Induction of Mr 92,000 Type IV Collagenase Expression in a Squamous Cell Carcinoma Cell Line by Fibroblasts

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ABSTRACT

A previous investigation (Matsumoto et al., J. Oral Pathol. Med., 18: 498–501, 1989) has shown that the in vitro invasion of a collagen gel by squamous cell carcinoma can be substantially augmented in the presence of fibroblasts. Therefore, we undertook a study to determine if the production of collagenase(s) by a squamous cell carcinoma cell line, UM-SCC-1, was up-regulated by fibroblasts. Cocultivation of UM-SCC-1 cells with MDA-TU-138 fibroblasts, both established from the oral cavity, resulted in a dose-dependent increase in the activity of a Mr 92,000 gelatinase as shown by zymography. Augmented Mr 92,000 gelatinase activity was a consequence of the stimulation of the UM-SCC-1 cells by a soluble, fibroblast-derived factor since this effect could be reproduced with fibroblast-conditioned medium but not with glutaraldehyde-fixed fibroblasts. The increased Mr 92,000 gelatinolytic activity could be accounted for by an increase in Mr 92,000 type IV collagenase (MMP-9) protein, as demonstrated by Western blotting for this metalloproteinase. Trypsin treatment of the fibroblast-conditioned medium abolished its ability to increase MMP-9 secretion by UM-SCC-1 cells. Furthermore, fractionation of the fibroblast-conditioned medium revealed a Mr 3,000–10,000 soluble factor(s) which was responsible for the augmented production of MMP-9 by UM-SCC-1 cells. To determine if the increased production of MMP-9, in response to the fibroblasts, was a consequence of increased promoter activity, UM-SCC-1 cells were transiently transfected with a chloramphenicol acetyltransferase reporter driven by the MMP-9 promoter and plated on plastic or on a monolayer of MDA-TU-138 fibroblasts. A 4–5-fold stimulation of MMP-9 promoter activity was observed with UM-SCC-1 cells plated with the MDA-TU-138 fibroblasts, when compared with similarly transfected cells recultured on plastic. In conclusion, we have demonstrated that MMP-9 expression in a squamous cell carcinoma cell line is augmented by a fibroblast-derived protein(s). This finding indicates a role for stromal cells in the regulation of MMP-9 expression in squamous cell carcinoma. The ability of fibroblasts to regulate MMP-9 expression in tumor cells in vitro may explain the observation that the amount of Mr 92,000 type IV collagenase mRNA in tumor cells is highest at the tumor/stromal interface of resected squamous cell carcinoma.

INTRODUCTION

The cause of mortality in most malignancies is the dissemination of the disease to distant sites. Although the mechanism(s) which underlie the spread of cancer cells to secondary sites is still poorly understood, it is widely accepted that breach of the basement membrane dissolution is the type IV collagen-degrading gelatinase B (Mr 92,000 type IV collagenase, also referred to as MMP-9; Refs. 5 and 6). One of the first observations which suggested a role for this metalloproteinase in tumor invasion was that the release of this collagenase correlated with the metastatic phenotype of transformed rat embryo cells (5). Subsequent studies reported similar findings with rat 13762NF mammary adenocarcinoma (7). However, the most compelling evidence supporting a role for this type IV collagenase in tumor invasion comes from a recent study by Bernhard et al. (6), which showed that the overexpression of this metalloproteinase in nonmetastatic rat embryo cells conferred a metastatic phenotype upon these cells.

While it appears that this metalloproteinase contributes to the invasive phenotype of several cancers including SCC, the regulation of the expression of this gene is still poorly understood. Certainly, there is evidence that genetic factors including activated oncogenes, such as c-Ha-ras and v-src, induce the expression of this collagenase, and presumably this contributes to the basal expression of MMP-9 by the tumor cells (5, 8). However, the effect of neighboring stromal cells on the control of MMP-9 expression in tumor cells has yet to be examined. A study by Matsumoto et al. (1), which demonstrated that fibroblasts promoted the invasion of 5 SCC cell lines into a collagen gel, together with the observation that MMP-9 mRNA levels in the tumor cells of SCC are highest at the tumor/stroma interface (9), prompted us to investigate the regulation of MMP-9 production by fibroblasts. We show, herein, that a soluble fibroblast-derived protein augments MMP-9 production in a SCC cell line and that this is by way of increased MMP-9 promoter activity.

