A Protective Role for Matrix Metalloproteinase-3 in Squamous Cell Carcinoma

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ABSTRACT

Elevated expression of matrix metalloproteinase-3 (MMP-3/stromelysin-1) is associated with a variety of tumor types, although its in vivo functional role remains unclear. In human and murine squamous cell carcinoma (SCC), MMP-3 is expressed in the stromal compartment at all of the stages of tumor progression and is expressed by the malignant epithelial cells in late-stage, highly invasive tumors. To elucidate whether MMP-3 plays a causal role during SCC, wild-type and MMP-3 null mice were subjected to chemical carcinogenesis procedures by topical application of either the complete carcinogen 1-methyl-3-nitro-1-nitrosoguanidine or two-stage initiation and promotion with 7,12-dimethylbenz[a]anthracene and 12-O-tetradecanoylphorbol-13-acetate. Contrasting with our expectations, tumors originating on MMP-3 null mice had enhanced initial tumor growth rates as compared with control animals, although there was no difference in tumor onset or incidence. This elevated rate in growth was coupled with an elevated proliferative index and a reduced vascular density but with no significant effect on apoptosis. Tumors from MMP-3 null mice had a prevalence of undifferentiated spindle tumors as compared with controls, which was concomitant with a higher percentage of MMP-3 null mice evidencing surface lung metastases. Tumor progression in MMP-3 null mice was inversely associated with leukocyte infiltration, in which an overall reduction in tumor-associated macrophages and neutrophils was evident. We propose that MMP-3 is expressed as a protective response and plays an important role in host defense during SCC tumorigenesis.

INTRODUCTION

Carcinogenesis is governed by the coordinate acquisition of favorable genetic lesions and complex interactions between tumor and host tissue, which, if successful, ultimately leads to an aggressive, metastatic phenotype (1). These intricate exchanges between tumor and stromal cells consist of an antitumorigenic assault as the normal host tissue attempts to maintain normal architecture and protumorigenic signals in which the normal stroma aids establishment of the tumor (2, 3). The matrix metalloproteinase (MMP) family of zinc-dependent endopeptidases represents one class of proteins that facilitates host and tumor communication (2, 3). Originally implicated in tumor invasion because of their matrix-degrading abilities, MMPs currently are thought to function throughout tumorigenesis, orchestrating critical cellular functions through proteolysis of matrix and nonmatrix components (4, 5).

One MMP family member, MMP-3 (stromelysin-1/E.C.3.4.24.17), is a key candidate for regulating tumor and host interactions during tumorigenesis. MMP-3, an extracellularly secreted enzyme, is overexpressed in a wide variety of tumor types (6). It is found almost exclusively in the tumor stroma, and its widespread expression is not surprising because MMP-3 can be generated by fibroblasts, endothelial cells, and cells of immune lineage (7). Unlike tumors derived from glandular epithelium, tumors derived from squamous epithelium also express MMP-3 in the malignant epithelial cells in tumors that show an invasive phenotype and an advanced disease progression (6, 8–12). MMP-3 has wide substrate specificity for various extracellular matrix components, targeting most proteins and proteoglycans except the fibrillar collagens (6, 13). Nonmatrix substrates identified also include other proteases, growth factors, and cellular adhesion molecules, such as pro-MMPs, plasminogen, heparin binding—epidermal growth factor, pro-interleukin 1β (IL-1β), insulin-like growth factor binding protein-3, and E-cadherin (4, 5). The variety of potential substrates coupled with the widespread distribution suggests that MMP-3 could have extensive effects on tumor progression. Despite the absence of MMP-3 expression by glandular epithelium itself, the stromally localized MMP-3 has been shown to influence mammary tumorigenesis. Transgenic mice overexpressing MMP-3 in mammary epithelium develop hyperplasia and invasive carcinoma (14). Our understanding of the role of MMP-3 in normal and pathologic processes has been considerably aided with the development of the MMP-3 null mouse (15). As with most MMP-deficient mice, the MMP-3 null mouse is fertile and viable. Use of these mice has revealed diverse roles for MMP-3 (e.g., in wound repair, adipocyte differentiation, aneurysm formation, and immune-mediated tissue injury; refs. 16–20).

