zymed Laboratories; San Francisco, CA) in PBS for 30 min. MT1-MMP polyclonal antibody (AB8103) was diluted to 7 µg/ml in 10% normal goat serum in PBS and incubated with cells for 1 h. After washes, the cells were incubated with Texas-Red dye-conjugated goat antimouse secondary antibodies (Molecular Probes, Eugene, OR) for 1 h at 25°C. Slides were mounted in Prolong Antifade Medium (Molecular Probes) and stored at 4°C. Images were captured using an Olympus IX70 microscope using the Texas-Red filter and IP Lab software (Signal Analytics; San Jose, California).

**RESULTS**

**Degradation of Type I Collagen by Oral SCC Cells.** When seeded on a reconstituted film of fibrillar type I collagen, SCC-25 and SCC-15 cells, but not CAL 27 cells, degraded the subjacent collagen through to the plastic. Fig. 1A (top) shows a typical degradation assay in which SCC-25 cells were cultured as a central colony on collagen films in the absence or presence of 160 nM PMA. Collagen degradation was assessed at 24, 48, and 72 h after seeding. After 24 h, both basal and PMA-stimulated collagen degradation were visible. Degradation increased ~2–3-fold between day 1 and day 3 under both conditions and did not extend beyond the boundaries of the colonies (see also bottom right panel). Below in Fig. 1A, the stained residual
collagen under the PMA-treated cell buttons is shown at a higher magnification. The left and middle panels are the central portions of cell colonies after removal of cells, whereas the right panel shows the edge of the colony at day 3. Consistent with previous studies (25, 27), degradation occurred as focally discrete areas of lysis that merged over time. Enhancement of collagen degradation by PMA was already detectable within the first 24 h. In experiments (data not shown) in which PMA was removed after the first 24 h (media replaced with DMEM/BSA), there was still significant (~2-fold) enhancement of collagen breakdown at day 3 relative to 3 days without PMA. This result indicated that the effects of the first 24 h of PMA were maintained for at least 2 more days.

In the collagen dissolution assay, the SCC-15 cells behaved very similarly to the SCC-25 cells, with a comparable level of basal degradation visible at 24 h that progressed ~2.5-fold by day 3 (data not shown). PMA enhanced basal degradation from ~1.5-fold to 2.5-fold in these cells (data not shown).

In contrast to the SCC-25 and SCC-15 cells, the CAL 27 cells did not degrade the underlying collagen substrate in this assay (Fig. 1B), although they attached and spread on collagen as readily as the other two cell lines (data not shown). CAL 27 cells were assayed for collagen dissolution over 3 days, as in Fig. 1A. There was no detectable degradation by the cells at any time point, even when similar experiments were allowed to proceed for 7 days in the presence of PMA (data not shown). Lack of degradation by these cells was verified by phase contrast microscopy (data not shown).

Fig. 1C shows two independent assays of SCC-25 cells, illustrating the apparently complete inhibition of collagen degradation when natural and synthetic inhibitors of MMPs were included. In the top panel, collagen degradation after 3 days by PMA-treated SCC-25 cells (CTL, left well) was inhibited by the synthetic broad-spectrum MMP inhibitor BB-94 at 21 nM (right well). Similarly, the bottom panel shows PMA-treated SCC-25 cells in the absence (CTL, left well) or presence (right well) of 0.5 μg/ml TIMP-2. In contrast to the apparently complete inhibition by TIMP-2, TIMP-1 at 0.5 μg/ml (central well) resulted in only partial (50–60%) inhibition, illustrating the direct contribution of MT1-MMP to collagen degradation. Similar patterns of inhibition were also observed with SCC-15 cells (data not shown).