MATERIALS AND METHODS

Materials. Membrane inserts (1 μm pore size) were obtained from Becton Dickinson Labware (Franklin Lakes, NJ). Centricon columns, with various molecular weight cutoffs, were purchased from Amicon (Beverly, MA).

Cell Culture. All cells were maintained in McCoy’s 5A medium supplemented with 10% FBS. Serum-free medium used for the collection of conditioned medium consisted of McCoy’s 5A supplemented with 4 μg/ml transferrin, 5 μg/ml insulin, and 10 ng/ml EGF. UM-SCC-1 cells, derived from a SCC of the oral cavity, were obtained from Dr. Thomas Carey at the University of Michigan. MDA-TU-138 fibroblasts were isolated from the outgrowth of a SCC of the oral cavity. Immunocytochemical staining of the cells with an anti-collagen III antibody revealed strong reactivity, indicating their stromal origin. Additionally, karyotyping of the MDA-TU-138 fibroblasts revealed a normal karyotype. All experiments made use of MDA-TU-138 fibroblasts prior to passage 20.

Zymography. These were performed as described previously (10). Culture supernatants were denatured in the absence of reducing agent and electrophoresed in a 7.5% SDS-polyacrylamide gel containing 0.1% (w/v) gelatin. The gel was incubated at room temperature for 2 h in the presence of 2.5% Triton X-100 and subsequently at 37°C overnight in a buffer containing 10 mM CaCl2, 0.15 mM NaCl, and 50 mM Tris (pH 7.5). The gel was then stained for protein with 0.25% Coomassie and photographed on a light box. Proteolysis was detected as a white zone in a dark field.
Western Blotting. These were carried out as described elsewhere (10) but with modifications. Conditioned medium was denatured under reducing conditions and electrophoresed in a 7.5% SDS-polyacrylamide gel. The resolved proteins were transferred to a nitrocellulose membrane. The membrane was blocked with a solution containing 3.0% BSA and incubated sequentially with an anti-MMP-9 antiserum (1:2000 dilution) and an anti-rabbit-horseradish peroxidase conjugate. Reactive proteins were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL) as described by the manufacturer.

CAT Assays. UM-SCC-1 cells were, at 70% confluency, transfected with CAT reporter constructs fused to full-length or 5'-truncated MMP-9 and urokinase promoters (11–13). All transient transfections were performed in the presence of 5 μg of an expression vector bearing the β-galactosidase gene to correct for differences in transfection efficiencies. Briefly, DNA, in 1 ml of buffered saline (pH 7.44) (25 mM HEPES, 1 mM MgCl2, 0.1 mM CaCl2, 0.1 mM NaCl, 5 mM KCl, and 0.7 mM Na2HPO4) was mixed with 50 μg hexadimethrine bromide (Aldrich Chemicals, Milwaukee, WI) and added to the cells in 10% FBS. After 5 h, the cells were shocked for 3 min with 30% glycerol and cultured for 16 h. The cells were harvested and replated on plastic or with a near-confluent (90%) monolayer of MDA-TU-138 fibroblasts. After 24–48 h, the cells were harvested and lysed by repeated freeze-thaw cycles in a buffer containing 0.25 M Tris-Cl (pH 7.8). Transfection efficiencies were determined by assaying for β-galactosidase activity. CAT activity was subsequently measured by incubating cell lysate (normalized for transfection efficiency) at 37°C for 16 h with 4 μM [3H]chloroamphenicol and 1 mg/ml Acetyl-CoA. The mixture was extracted with ethyl acetate, and acetylated products were subjected to thin layer chromatography using chloroform:methanol (95:5) as a mobile phase.

RESULTS

Cocultivation of a SCC Cell Line with MDA-TU-138 Fibroblasts Leads to Elevated Gelatinase Activity. As reported previously (10), UM-SCC-1 cells produce M, 92,000 gelatinase (M, 92,000 type IV collagenase, also referred to as MMP-9) as evident by zymography and Western and Northern blotting experiments, whereas MDA-TU-138 fibroblasts, which are deficient in this enzyme, secrete a M, 72,000 gelatinase which is indistinguishable in size from MMP-2 (Fig. 1). When a constant number of UM-SCC-1 cells were cocultured with an increasing number of MDA-TU-138 fibroblasts, a dose-dependent increase in M, 92,000 gelatinase activity (Fig. 1) was evident. In contrast, M, 72,000 gelatinase activity was not augmented by cocultivation of the two cell populations (compare 100% confluent fibroblasts alone (Fig. 1, last lane) with coculture of UM-SCC-1 and fibroblasts (Fig. 1, second to last lane). The increase in MMP-9 is unlikely to be a consequence of a stimulation of UM-SCC-1 growth rate in the presence of fibroblasts, since the number of cells in cocultures did not exceed the sum of the cell number of UM-SCC-1 cells and fibroblast cells in independently maintained cultures.