Murine models of chemically induced squamous cell carcinoma (SCC) have been instrumental in defining key events occurring throughout tumor progression. We previously have shown that MMP-3 is expressed in the stromal component of benign and early-stage SCC and that the malignant epithelial cells acquire MMP-3 expression concomitant with progression to the metastatic spindle cell phenotype of SCC (21). This pattern of expression is consistent with that observed in human SCC, in which MMP-3 expression levels are correlative with disease progression, and tumor expression of MMP-3 is associated with invasive SCC (6, 8–12). Because of the correlation between disease progression and elevated levels of MMP-3, as well as the acquisition of tumor expression of MMP-3 with an invasive phenotype, we hypothesized that MMP-3 plays a causal role in SCC progression and particularly in generating a metastatic phenotype. To test this hypothesis, MMP-3 null and wild-type animals were subjected to standard chemical-induced SCC procedures, and the responses were compared.

MATERIALS AND METHODS

Animal Models. MMP-3 null mice were generated by homologous recombination as described previously and maintained on a 129/SvEv background (15, 17). Isogenic 129/SvEv mice (129/SvEvTac; Taconic, Germantown, NY) were used as wild-type controls. Mice were housed, fed, and treated in accordance with the guidelines approved by the Committee for Protection of Animal Subjects at Vanderbilt University Medical Center. At 8 weeks of age, MMP-3 null and wild-type mice were shaved on the dorsal area 2 days before initiating chemical carcinogenesis treatments and thereafter as needed. Two separate chemically induced carcinogenesis regimens were followed. Dorsal skin of MMP-3 null (n = 20 females) and wild-type mice (n = 10 females and 10 males) was treated weekly for up to 36 weeks with 1.6 mmol 1-methyl-3-nitro-1-nitroso-guanidine (MNNG; Sigma Chemical Co., St. Louis, MO; 22). In a separate study, MMP-3 null (n = 5 males and 5 females) and wild-type...
ace tone were applied twice weekly for 25 weeks and terminated 48 hours before tumors were harvested (21). Mice were examined weekly for the presence of skin tumors, and perpendicular measures of tumor length and width were gauged with calipers (Manostat Corporation, New York, NY). Tumor volumes were estimated according to the formula $V = (L/2) \times (W)^2$, where $V$ = volume, $L$ = length, and $W$ = width. Mice were sacrificed on completion of the DMBA-TPA chemical induction protocol at week 25. For the MNNG study, mice were sacrificed on completion of the protocol at week 36; however, mice showing signs of morbidity were sacrificed as necessary (weeks 30 to 35 of the study) in accordance with guidelines approved by the Committee for Protection of Animal Subjects at Vanderbilt University Medical Center. At autopsy, lungs were inflated and fixed with Bouin’s fixative (VWR International, West Chester, PA) and visually examined for the presence of metastases (22). Papillomas and carcinomas were dissected and immediately fixed in 4% paraformaldehyde before paraffin embedding (21).

**Histopathologic Analysis.** H&E staining was performed on paraffin-embedded sections (5 μm) as described previously (21). The sections were examined in a blinded manner by two observers (L.E.K. through the Vanderbilt University Skin Diseases Research Core Center and H.C.C.) for evidence of tumor promotion (“dark cells”), presence of atypia and invasion, and evaluation of the likely biological aggressiveness of the tumors [epithelioid SCC versus spindle cell-type SCC (SpSCC)]. These data were recorded for subsequent statistical analysis of the data after unblinding. Areas of fibrosis were determined on paraffin-embedded sections stained with trichrome by Gomori’s method.

