Objective: To elucidate tumor-stromal interactions during tumor invasion by assessing the expression of proteolytic enzymes by carcinoma-associated fibroblasts (CAFs) in vivo using complementary DNA (cDNA) array analysis.

Methods: Tumor-associated stroma was isolated from tumor and adjacent mucosal specimens of the same patient by laser capture microdissection, and the messenger RNA (mRNA) was assessed by cDNA microarray specific for proteolytic enzymes and their inhibitors. Protein overexpression was then analyzed by immunoblotting of primary fibroblast isolates derived from skin, mucosa, and tumor specimens.

Results: Array analysis of 4 tumor and 4 adjacent mucosal samples demonstrated significant (2.6-fold) overexpression of membrane type 1 matrix metalloproteinase (MT1-MMP) but not of serine proteases or other matrix metalloproteinases. Analysis of normal dermal fibroblasts, normal mucosal fibroblasts, and CAFs similarly demonstrated up-regulation of MT1-MMP.

Conclusions: These results suggest that MT1-MMP mRNA is specifically up-regulated in CAFs in vivo whereas MT1-MMP protein is specifically up-regulated in CAFs in vitro. Known to induce tumor cell invasion when expressed in tumor cells, CAF expression of MT1-MMP may be important in the stromal response to tumor cells that characterizes the desmoplastic reaction.

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ARCINOMAS DEVELOP FROM cells that have undergone genetic mutations resulting in the deregulation of normal growth-controlling mechanisms. Research on the origins of cancer has focused on the study of tumor cells, and has considered tumorigenesis as an independent process governed by genes carried by the tumor cells. The desmoplastic response characteristic of head and neck carcinomas consists of fibroblasts, inflammatory cells, and endothelial cells. The secretion of growth factors, matrix proteins, and proteases by fibroblasts and tumor cells creates a tumor microenvironment that facilitates tumor growth and invasion. Identification of protease elaboration in breast cancers by in situ hybridization was followed by similar discoveries in other tumors, including head and neck cancers.

Fibroblast-derived proteases (rather than those expressed by tumor cells) are considered necessary for extracellular matrix remodeling and play a role in wound healing. The proliferation of carcinoma-associated fibroblasts (CAFs), with the growth factors, proteases, and other proteins that these stromal cells secrete, significantly influences cancer cell invasion and metastasis. Studies have demonstrated that CAFs can affect tumor cell invasion, growth, and transformation. If CAFs can promote malignant transformation, they may be a target for medical intervention. New therapeutic strategies targeting the supporting elements in the tumor microenvironment are a novel approach to inhibiting tumor growth. Exploitation of these new treatments is only possible with a better understanding of the mechanism(s) by which fibroblasts support tumor cell growth and invasion.

We sought to identify proteases and their inhibitors that are elaborated by head and neck CAFs during the desmoplastic response in order to define precise molecular targets, and focused on genes expressing matrix metalloproteinases (MMPs) and serine proteases. In investigations on stromal contributions to malignant transformation of epithelial cells, MMPs have been found to play an important role in tumor-host communication. This is especially true at the intimate “invasive front” of an infiltrating tumor. The MMPs consist of a group of more than 25 structurally related enzymes that are characterized by their Zn²⁺-dependent proteolytic activity. They
have been implicated in extracellular matrix degradation, the modulation of inflammatory responses and local immune function, tissue remodeling, angiogenesis, and metastatic spread.

METHODS

LASER CAPTURE MICRODISSECTION

The patients gave consent and the procedure was performed according to institutional guidelines. Specimens from adjacent mucosal tissue and squamous cell carcinoma were obtained intraoperatively from the oral cavity and oropharynx of previously untreated patients who were undergoing tumor ablation. Mucosal tissue was obtained from uninvolved intraoral mucosa and confirmed by histologic analysis at the time of microdissection. The specimens were embedded in carbamyl transferase medium (Tissue-Tek OCT Compound; Miles Scientific, Naperville, Ill) and snap-frozen at −80°C. Frozen sections were cut, stained with hematoxylin, and dissected using the Arcturus PixCell I Laser Capture Microdissection System (Arcturus Engineering, Mountain View, Calif). Stromal tissue (˃5×10⁵ cells from each sample) was selectively collected from tumor tissue and adjacent mucosa using laser capture microdissection as described previously.¹¹ Laser capture microdissection conditions were as follows: laser spot size, 15 μm; laser power, 65 mW; and laser pulse duration, 2 milliseconds.