MDA-TU-138 Fibroblasts Release a Soluble Factor Which Increases the Amount of MMP-9 Protein Secreted by UM-SCC-1 Cells. To determine if the increase in M, 92,000 gelatinase activity was mediated by a soluble factor, UM-SCC-1 and MDA-TU-138 fibroblasts were cultured in chambers separated by a solute-permissive membrane. The conditioned medium from both chambers was pooled and analyzed by gelatin zymography. Fig. 2 shows an increase in M, 92,000 gelatinase activity in response to an increasing number of MDA-TU-138 fibroblasts. In contrast, cocultivation of the two populations of cells did not result in augmented M, 72,000 gelatinase activity (Fig. 2, compare last two lanes).

To determine if the MDA-TU-138 fibroblasts were the source of the soluble M, 92,000 gelatinase-stimulating factor, conditioned medium from these cells was added to cultured UM-SCC-1 cells. After 1 day of culture, the UM-SCC-1 culture supernate (conditioned medium) was collected and assayed for gelatinase activity and for MMP-9 protein by zymography and Western blotting, respectively. A dose-dependent increase in M, 92,000 gelatinase activity (Fig. 3A), which was paralleled by an increase in the amount of MMP-9 protein (Fig. 3B), could be detected in the culture supernates of the UM-SCC-1 cells stimulated with MDA-TU-138 fibroblast-conditioned medium. It is unlikely that the elevation of M, 92,000 gelatinase activity is a consequence of increased cell growth since we found no evidence of increased proliferation of UM-SCC-1 cells in the presence of fibroblast-conditioned medium. The induction of M, 92,000 gelatinase activity was evident in two of three established SCC cell lines tested (data not shown), suggesting that the response of UM-SCC-1 cells may be representative of a subpopulation of tumor cells present in these malignancies. Thus, these data suggest that a soluble factor present in the MDA-TU-138 fibroblast-conditioned medium augments MMP-9 protein levels in cultures of UM-SCC-1 cells. To further investigate this contention, UM-SCC-1 cells were plated with viable (PBS-treated) or glutaraldehyde-fixed fibroblasts, and conditioned medium was collected from the cocultures. The viable fibroblasts stimulated M, 92,000 gelatinase activity in the cocultures. In contrast, the glutaraldehyde-fixed fibroblasts (Fig. 4), which failed to release M, 72,000 gelatinase, did not augment M, 92,000 gelatinase secretion by UM-SCC-1 cells.

Trypsin Sensitivity and Estimation of the Molecular Size of the MMP-9-Inducing Fibroblast-derived Soluble Factor. To determine if the MMP-9-inducing activity derived from the MDA-TU-138 fibroblasts was a protein(s), conditioned medium from the stromal cells was treated with trypsin. Trypsin activity was subsequently neutralized with soybean trypsin inhibitor. Untreated or trypsin-treated fibroblast-conditioned medium was added to 80% confluent
fibroblasts, compared with similarly transfected cells plated on plastic (Fig. 7, A and B). The inductive effect of the fibroblasts on MMP-9 promoter activity in UM-SCC-1 cells did not reflect a general increase in transcriptional activation in these cells since we did not observe a similar stimulation when using the urokinase promoter fused to a CAT reporter (Fig. 7C). These data suggest that the fibroblast-dependent increase in MMP-9 production by UM-SCC-1 cells reflects, at least in part, increased activity of the metalloproteinase promoter.