**Immunohistochemistry.** Five-micrometer paraformaldehyde-fixed paraffin-embedded sections were analyzed by immunohistochemistry as described previously (22). Papillomas induced by DMBA-TPA treatment or stage-matched MNNG-induced SCC and/or SpSCC were evaluated. The following primary antibodies were used: rabbit polyclonal anti-keratin 5 (1:1000; Covance Research Products Inc., Denver, PA), rabbit polyclonal anti-keratin 6 (1:1000; Covance Research Products Inc.), mouse ascites anti-pankeratin (1:400; Sigma-Aldrich Corp., St. Louis, MO), mouse monoclonal anti-proliferating cell nuclear antigen (PCNA; 1:400; Zymed Laboratories, San Francisco, CA), rat monoclonal anti-platelet/endothelial cell adhesion molecule (PECAM)/CD31 (1:100; Pharmingen, San Diego, CA), rat monoclonal antineutrophil (1:200; Serotec Inc., Raleigh, NC), rat monoclonal anti-CD3 (1:200; Serotec Inc., Raleigh, NC), and rat monoclonal anti-CD3ε (1:200; Santa Cruz Biotechnology, Santa Cruz, CA). Negative controls were performed using appropriate species- and isotype-matched immunoglobulins. Sections then were incubated with appropriate secondary antibody (Vector Laboratories, Burlingame, CA), and antibody binding was detected using ABC Elite Method (Vector Laboratories, Burlingame, CA), rat monoclonal anti-platelet/endothelial cell adhesion molecule (PECAM)/CD31 (1:100; Pharmingen, San Diego, CA), rat monoclonal antineutrophil (1:200; Serotec Inc., Raleigh, NC), rat monoclonal anti-CD3ε (1:200; Santa Cruz Biotechnology, Santa Cruz, CA). Negative controls were performed using appropriate species- and isotype-matched immunoglobulins. Sections then were incubated with appropriate secondary antibody (Vector Laboratories, Burlingame, CA), and antibody binding was detected using ABC Elite Method (Vector Laboratories) with diaminobenzidine as the substrate. Sections were then performed with appropriate secondary antibody (Vector Laboratories, Burlingame, CA), and antibody binding was detected using ABC Elite Method (Vector Laboratories) with diaminobenzidine as the substrate. Sections then were counterstained with hematoxylin to visualize nuclei. The Mouse Pathology and Immunostaining Core, Vanderbilt University Medical Center (Nashville, TN) performed the immunohistochemical analysis for keratin 10 and keratin 14. Sections stained with keratin 5, keratin 6, keratin 10, keratin 14, or pankeratin were evaluated for presence or absence of antigen. In the case of PCNA immunostaining, an average of 1000 nuclei were evaluated for six to eight stage-matched tumors per experimental group. Quantitation of PECAM/CD-31 immunostaining was performed morphometrically using Image Pro-Plus software (Media Cybernetics Inc., Silver Spring, MD) to determine the area of positive staining for six to eight stage-matched tumors per experimental group. Counting multiple arbitrary areas defined by Metamorph Imaging System (Universal Imaging Corporation, Downingtown, PA) of six to eight stage-matched tumors per experimental group was done to assess neutrophil and CD3ε immunostainings.

**Apoptosis Analysis.** Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) was performed on paraformaldehyde-fixed, paraffin-embedded sections (5 μm) using the Apo-Tag Kit (Intergen, Purchase, NY) according to manufacturer’s directions. Papillomas induced by DMBA-TPA treatment or stage-matched MNNG-induced SCC were evaluated. Antibody binding was detected using ABC Elite Method (Vector Laboratories) with diaminobenzidine as the substrate, and sections were counterstained with contrast green to visualize cells. An average of 1500 nuclei were evaluated for TUNEL positivity for six to eight stage-matched tumors per experimental group.