RNA EXTRACTION AND AMPLIFICATION, AND cDNA ARRAY

Total RNA was extracted using 0.1 mL of RNA STAT-60 (ISO-TEX Diagnostics, Friendswood, Tex) according to the manufacturer’s protocol, and RNA was resuspended in RNase-free water. Biotinylated complementary DNA (cDNA) probes were then generated from RNA isolates using linear polymerase chain amplification. The Nonrad GEArray Ampolabeling kit (SuperTex Diagnostics, Friendswood, Tex) was used to generate biotin-labeled probes. These probes were then hybridized to the GEArray Q Series Human Tumor Metastasis Gene Array kit (SuperArray Bioscience Corp, Bethesda, Md) according to the manufacturer’s protocol. Briefly, the cDNA probes generated by linear amplification of the messenger RNA (mRNA) were denatured, and hybridization was conducted according to the manufacturer’s protocol. Briefly, the nylon membranes were incubated overnight at 60°C with the biotin-labeled probe and washed twice in 1% sodium saline citrate (SSC) and twice in 1% sodium dodecyl sulfate (SDS) for 60 minutes each, then once in 0.1× SSC and twice in 0.5% SDS for 20 minutes each, all at 60°C. The membranes were exposed to film at room temperature with intensifying screens. The relative expression level of each gene was determined for 20 minutes each, all at 60°C. The membranes were exposed to film at room temperature with intensifying screens.

Assay Reagent Kit (Pierce Chemical Company, Rockford, Ill), containing 96 specific cDNAs (300-600 base pairs) known to be involved in tumor growth and metastasis. The probe genes evaluated were matrix metalloproteinases MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-13, MMP-14, MMP-15, MMP-16, tissue inhibitor of metalloproteinases TIMP-1, TIMP-2, and TIMP-3, thrombospondin 1, plasminogen activator inhibitor 1 (PAI-1), elastase, cathepsin B, cathepsin L, cathepsin D heparanase, urokinase (uPA), and urokinase receptor (uPAR). Reference genes included 18S, glyceraldehyde phosphate dehydrogenase (GAPDH), and β-actin. The cDNA probes generated by linear amplification of the messenger RNA (mRNA) were denatured, and hybridization was conducted according to the manufacturer’s protocol. Briefly, the nylon membranes were incubated overnight at 60°C with the biotin-labeled probe and washed twice in 1% sodium saline citrate (SSC) and twice in 1% sodium dodecyl sulfate (SDS) for 60 minutes each, then once in 0.1× SSC and twice in 0.5% SDS for 20 minutes each, all at 60°C. The membranes were exposed to film at room temperature with intensifying screens. The relative expression level of each gene was determined through densitometric scanning after digital capture with a Coolpix 4500 camera (Nikon USA, Melville, NY).

DATA ANALYSIS

Data are presented as relative densitometric units corrected for equal RNA loading based on the corresponding GAPDH and β-actin mRNA levels, as described for cDNA array analysis.¹¹ Densitometry readings were averaged among the samples using a software program (Jimage; http://rsb.info.nih.gov/nih-image). We defined the level of expression of a gene by comparing its expression in tumor stroma and in healthy stroma: it was up-regulated if the ratio was 2.0 or greater, down-regulated if the ratio was 0.5 or less, and unchanged if the ratio was between 0.5 and 2.0. The relative expression levels of each marker for the 4 patients were compared with the relevant values (those of GAPDH and blank) by the Wilcoxon test. The linear rank statistics based on the Wilcoxon scores were used to compute exact P values. P<.05 (2-sided) was considered statistically significant.

CELL CULTURE

Normal dermal fibroblasts (NDFs), normal mucosal fibroblasts (NMFs), and CAFs were isolated from primary culture (as described below) and maintained in Dulbecco modified Eagle medium (DMEM) (Invitrogen, Grand Island, NY) with 20% fetal bovine serum and antibiotics (100 U/mL of penicillin and 100 μg/mL of streptomycin sulfate).