Serine and cysteine proteinases may contribute to the degradation of ECM components directly or indirectly by activation of latent MMPs (7, 36). To determine whether these classes of proteinases participate in the extracellular degradation of collagen fragments, we tested a variety of inhibitors in assays with SCC-25 and SCC-15 cells. We found that the serine proteinase inhibitor aprotinin (200 μg/ml; Fig. 1D), the plasmin inhibitor EACA (2–10 nm), and the cysteine proteinase inhibitor E-64 (10 μg/ml; data not shown) were each ineffective at blocking collagen dissolution. Likewise, leupeptin (10 μg/ml), an inhibitor of serine and cysteine proteinases with trypsin-like specificity, was also ineffective (data not shown). Taken together, these data show that subjacent collagen degradation by SCC-25 and SCC-15 cells occurred in the absence of growth factors, cytokines, and exogenous proteinases, was enhanced by PMA, and was strictly MMP-mediated.

**Activation of Endogenous MMP-2 (Gelatinase A) by Culture on Collagen Films.** Degradation was confined to the subjacent collagen, which implicated cell surface associated MMPs as opposed to MMPs in the bulk conditioned media. We began MMP profiling by identification of endogenous gelatinases in SCC-25 cells and examined their regulation by culture on type I collagen films. Fig. 2A shows a zymographic analysis of cell membranes, lysates, and conditioned media of SCC-25 cells cultured on either tissue culture plastic (Lanes 1, 3, and 5) or collagen I films (Lanes 2, 4, and 6). Endogenous gelatinases comigrated with purified human proMMP-2 (72 kDa gelatinase A) and proMMP-9 (92 kDa gelatinase B; Chemicon zymography standards) in control Lanes (not shown). Culture on collagen increased total MMP-2 levels and up-regulated the activation of proMMP-2 to the fully active 62 kDa species relative to culture on plastic. (The molecular masses of 66, 64, and 62 kDa referred to below are those of the nonreduced pro-, intermediate, and active MMP-2 species, respectively, in zymograms.) The identity of the 66 kDa gelatinase in SCC-25 cells was verified as proMMP-2 using specific antibodies in Western blot analysis (data not shown).

The activation of MMP-2 by culture on or within three-dimensional gels of type I collagen was described in various types of cells (37–41) and has been attributed to increased MT1-MMP expression and activity. The requirement for MMP activity was confirmed in our system by treatment of collagen-cultured cells with BB-94 or TIMP-2 before harvesting for zymography (data not shown). The experiment in Fig. 2B confirmed the requirement for fibrillar type I collagen in inducing MMP-2 activation, as observed by others (39–41). SCC-25 cells were plated in parallel on tissue culture plastic (Fig. 2B, Lane 1) and on dishes coated with poly-D-lysine (Lane 2), collagen I film (Lane 3), laminin-1 (Lane 4), fibronectin (Fib; Lane 5), or a thin coat (10 μg/ml) of type I collagen (Thin coat coll; Lane 6). Molecular size standards are indicated to the left. Positions of proMMP-9 and pro- and active MMP-2 are marked on right. Experiments shown are representative of more than three.
Detection of MT1-MMP in Oral SCC Cells. We next examined the cell lines for expression of MT1-MMP to examine its role in collagen degradation. Three major forms of MT1-MMP have been described in cells, including a proform of M₄ 63,000 (sometimes seen as M₄ 63,000/65,000 doublet), an active species at M₄ 55,000–60,000, and a catalytically inactive truncated fragment of M₄ 43,000–45,000, which includes the hinge domain (40, 42, 45). To identify the forms present in the oral SCC cell lines, we analyzed cell membranes and lysates by Western immunoblot using a polyclonal antibody (AB815) to the hinge domain. Fig. 3A shows typical Western blots of membranes from PMA-treated cells cultured on plastic (Lanes 1 – 3) and of lysates from collagen cultured cells (Lanes 4 – 6). In membranes from both HT-1080 fibrosarcoma cells (used as a positive control; Lane 1) and SCC-25 (Lane 2), a strong band of immunoreactivity was observed at M₄ 57,000 (thick arrow), which corresponds in molecular weight to the active species (40, 45). This band was present at greatly reduced levels in the CAL 27 membranes (Lane 3), although equal quantities of total membrane protein were loaded per lane. A minor band of M₄ 63,000 (open arrow) was also detectable in Lanes 1 – 3, possibly the pro-form. Other membrane preparations (SCC-25 and CAL 27; data not shown) have contained variable levels of proMT1-MMP at M₄ 63,000/65,000 as seen in Lanes 4 – 6, although the active M₄ 57,000 form in SCC-25 was always predominant. Consistent with previous studies, PMA-treated HT-1080 cells contained a lower molecular weight fragment of MT1-MMP corresponding to the M₄ 43,000 truncated form (Fig. 3A, Lane 1, thin arrow; Refs. 42, 43, 45). A slightly smaller fragment was also observed in Lanes 1 – 3 (more prominent in CAL 27), perhaps the result of additional processing of the M₄ 43,000 form. In separate Western blots of lysates from the three oral SCC cell lines (Fig. 3A, Lanes 4 – 6), the proMT1-MMP doublet at M₄ 63,000/65,000 was prominent. To confirm that the doublet was proMT1-MMP, Western blots of SCC-25 and CAL 27 lysates that had been probed with AB815 were stripped and reprobed with a mouse monoclonal antibody (AB8101; Chemicon); AB8101 reacted with a band corresponding to the lower band of the doublet in both cell lines (data not shown). Therefore pro-, active, and processed species of MT1-MMP are detectable in the oral SCC cells. Membrane preparations of SCC-25 cells are enriched in the active species.