**Statistical Analysis.** Kaplan-Maier plots were analyzed using the log-rank test. Tumor growth patterns were derived from the change in total tumor count and area. Similarity of the tumor growth patterns was assessed using the log-rank test. Tumor volume measurements were performed using Image Pro-Plus software (Media Cybernetics Inc., Silver Spring, MD) to determine the area of positive staining for six to eight stage-matched tumors per experimental group. Counting multiple arbitrary areas defined by Metamorph Imaging System (Universal Imaging Corporation, Downingtown, PA) of six to eight stage-matched tumors per experimental group was done to assess neutrophil and CD3ε immunostainings.

(n = 5 males and 5 females) mice were subjected to a single topical application of a solution containing 25 μg 7,12-dimethylbenz[a]anthracene (DMBA; Sigma Chemical Co.) dissolved in 100 μL of acetone directly applied to shaved skin. One week after the first treatment, 5 μg 12-O-tetradecanoylphor- bol-13-acetate (TPA; LC Laboratories, Woburn, MA) dissolved in 100 μL of acetone were applied twice weekly for 25 weeks and terminated 48 hours before tumors were harvested (21). Mice were examined weekly for the presence of skin tumors, and perpendicular measures of tumor length and width were gauged with calipers (Manostat Corporation, New York, NY). Tumor volumes were estimated according to the formula $V = (L/2) \times (W)^2$, where $V$ = volume, $L$ = length, and $W$ = width. Mice were sacrificed on completion of the DMBA-TPA chemical induction protocol at week 25. For the MNNG study, mice were sacrificed on completion of the protocol at week 36; however, mice showing signs of morbidity were sacrificed as necessary (weeks 30 to 35 of the study) in accordance with guidelines approved by the Committee for Protection of Animal Subjects at Vanderbilt University Medical Center. At autopsy, lungs were inflated and fixed with Bouin’s fixative (VWR International, West Chester, PA) and visually examined for the presence of metastases (22). Papillomas and carcinomas were dissected and immediately fixed in 4% paraformaldehyde before paraffin embedding (21).

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volume (mm$^3$) for each tumor measured weekly from each tumor’s onset as described previously (22). The linear range was defined as the rate between first appearance of tumors and the week at which the first plateau of each curve was reached. Immunohistochemical results were analyzed by a nonparametric (Mann-Whitney) method when indicated. All of the statistical analyses were performed using Statview software (SAS Institute, Cary, NC). We examined sex as a source of variation for certain statistical models (i.e., tumor onset, tumor frequency, and tumor growth) and noted no differences between sexes. As such, all of the statistical analyses shown were done on all of the samples with genotype as the major source of variation and are not matched according to sex differences.

RESULTS

Tumors Originating in MMP-3 Null Animals Have a Faster Rate of Growth and Disease Progression. To test the hypothesis that MMP-3 plays a causal role in generating metastatic SpSCC, MMP-3 null and wild-type mice were subjected to topical application of the complete carcinogen MNNG for 36 weeks. MNNG has been shown to enhance progression of SCC to the metastatic spindle cell type compared with other methods of chemical initiation and progression, allowing for a better chemical assessment of the effects on tumor progression. Individual skin lesions were recorded when they were at least 1 mm in diameter and present for at least two consecutive weeks.

All of the mice subjected to MNNG treatment developed papillomas irrespective of their MMP-3 status. Papillomas began to appear at week 18 in wild-type mice and at week 19 in MMP-3 null mice (Fig. 1A). All of the wild-type and MMP-3 null mice had tumors by weeks 26 and 29, respectively, and the overall tumor latency did not significantly differ between the two experimental groups (log-rank test, $P = \text{NS}$). Furthermore, by week 26 of MNNG treatment, the average number of tumors per mouse was statistically higher in wild-type than in MMP-3 null animals (Fig. 1B). However, when papillomas are separated from carcinomas, by the end of the study wild-type animals had significantly more papillomas per mouse than did MMP-3 null counterparts (wild-type mice 2.85 ± 2.25 papillomas/mouse versus MMP-3 null 1.21 ± 1.03 papillomas/mouse; $P = 0.046$), whereas wild-type animals had a trend toward the development of fewer carcinomas than did MMP-3 null animals (wild-type mice 1.0 ± 0.85 carcinomas/mouse versus MMP-3 null 1.55 ± 0.61 carcinomas/mouse; $P = \text{NS}$).