ISOLATION OF FIBROBLASTS FOR IN VITRO EVALUATION

Carcinoma-associated fibroblasts, NMFs, and NDFs were isolated from patients undergoing curative resection of head and neck squamous cell carcinoma (HNSCC) and separated from epithelial cells by differential trypsinization using a modified protocol, as described.¹²,¹¹ At the time of tumor resection, specimens from a tumor, adjacent mucosa, and healthy skin were obtained for culture. Specimens were obtained from oral tongue tumors in 3 patients. Neck skin or grossly normal buccal mucosa was removed for analysis in 1 patient. Specimens obtained for culture (n=3) were from patients different from those undergoing microdissection (n=4). Briefly, specimens were minced, washed in 70% ethanol and then in PBS, then dried on 6-well culture plates in triplicate. Specimens were incubated for 21 to 28 days in DMEM supplemented with 20% fetal calf serum, 2mM l-glutamine, 100 U/mL of penicillin, 1 mg/mL of streptomycin, and 2 μg/L of amphotericin B. Cells were then transferred by brief trypsinization (0.25% for 30 seconds with gentle agitation at room temperature) to 6-well dishes and grown to confluence (passage 0). Cells were then passaged by differential trypsinization and plated onto coverslips (passage 1) and subsequently 100-mm dishes. Cells grown on coverslips were used to perform immunohistochemical analysis for vimentin and for cytokeratins 8 and 14 on passages 0, 1, and 2 to determine the presence of tumor cells. Epithelial cells were not identified in fibroblast populations after passage 1. All experiments used cells between passages 1 and 3.

WESTERN BLOTTING

Isolated sets of NDFs, NMFs, and CAFs (n=3) obtained from the same patient as described above were then assessed by Western blotting. Specimens were homogenized in radioimmunoprecipitation assay (RIPA) buffer with 1mM phenylmethylsulfonyl fluoride (PMSF) and 15 μg/mL each of aprotinin and pepstatin. After protein determination using the BCA Protein Assay Reagent Kit (Pierce Chemical Company, Rockford, Ill), a total of 40 μg of protein per lane was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Invitrogen Corp, San Diego, Calif) under reducing conditions on a precast 12% tris-glycine gel (Invitrogen Corp); after electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Millipore Corp, Bedford, Mass). Cell lysates from HT1080 cells and LX1 tumor cells were used as positive con-
controls for MT1-MMP and reversion-inducing cystein-rich protein with Kazal motifs (RECK), respectively. Immunoblotting was performed using anti–MT1-MMP specific monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) and anti-RECK specific monoclonal antibody (MBL, Watertown, Mass). Secondary antimouse antibody was used, followed by chemiluminescence detection. Mouse monoclonal antibody to /H9252-actin (anti–/H9252-actin antibody, Santa Cruz Biotechnologies) was then used to correct for uneven loading on SDS-PAGE.

RESULTS

IN VIVO ASSESSMENT OF PROTEASES

Laser capture microdissection was used to isolate stroma from surrounding nests of tumor cells in untreated HNSCC tumors and from adjacent mucosal epithelium from 4 paired patient samples (Table). Isolation attempts focused on the capture of loose connective stromal tissue for analysis rather than inflammatory infiltrate or endothelial cells. Furthermore, stroma was primarily isolated from the invasive edge of the tumor to avoid necrotic and/or hypoxic tissues (Figure 1). Isolated RNA underwent linear amplification prior to cDNA array analysis (Figure 2).

Analysis of in vivo protease and protease inhibitor expression of paired normal mucosal stroma and tumor-associated stroma in 4 patients did not reveal expression of the serine protease family (eg, urokinase), elastase, or cathepsins B, D, or L (data not shown). Although

| Characteristics of 4 Patients Who Underwent Laser Capture Microdissection |
|-----------------------------|------------------|----------------|----------------|
| Patient No. | TNM Stage | Tumor Site | Subsite |
| 1 | T3 N2b M0 | Oropharynx | Tonsil |
| 2 | T3 N0 M0 | Oral cavity | Oral tongue |
| 3 | T4 N2a M0 | Oropharynx | Tonsil |
| 4 | T3 N0 M0 | Oropharynx | Tonsil |