A sensitive procedure for surface biotinylation and immunoprecipitation provided data about cell surface expression of MT1-MMP under conditions of the collagen degradation assay. AB815 immunoprecipitated active MT1-MMP efficiently from lysates of nonbiotinylated SCC-25 cells (data not shown). The Western blot in Fig. 3B (left panel) shows the typical pattern of bands present in immunoprecipitates from surface biotinylated cells, detected with avidin-HRP and ECL. Surface proteins on adherent SCC-25 cells were biotinylated, and then membrane extracts were immunoprecipitated with AB815 as described (Fig. 3B, Lane 2). Membrane extracts of biotinylated HT-1080 cells (Lane 1) were included for comparison. In both immunoprecipitates, avidin-HRP revealed a biotinylated band corresponding in molecular weight to the M₄ ~57,000 – 60,000 active form of MT1-MMP. The truncated M₄ 43,000 fragment (arrowhead) also stained prominently in HT1080 cells. Separate aliquots of the corresponding nonimmunoprecipitated membrane extracts were Western blotted and probed with AB815 (Fig. 3B, Lanes 4 and 5; HT-1080 and SCC-25, respectively) to compare the migration of the active species with the biotinylated bands (double-headed arrow). In comparison, the proform (open arrow) present in lysate from SCC-25 cells (Fig. 3B, Lane 6) was clearly separated from the active form in these gels. We conclude that the major surface biotinylated, immunoprecipitated band from SCC-25 cells is a processed (and most likely active) form of MT1-MMP.

Effects of PMA on MMP-2 Activation and MT1-MMP Processing. PMA amplifies the basal degradation of collagen, apparent after the first 24 h. To determine whether PMA also enhanced collagen-induced MMP-2 activation, SCC-25 cells were cultured on col-
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Fig. 4. Enhanced MMP-2 activation and MT1-MMP processing by PMA. A, zymographic analysis of lysates (60 μg/lane) of SCC-25 cells cultured on collagen in the absence (Lanes 1 and 3; 0.016% DMSO) or presence (Lanes 2 and 4) of 160 nM PMA (0.016% DMSO). Samples of Lanes 1 and 2 were collected after 6 h, and samples of Lanes 3 and 4 were collected after 30 h. Molecular size standards are indicated to the left. Positions of proMMP-9 and of pro- and active MMP-2 are marked on right. Zymogram shown is representative of three. B, time course of surface MT1-MMP expression in collagen-cultured SCC-25 cells in the absence (Lanes 1, 3, 5, and 7; 0.016% DMSO) or presence (Lanes 2, 4, 6, and 8) of PMA. Cells were plated in DMEM+/+ and incubated overnight, then media changed to DMEM/BSA supplemented with PMA or DMSO (vehicle) as indicated. After 5 min (Lanes 1 and 2), 30 min (Lanes 3 and 4), 6 h (Lanes 5 and 6), and 24 h (Lanes 7 and 8), cells were surface biotinylated and lysed. Five hundred μg of lysate/sample were immunoprecipitated with AB815. Eluted and reduced immunoprecipitates were resolved by 10% SDS-PAGE, Western blotted, and probed with avidin-HRP/ECCL. Lane 8, same sample as in Lane 8, immunoprecipitated with rabbit IgG as negative control. Molecular size standards are indicated to the left. * left, indicates position of nonspecific band. Experiment shown is representative of two independent experiments. Thin arrow, active MT1-MMP. Thick arrow, truncated MT1-MMP. Ab, absence (+) or absence (−) of immunoprecipitating antibody (AB815).