We evaluated whether MMP-3 status affected tumor growth or progression. Tumor volume was assessed weekly, and from these data we determined the initial tumor growth rates. Surprisingly, tumors originating on MMP-3 null animals had a significantly faster rate of growth than those originating on wild-type animals (Fig. 1C). However, the average rate of tumor burden (additive volume of each tumor/mouse) did not significantly differ between wild-type and MMP-3 null animals (data not shown). Each tumor following 30 to 36 weeks of MNNG treatment was harvested, and individual tumors were histologically graded by H&E to determine stage of progression by morphology (Fig. 2A). Papillomas also were distinguished immunohistochemically by loss of the differentiation-specific suprabasal marker, keratin 10. For confirmation of epidermal origin of tumors, essential for spindle cell phenotype of SCC, tumors were analyzed by immunohistochemistry for retention of basal or known tumor-induced squamous keratins (keratin 5, keratin 6, pankeratin). Areas of fibrosis were defined histologically by staining with Gomori’s trichrome.
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Tumor growth depends on the development of a blood vasculature to bring in nutrients critical to sustain growth (2, 3). Because overall tumor growth and tumor proliferation were affected by the absence of MMP-3, neovascularization was evaluated in stage-matched MNNG tumors induced in the two experimental groups. Blood vessel density was examined using the cell surface endothelial marker PECAM/CD31 (Fig. 4C). Unexpectedly, MMP-3 null tumors from invasive stages of tumorigenesis generated by MNNG had a higher percentage of nuclear PCNA staining but similar levels of apoptosis as compared with wild-type tumors (Fig. 4A). By contrast, although MMP-3 null tumors had a trend toward a higher percentage of cells that were TUNEL positive, these data did not significantly differ between the two groups (Fig. 4B). Furthermore, MMP-3 null tumors from invasive stages of tumorigenesis generated by MNNG had a higher percentage of nuclear PCNA staining but similar levels of apoptosis as compared with stage-matched wild-type controls (Fig. 4A and B). However, hyperplastic epidermis adjacent to the tumor areas from wild-type and MMP-3 null tumors had similar levels of PCNA positivity (wild-type 38.20 ± 13.58, n = 9 versus MMP-3 null 41.24 ± 7.50, n = 7, average percentage PCNA-positive nuclei ± SD; P = NS); likewise, proliferation indices were similar in resting epidermis (data not shown). Thus, the absence of MMP-3 expression correlates with a greater number of tumor cells evidencing proliferative capacity.

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The results of the MNNG experiments, which were designed to study the late stages of SCC progression, suggested that loss of MMP-3 altered early stages of SCC progression. In a study designed to focus on early-stage SCC progression, we chose the less aggressive protocol of classical two-stage carcinogenesis using DMBA as the initiator, followed by 25 weeks of promotion with TPA. This two-stage chemical carcinogenesis procedure is less aggressive than weekly carcinogen treatments and extends the early stages of tumorigenesis. The study was ended at 25 weeks to obtain tumors at the papilloma stage of progression to use in further analyses. At week 25, 90% (9 of 10) of the wild-type and MMP-3 null mice had tumors (Fig. 3A). In agreement with the MNNG study, there was no statistical difference in the overall tumor latency (Fig. 3A). Likewise, there was no difference in the average number of papillomas per mouse between the wild-type and MMP-3 null animals during the course of DMBA-TPA study (Fig. 3B). However, in evaluating the change in tumor volume from time of initial appearance, tumors originating in MMP-3 null animals had a faster initial growth rate as compared with wild-type animals (Fig. 3C). Collectively, these results suggest that MMP-3 expression slows down tumor growth early in tumorigenesis and delays tumor progression.