Figure 1. Laser capture microdissection was performed on normal (A) and tumor-associated stroma (B). Loose connective tissue was isolated from tumors at the tumor-host interface and from normal stroma adjacent to histologically normal mucosal epithelium in 4 patients, and a representative sample from each is shown. Approximately 5000 “spots” per specimen were captured for messenger RNA isolation.
MMP11 was elevated in one sample, expression of MT-1 MMP (MMP14) mRNA was consistently elevated (2.6-fold) in all 4 patient samples of tumor-associated stroma (Figure 3). Expression of other MMPs or TIMPs were also assessed by cDNA array, but were not found to be overexpressed by either normal or tumor-associated stroma (Figure 3 and Figure 4).

IN VITRO ASSESSMENT OF MT1-MMP AND RECK

To assess CAF expression of MT1-MMP protein, CAFs, NDFs, and NMFs were isolated from 3 patients undergoing oral cavity tumor resection. Fibroblasts were isolated by differential trypsinization and immunohistochemical analysis was performed to confirm isolation of fibroblasts (Figure 5). Fibroblast cell lysates were assessed for MT1-MMP expression by Western blotting, which demonstrated elevated MT1-MMP expression (2.1-fold) (Figure 6). Because the cDNA array did not include RECK,
a membrane-bound MMP inhibitor (inhibitor of MT1-MMP activity and MMP9 expression) only recently identified, the presence of this protein was assessed in cultured fibroblasts (Figure 5). The expression of RECK in all 3 sets of isolated fibroblasts was elevated in NDFs compared with CAFs (2.4-fold) (Figure 5).

COMMENT

As in wound healing, fibroblasts in tumors express many of the molecular components of the extracellular milieu, including collagens, proteases, growth factors, and proteoglycans. The remodeling of the tumor microenvironment by CAFs is thought to modulate tumor cell proliferation and invasion. Our analysis of protease expression in vivo by laser capture microdissection and cDNA array analysis suggests that MT1-MMP is the primary protease up-regulated in the stromal cells of head and neck tumors. Further in vitro assessment of isolated CAFs similarly identified up-regulation of MT1-MMP.

Immunohistochemical studies by others have suggested that MT1-MMP is highly expressed in HNSCC tumors and is often localized to the stroma. Previous studies have found that up-regulation of MT1-MMP in tumors correlates with poor patient outcome and lymph node metastasis. The most striking finding in this study was the absence of other MMPs expressed in vivo by fibroblasts. Analysis of head and neck tumors for MMPs has consistently demonstrated that multiple members of the protease family are expressed, including MMP1, MMP2, MMP3, MMP9, urokinase, PAI1s, and TIMPs. Our study suggests that other MMPs expressed by HNSCC tumors are more likely derived from tumor cells (MMP1), or present ubiquitously in the serum and interstitial fluid (MMP2).

The analysis of CAFs isolated from tumor fibroblasts expressed more MT1-MMP compared with normal mucosal and dermal fibroblasts. Confirmation by immunohistochemistry is being conducted; however, multiple previous studies have performed this analysis and identified anti–MT1-MMP immunoreactivity in the stroma. Confirmation of protein expression by isolation of CAFs from tumors was performed on the basis of studies showing that isolated CAFs in other cancers maintain a transformed phenotype. However, expression of protein by isolated fibroblasts may not recapitulate the in vivo setting. Furthermore, the isolation of fibroblasts from surrounding epithelial cells may significantly alter their patterns of protease expression.

As early as the 1980s, chemotherapeutic trials using nonselective MMP inhibitors were attempted but halted owing to severe adverse effects, technical difficulty, and lack of efficacy in human trials. It was concluded that the lack of tissue- and stage-specific target MMPs limited efficacy. Targeting the specific MMPs in the tumor or surrounding stroma may improve results.

Reciprocal communication between tumor cells and fibroblasts is thought to promote a microenvironment that favors epithelial tumor cell growth and invasion. In this study, we have compared the proteases elaborated by CAFs with those of normal stromal tissue to identify an important mediator of tumor-stromal interactions in vivo. Membrane type 1 metalloproteinase may play a central role in the remodeling of the tumor extracellular matrix.

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