These data show that PMA treatment of SCC-25 cells accelerates collagen-induced MMP-2 activation, apparent by the first 24 h, and this corresponds in time to the appearance on cell surfaces of a Mr 43,000–processed form of MT1-MMP.

Comparison of CAL 27 and SCC-15 with SCC-25 Cells. We next examined the SCC-15 and CAL 27 cells for collagen-induced MMP-2 activation and MT1-MMP expression. Each cell line was plated in parallel onto plastic or collagen-coated wells and cultured for 48 h in the presence or absence of PMA. Lysates from each sample were analyzed by gelatin zymography (Fig. 5A). SCC-25 cells (Lanes 1–4) showed the characteristic increase in proMMP-2 activation on collagen relative to plastic (Lane 3 versus Lane 1) with enhancement by PMA (Lane 4 versus Lane 3). Intermediate forms of MMP-2 (Mr 64,000) are also visible in these gels. Likewise, collagen culture of SCC-15 cells resulted in a pronounced increase in endogenous proMMP-2 activation (Fig. 5A, Lane 11 versus Lane 9) and an apparent increase in levels of total MMP-2. PMA enhanced the activation of MMP-2 on both plastic and collagen in these cells (Lanes 10 versus Lane 9 and Lane 12 versus Lane 11). Synthesis of MMP-9 was stimulated by PMA in both SCC-25 (Lanes 2 and 4) and SCC-15 cells (Lanes 10 and 12), as observed in other systems (15), and active MMP-9 was detectable in PMA-treated SCC-15 cells (Lanes 10 and 12). In marked contrast, lysates from CAL 27 cells (Fig. 5A, Lanes 5–8) contained no detectable gelatinase activity under any conditions. In similar experiments, the CAL 27 cells did not express or activate gelatinases on other ECM substrates including laminin-1 and fibronectin, although proforms of MT1-MMP were detectable by Western blot in each sample (data not shown).

Cell surface expression of MT1-MMP was compared in the three cell lines by surface biotinylation and immunoprecipitation (Fig. 5B). Cells were cultured on collagen for 24 h in the presence of PMA and then surface biotinylated, lysed, and immunoprecipitated with AB815. Equal quantities of protein (1000 μg/sample) were immunoprecipitated from SCC-25 and CAL 27 lysates (Fig. 5B, Lanes 1 and 2), whereas only 400 μg could be immunoprecipitated from SCC-15 lysate (Lane 3). Immunoprecipitates were electrophoresed, transblotted, and probed with avidin-HRP. In both SCC-25 (Lane 1) and SCC-15 (Lane 3) cells, active (Mr 57,000) MT1-MMP was prominently stained (thin arrow). The lower molecular weight Mr 43,000 band was also visible in the SCC-15 sample (Lane 3, thick arrow), although it was not detectable in the SCC-25 sample in this experiment. In contrast to the strong staining of active MT1-MMP in SCC-25 and SCC-15 cells, this band was barely visible in the CAL 27 cells (Lane 2) under the same conditions. Parallel cultures of CAL 27 cells, which continued for 48 h before biotinylation, did not contain increased levels of surface-labeled Mr 43,000 MT1-MMP (data not shown). These data are also consistent with the Western blot of membrane samples (Fig. 5A), which showed reduced expression of active MT1-MMP in CAL 27 cell membranes relative to SCC-25 membranes.