**Fig. 2.** A soluble factor mediates the enhanced Mr 92,000 gelatinase activity evident in cocultures of UM-SCC-1 cells and MDA-TU-138 fibroblasts. UM-SCC-1 cells (100,000) were cultured on a 1-μm solute-permissive membrane. The membrane inserts, with UM-SCC-1 cells, were suspended in 35-mm dishes containing medium alone (first lane) or MDA-TU-138 fibroblasts at varying levels of confluency. After 24 h of culture, both chambers were washed with serum-free medium and replenished with fresh serum-free medium. After another 24 h, aliquots of equal volume from conditioned medium pooled from both chambers were analyzed for gelatinolytic activity by zymography. The position of the Mr 92,000 (92 kDa) and Mr 72,000 (72 kDa) gelatinases are shown. Fibroblasts lane, conditioned medium collected from 100% confluent MDA-TU-138 fibroblasts grown alone. Representative data of two separate experiments are shown.

UM-SCC-1 cells. After 24 h, the UM-SCC-1 culture supernate was harvested and analyzed by zymography (Fig. 5). Trypsin-treated fibroblast-conditioned medium did not stimulate MMP-9 activity in the cultured UM-SCC-1 cells. Thus, these data suggest that the soluble MMP-9-inducing factor(s) released from the fibroblasts consists of one or multiple proteins.

To estimate the molecular size of the MMP-9-inducing factor, fibroblast-conditioned medium was fractionated on gel filtration columns with varying cutoff membranes. The eluant (flow-through) was collected and tested for its ability to increase MMP-9 activity in cultures of UM-SCC-1 cells (Fig. 6). The flow-through generated with the Mr 3,000 cutoff filter did not augment MMP-9 activity in culture supernates of UM-SCC-1 cells. In contrast, increased Mr 92,000 gelatinase activity was observed with UM-SCC-1 cells stimulated with the flow-through from a Mr 10,000 membrane. Thus, these data suggest that the MMP-9-stimulating activity in fibroblast-conditioned medium has a molecular weight between 3,000 and 10,000.

**Stimulation of MMP-9 Promoter Activity in UM-SCC-1 Cells Cultured in the Presence of MDA-TU-138 Fibroblasts.** Increased levels of MMP-9 in response to a divergent set of stimuli has been ascribed to increased transcriptional activation of the gene (8, 11). Accordingly, to determine the role of increased MMP-9 promoter activity in the induction of metalloproteinase synthesis by the MDA-TU-138 fibroblasts, UM-SCC-1 cells were transiently transfected with a CAT reporter gene driven by the full length (670 base pairs) MMP-9 promoter or the minimal (73 base pairs) promoter. After 24 h, the cells were replated either on plastic or with MDA-TU-138 fibroblasts. The cells were harvested 1 day later and assayed for CAT activity.

A strong (4—5-fold) induction of CAT activity was observed with UM-SCC-1 cells transfected with the full-length promoter-CAT reporter (but not the minimal promoter) and plated with the MDA-TU-138 fibroblasts, compared with similarly transfected cells plated on plastic (Fig. 7, A and B). The inductive effect of the fibroblasts on MMP-9 promoter activity in UM-SCC-1 cells did not reflect a general increase in transcriptional activation in these cells since we did not observe a similar stimulation when using the urokinase promoter fused to a CAT reporter (Fig. 7C). These data suggest that the fibroblast-dependent increase in MMP-9 production by UM-SCC-1 cells reflects, at least in part, increased activity of the metalloproteinase promoter.

**Fig. 3.** A soluble fibroblast-derived factor increases Mr 92,000 gelatinase activity via an increased amount of protein. UM-SCC-1 cells were grown to 70% confluency in 10% FBS and washed two times with serum-free medium. The cells were replenished with serum-free medium (Control) or fibroblast-conditioned medium (CM) undiluted (1.0) or diluted 2-, 4-, and 8-fold in serum-free medium. After 1 day, the culture supernates were collected and clarified by centrifugation; then the UM-SCC-1 cells were counted with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Aliquots of the culture supernates, normalized to cell number, or undiluted fibroblast-conditioned medium were subjected to zymography (A) and Western blotting (B) for MMP-9 protein. The experiment was repeated twice.