Reduced expression of MT1-MMP on the surface of CAL 27 cells was also demonstrated by immunofluorescence staining (Fig. 5C). SCC-25, CAL 27, and SCC-15 cells cultured on glass coverslips were stained with a polyclonal antibody against the hemopexin domain of MT1-MMP (AB8103) and then Texas-Red-labeled secondary antibody. High levels of membrane staining as well as stippled intracellular staining were evident in SCC-25 cells (Fig. 5C, top). A similar intensity of immunofluorescence was observed in SCC-15 cells, with punctate membrane staining and stippled intracellular staining (bottom panel). In comparison, the CAL-27 cells showed much less total MT1-MMP staining (middle panel), with limited areas of weak punctate staining in membranes. The immunofluorescence data are in agreement with the surface biotinylation experiments (Fig. 5B), which
Fig. 5. SCC-15 cells but not CAL 27 cells activate endogenous MMP-2 upon collagen culture and express detectable cell surface MT1-MMP. A, cells from each line were plated in DMEM/BSA onto either plastic (PL; Lanes 1, 2, 5, 6, 9, and 10) or collagen-coated dishes (Coll; Lanes 3, 4, 7, 8, 11, and 12). After 3 h, either DMSO (0.016%; Lanes 1, 3, 5, 7, 9, and 11) or PMA (160 nM; Lanes 2, 4, 6, 8, 10, and 12) were added and culture continued for 2 days. Lysates were then prepared and analyzed by zymography (60 µg/lane). Molecular size standards are indicated to the left. Positions of pro- and active MMP-9, and pro-, intermediate, and active MMP-2 are marked on right. Experiments shown are representative of more than three. B, surface biotinylation and immunoprecipitation of MT1-MMP in SCC-25 (Lane 1), CAL-27 (Lane 2), and SCC-15 cells (Lane 3). Cells were plated on collagen-coated dishes and incubated overnight in DMEM/+. Dishes were rinsed and media changed to DMEM/BSA supplemented with 160 nM PMA. After 24 h, cells were surface biotinylated, lysed, and immunoprecipitated with AB815. One thousand µg/sample were immunoprecipitated in SCC-25 (Lane 2; SCC-25 and CAL-27, respectively), and 400 µg were immunoprecipitated in Lane 3 (SCC-15). Lane 4 (CTRL) is the same sample as in Lane 1, “immunoprecipitated” (1000 µg) with protein A-Sepharose alone as negative control. Eluted and reduced immunoprecipitates were resolved by 10% SDS-PAGE, Western blotted, and probed with avidin-HRP/ECL. Molecular size standards are indicated to the left. Positions of nonspecific bands, based on their positions in other negative control immunoprecipitations. Thin arrow, active MT1-MMP. Thick arrow, M, 43,000 truncated MT1-MMP fragment (Lane 3). Ab refers to presence (+) or absence (−) of immunoprecipitating antibody (AB815). Experiment shown is representative of three. C, immunofluorescence staining of MT1-MMP in adherent SCC-25 (top panel), CAL-27 (middle panel), and SCC-15 (bottom panel) cells. Cells were seeded on glass coverslips in DMEM/+. Images were captured on an Olympus IX70 microscope using the Texas-Red filter. The low level of staining in CAL 27 cells contrasts with the high level of membrane and intracellular staining of SCC-25 and SCC-15 cells. Negative controls (no primary antibody) showed no detectable immunofluorescence (data not shown).
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from the same cultures (Lanes 5 and 6) and lysates (Lanes 7 and 8) revealed much lower levels of active MMP-2 in CMK treated cells compared with controls (Lane 6 versus Lane 5; and Lane 8 versus Lane 7, respectively). Therefore, we conclude that CMK inhibits collagen degradation through the inhibition of proMT1-MMP processing.

Expression of MMP-1 and MMP-13 in Oral SCC Cells. Finally, the cell lines were examined for expression of MMP-1 and MMP-13, which are secreted interstitial collagenases implicated previously in the progression of oral SCC (8, 19). Fig. 7 shows a representative Western blot of lysates from SCC-25 and CAL 27 cells collected after 2 days of culture on either fibronectin (FN) or collagen films (Coll) in the absence of PMA, which was probed sequentially with the indicated antibodies. In these experiments, culture on fibronectin yielded results equivalent to those on tissue culture plastic. The top panel of Fig. 7 shows M, 52,000 proMMP-1 in lysates of both cell lines under either condition. Likewise, stripping and reprobing of this blot with anti-MMP-13 Ab revealed expression of M, 60,000 proMMP-13 in both cell lines (Fig. 7, middle panel). Interestingly, in the CAL 27 cells, MMP-13 was apparently induced several-fold by collagen culture relative to culture on fibronectin (Lane 4 versus Lane 3). Lysates of SCC-15 cells also had comparable levels of proMMP-1 and proMMP-13 under all culture conditions (data not shown). In separate experiments (data not shown), Western blots of 20× concentrated serum-free conditioned media (48 h) detected secreted proMMP-1 and proMMP-13 from all three cell lines when probed with monoclonal Abs IM35L (anti-MMP-1) and ID3 (anti-MMP-13). As seen in lysates, collagen culture up-regulated the secretion of proMMP-13 from CAL 27 cells (data not shown). Taken together, these data reveal constitutive expression and secretion of proMMP-1 and proMMP-13 in the collagen-degrading SCC-25 and SCC-15 cells as well as the nondegrading CAL 27 cells, although active species were not detectable by Western blot even in the presence of PMA (data not shown).

DISCUSSION

Three cell lines derived from the same histological site, SCC of the tongue, were assessed for their interstitial collagen-degrading capacities. The assay used was originally developed to examine dissolution of native reconstituted type I collagen fibrils by mucosal keratinocytes and human fibroblasts (24–27), and data from these studies implicated the secreted collagenases. Unlike our experimental system, several of these previous studies included trypsin or plasminogen in some assay wells, which stimulated secretion and/or activation of procollagenase-1 and concomitantly up-regulated degradation (24, 26). Undoubtedly, cells possess more than one mechanism for matrix degradation and exogenous trypsin or plasminogen may mobilize a set of MMPs distinct from those operating in our assays. More recently, the
importance of membrane localization of MMP activity has been demonstrated in several assays of cellular invasion and morphogenesis (47, 48). Therefore, we omitted exogenous activators of secreted collagenases and focused our efforts on identifying membrane-associated collagenolytic activity in cells from a highly invasive type of cancer, oral SCC.

The SCC-25 and SCC-15 cells degraded subjacent collagen fibrils over a period of 3 days in the absence of exogenous growth factors, cytokines, or proteases, which have been used to induce/activate secreted MMPs in other systems (14, 16, 17, 24–26, 49). Basal collagen degradation was enhanced ∼1.5–3-fold by the tumor-promoting phorbol ester, PMA. Both basal and PMA-stimulated collagen degradation were strictly MMP-mediated, as observed with keratinocytes and fibroblasts (25, 26). The CAL 27 cells, in contrast, seemed incapable of collagen dissolution even in the presence of PMA. All three cell lines had in common the constitutive expression of collagenase-1 (MMP-1) and collagenase-3 (MMP-13), which are secreted MMPs with activity against triple-helical collagens (7, 50). However, only the collagen-degrading SCC-25 and SCC-15 cells expressed detectable levels of active MT1-MMP on their surfaces, along with gelatinases A and B (MMP-2 and -9). Concomitantly, these cells were capable of MT1-MMP-mediated proMMP-2 activation, which was induced by collagen culture and further enhanced by PMA. When processing of the MT1-MMP zymogen was inhibited by the furin inhibitor peptide, collagen degradation was blocked. Taken together, we conclude that pericellular collagenolytic activity in oral SCC cells is mediated by membrane-associated MMPs, and that MT1-MMP is essential.