**Fig. 4.** Cell-cell contact is an insufficient stimulus for MMP-9 induction. MDA-TU-138 fibroblasts were grown in 35-mm dishes to the indicated level of confluency and treated for 5 min with PBS or 2.5% glutaraldehyde in PBS. The cells were washed extensively with PBS. UM-SCC-1 cells (250,000) were plated on plastic or with the fibroblast monolayers; 24 h later, they were washed and changed to serum-free medium. Conditioned medium was harvested and clarified by centrifugation 1 day later. Aliquots of the conditioned supernatants were subjected to gelatinase activity by zymography. Fibroblasts lane, conditioned medium collected from fibroblasts cultured alone. The position of the Mr 92,000 (92 kDa) and Mr 72,000 (72 kDa) gelatinases are shown. Data are typical of three separate experiments.
mechanisms (autocrine loops and oncogene activation) in driving MMP-9 expression (8, 11, 13–15), largely ignoring the contribution of stromal:tumor cell interactions to this process. We report, for the first time, on the induction of MMP-9 expression in a SCC cell line by a fibroblast-derived protein(s). This finding may explain, at least in part, two previous observations from other investigators: (a) fibroblasts stimulate the invasion of SCC cells into a collagen gel (1); and (b) that the level of MMP-9 mRNA in tumor cells is highest at the tumor/stromal interface of SCC (9).

In an earlier investigation, Henry et al. (16) had observed augmented degradation of type IV collagen by Lewis lung carcinoma in the presence of conditioned medium from peritoneal macrophages. However, the identity of the collagenase was not established nor was it clear whether the increased collagenolysis was a consequence of altered kinetics of the proteolytic cleavage or reflected the production of more enzyme by the Lewis lung carcinoma. In a more comprehensive study, Gohji et al. (17) found an up-regulation of the Mr 72,000 type IV collagenase (MMP-2) activity in human renal cell carcinoma.

**DISCUSSION**

In order for tumor cells to demonstrate an invasive phenotype, they must first breach the basement membrane which separates the epithelial and stromal compartments. Towards this end, tumor cells elaborate one or multiple proteases, which degrade key components of the basement membrane (2). The M, 92,000 type IV collagenase (MMP-9) has been implicated in this process by virtue of its ability to degrade type IV collagen present in the basement membrane (12). Consequently, several studies have addressed the mechanism(s) by which the expression of this metalloproteinase is regulated in certain malignancies including SCC of the head and neck. However, most of these investigations have focused on the role of constitutive.
in response to fibroblasts or fibroblast-conditioned medium. While these reports document an inductive effect of a non-tumor cell population on collagenase activity/production by a tumor cell population, other reports have suggested that this stimulation can, in fact, be in the opposite direction. Thus, Himelstein et al. (18) presented convincing data that MMP-9 production in rat embryo fibroblasts was induced by metastatic (but not by non-metastatic) tumor cells. In a similar vein, Goslen et al. (19) demonstrated that collagenase production by skin fibroblasts could be stimulated with extracts of basal cell carcinomas. This being attributed to a macromolecule of Mr ~19,000.

We considered the possibility that the MDA-TU-138 fibroblast-dependent augmentation of MMP-9 expression in the UM-SCC-1 cell line was a consequence of cell-cell contact. Certainly, physical cell-cell interactions have been shown to be critical for both the induction of protease expression in cocultures of rat embryo fibroblasts and transformed rat embryo cells (18), as well as the fibroblast-dependent activation of MMP-2 produced by lung tumor cells (20). However, for cultured UM-SCC-1 cells, it is unlikely that cell-cell interaction is sufficient for the elevated production of MMP-9 for several reasons: (a) addition of the fibroblast-conditioned medium to the UM-SCC-1 cells reiterated the induction of MMP-9 protein seen with coculturing of the two cell populations; (b) this stimulation of MMP-9 production was preserved following separation of the tumor and fibroblast cells by a porous filter; (c) culturing of the SCC cells on glutaraldehyde-fixed fibroblasts completely abolished the stimulation of MMP-9 production, suggesting that cell-cell interaction was, by itself, insufficient for the induction of MMP-9. Thus, the most plausible explanation is that the stromal cells produce a soluble factor which stimulates MMP-9 production in the tumor cells. Indeed, several cytokines as well as extracellular matrix components (21, 22), such as type I collagen, can substantially augment MMP-9 production in diverse cell types including keratinocytes. Although the identity of the fibroblast-derived endogenous protein(s) responsible for the elevated production of MMP-9 in the SCC tumor cells remains to be determined, our gel filtration studies indicate that a relatively small (Mr 3,000–10,000) protein(s) is responsible for the stimulation.

In summary, we have found that the expression of the Mr 92,000 type IV collagenase (MMP-9) in a SCC cell line is up-regulated by a soluble fibroblast-derived protein(s). This finding argues for a role of stromal cells in regulating the proteolytic potential of invasive SCC via the modulation of a key metalloproteinase implicated in extracellular matrix degradation.

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