Tumor-promoting phorbol esters such as PMA induce the transcriptional activation of several secreted MMPs and MT1-MMP (7, 15, 32, 51, 52). The up-regulation of endogenous MMPs by PMA allowed us to detect processing of cell-surface MT1-MMP to the Mr 43,000 species, providing evidence of its functional activity as seen in fibroblasts and ovarian carcinoma cells (40, 42).

Tumor cell invasion of ECM barriers is a highly complex process involving multiple interactions with host components (3, 4). In particular, dynamic adhesion of tumor cells to ECM, proteolytic modification of the matrix, and migration through the proteolyzed region must be coordinated both spatially and temporally for effective invasion to occur. Experimental analysis of such a complex process can be simplified somewhat by focusing on one aspect of invasion, i.e., proteolysis of matrix barriers or chemotactic migration of tumor cells. Our collagen degradation assay provides an assessment of the tumor cells’ proteolytic capacities in the absence of a migratory stimulus. Interstitial collagens are the most abundant proteins of the ECM, and the endogenous capacity of tumor cells to degrade this matrix may confer an advantage over nondegrading cells in situations where available growth factors and cytokines were limiting. Head and neck SCCs, which include oral SCC, are highly invasive cancers and a major cause of cancer morbidity and mortality (1, 2, 53). Therefore, understanding the molecular mechanisms by which oral SCC cells proteolyze and invade interstitial collagen barriers is of vital importance. Preliminary data using Transwell invasion assays have shown that although all three cell lines can migrate at comparable rates over uncoated filters toward a chemotactic stimulus (10% fetal bovine serum in DMEM), their capacity to invade a film of type I collagen and translocate to the underside of the filter correlates with their capacity to proteolyze type I collagen in the two-dimensional degradation assay (data not shown). Furthermore, as with collagen degradation, invasion of collagen by SCC-25 and SCC-15 cells is inhibited >99% by TIMP-2 and only 50–60% by TIMP-1 (data not shown), supporting the hypothesis that active MT1-MMP is essential for this process.

The SCC-25 cells were shown in the current and in previous studies to express MMPs -1, -2, -3, and -9 under routine culture conditions (54–56). In addition, we demonstrated constitutive expression of MT1-MMP and MMP-13 along with the same repertoire of MMPs in the SCC-15 cells. Upon discovering that MT1-MMP and MMP-2 were reduced or absent in the nondegrading CAL 27 cells, we examined their regulation in the degrading cells by collagen culture and PMA, conditions which mimicked the degradation assay. Culture on type I collagen films resulted in increased production and activation of cell-associated MMP-2 in both SCC-25 and SCC-15, which was an indirect indicator of increased MT1-MMP activity. PMA accelerated this process within 24 h and concomitantly up-regulated the processing of MT1-MMP to a Mr 43,000 fragment. Because MT1-MMP has been demonstrated to proteolyze type I collagen in cellular assay systems (48), we hypothesized that MT1-MMP may be responsible for collagen degradation by the oral SCC cells either alone or in concert with MMP-2. In support of this hypothesis, inhibition of degradation by TIMP-2 seemed complete, whereas inhibition by TIMP-1 was only 50–60%. Because TIMP-1 is known to be a poor inhibitor of MT1-MMP (57), residual degradation in the presence of TIMP-1 is directly mediated by MT1-MMP. Blocking the processing of the MT1-MMP zymogen to its active form with the furin inhibitor peptide resulted in near complete (>99%) inhibition of collagen degradation, providing additional evidence of the involvement of MT1-MMP. Taken together, we conclude that MT1-MMP has a direct role in the proteolysis of type I collagen, although the concerted action
of MT1-MMP with other MMP(s)—possibly MMP-2—is required for complete clearance of collagen through to the plastic.

Culture of several different cell types on or within three-dimensional type I collagen gels has been shown to induce the MT1-MMP-mediated activation of proMMP-2 (17, 38–41, 58). In most of these cells, collagen culture up-regulates MT1-MMP mRNA and/or protein levels. These in vitro data, together with in vivo data from invasive tumors showing coexpression of MT1-MMP with MMP-2 and/or collagen I has led to the proposal that this mechanism contributes to tumor invasion and metastasis (18, 58–61). Recent studies with tumor tissue from head and neck SCCs (including oral SCC) have shown overexpression of MT1-MMP mRNA in tumor tissue relative to corresponding normal tissue (20), and MT1-MMP protein was detected in the tumor cells (12, 20). High expression levels of MT1-MMP protein, along with its colocalization with MMP-2, was linked to the more invasive and metastatic cases (12). Therefore, the collagen-induced proMMP-2 activation observed in vitro in the SCC-25 and SCC-15 cells may reflect their aggressiveness in vivo. The increased processing of MT1-MMP to a Mₙ 43,000 fragment when PMA was added to collagen cultured cells is similar to the effects of PMA on HT-1080 cells, and the presence of this form has been correlated with increased activation of MMP-2 (40, 42). Our studies have now demonstrated this correlation in the oral SCC cells as well. Because this fragment is catalytically inactive, it is believed to represent down-regulation of the MT1-MMP cycle of activity on the cell surface (42, 43, 62).

MT1-MMP and stromelysin-3 are unique among the MMPs in having an RXXR recognition motif for furin and furin-like convertases at the COOH-terminal end of the propeptide domain (21, 63). Processing of proMT1-MMP by furin (64) is believed to be the major mechanism for conversion of thezymogen to its active form (65, 66). In previous studies, the furin inhibitor peptide CMK at 10–100 µM reduced intracellular processing of proMT1-MMP and inhibited both MMP-2 activation and in vitro invasion (38, 44, 46). In our system, additional confirmation of the involvement of MT1-MMP in pericellular collagen degradation came from the clear dose-dependent inhibition of degradation in the presence of the furin inhibitor peptide. The corresponding increase in proMT1-MMP and reduced active species in peptide-treated cells confirmed the mechanism of action of the inhibitor.

We cannot explain the relative lack of active, surface-expressed MT1-MMP on CAL 27 cells with the available data. Using RT-PCR, furin mRNA was detected in all three cell lines (data not shown). We also amplified and sequenced RT-PCR products corresponding to MT1-MMP mRNA (first 732 nucleotides) from SCC-25 and CAL-27 cells and found that the sequences showed no mutations or other changes in the propeptide-coding region relative to wild-type human cells and found that the sequences showed no mutations or other changes in the propeptide-coding region relative to wild-type human cells. Tumors showing coexpression of MT1-MMP with MMP-2 and/or collagen I has led to the proposal that this mechanism contributes to tumor invasion and metastasis (18, 58–61). Recent studies with tumor tissue from head and neck SCCs (including oral SCC) have shown overexpression of MT1-MMP mRNA in tumor tissue relative to corresponding normal tissue (20), and MT1-MMP protein was detected in the tumor cells (12, 20). High expression levels of MT1-MMP protein, along with its colocalization with MMP-2, was linked to the more invasive and metastatic cases (12). Therefore, the collagen-induced proMMP-2 activation observed in vitro in the SCC-25 and SCC-15 cells may reflect their aggressiveness in vivo. The increased processing of MT1-MMP to a Mₙ 43,000 fragment when PMA was added to collagen cultured cells is similar to the effects of PMA on HT-1080 cells, and the presence of this form has been correlated with increased activation of MMP-2 (40, 42). Our studies have now demonstrated this correlation in the oral SCC cells as well. Because this fragment is catalytically inactive, it is believed to represent down-regulation of the MT1-MMP cycle of activity on the cell surface (42, 43, 62).

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In conclusion, we postulate that tumor cells with an endogenous capacity for collagenolysis would be more independent of host factors at an early stage of progression and have an advantage over tumor cells without this capacity. Our data illustrate the potential importance of tumor cell-derived MT1-MMP in progression of oral SCC cells.

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REFERENCES

COLLAGEN DEGRADATION BY ORAL SQUAMOUS CELL CARCINOMA


Membrane Type I-Matrix Metalloproteinase-Mediated Degradation of Type I Collagen by Oral Squamous Cell Carcinoma Cells