Proliferation and Angiogenesis Are Affected by Changes in MMP-3 Expression. Analysis of tumor size revealed that tumors from MMP-3 null animals had a faster rate of growth than those from wild-type counterparts. Changes in gross tumor size can be caused by many reasons, including fluctuations in tumor cell number, edema, or fibrosis. We next investigated whether this selective growth advantage was caused by a change in tumor cell proliferation or survival. Papillomas induced by DMBA-TPA treatment in MMP-3 null and wild-type animals were analyzed for proliferation status and apoptosis by the presence of nuclear PCNA- and TUNEL-positive cells, respectively. The percentage of nuclei positive for PCNA staining was significantly higher in the MMP-3 null tumors as compared with wild-type tumors (Fig. 4A). By contrast, although MMP-3 null tumors had a trend toward a higher percentage of cells that were TUNEL positive, these data did not significantly differ between the two groups (Fig. 4B). Furthermore, MMP-3 null tumors from invasive stages of tumorigenesis generated by MNNG had a higher percentage of nuclear PCNA staining but similar levels of apoptosis as compared with stage-matched wild-type controls (Fig. 4A and B). However, hyperplastic epidermis adjacent to the tumor areas from wild-type and MMP-3 null tumors had similar levels of PCNA positivity (wild-type 38.20 ± 13.58, n = 9 versus MMP-3 null 41.24 ± 7.50, n = 7, average percentage PCNA-positive nuclei ± SD; P = NS); likewise, proliferation indices were similar in resting epidermis (data not shown). Thus, the absence of MMP-3 expression correlates with a greater number of tumor cells evidencing proliferative capacity.

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tumors did not have a higher percentage of necrotic lesions typical of tumors that have outgrown their nutritional needs. Likewise, there was no change in apoptosis as evaluated by TUNEL analysis between the experimental groups (Fig. 4B). Thus, angiogenesis positively correlated with MMP-3 expression.

Absence of MMP-3 Is Correlated with a Reduction in Leukocyte Infiltration. Potential roles for MMP-3 in immune cell function can be surmised from a variety of data. For example, a number of identified MMP-3 substrates are known as regulators of immune cell function (4, 5). We also have shown previously that MMP-3 null animals have a reduction in infiltrating monocytes in a murine herniated disk model (23). We investigated whether macrophage infiltration was altered in size-matched papillomas from MMP-3 null and wild-type animals. All of the papillomas from wild-type animals had macrophages associated with the tumor stromal boundary (10 of 10 tumors; Fig. 5A). In contrast, no macrophages were detected in a majority of papillomas from MMP-3 null animals (six of eight tumors; Fig. 5B), with a minority of papillomas having a modest number of associated macrophages (two of eight tumors; Fig. 5C). We extended this observation by asking whether MMP-3 deficiency globally affected the tumor presence of other immune cell types. Neutrophils were significantly fewer in number in MMP-3 null tumors as compared with wild-type tumors (Fig. 5D). This overall reduction in neutrophil infiltration was maintained in MMP-3 null tumors of advanced, spindle cell-type progression. Examination of CD3ε-positive T cells revealed similar levels were present in wild-type and MMP-3 null tumors (Fig. 5E). Collectively, these results suggest that MMP-3 expression is closely associated with the ability of macrophages and neutrophils to infiltrate the tumor.

DISCUSSION

There is substantial correlative and experimental evidence to suggest that MMPs play a causal role in tumor cell invasion and metastasis. Despite the close association between MMP-3 expression and tumor progression, the results in the current study suggest a preventative role for MMP-3 during SCC. MMP-3 expression correlated with slower-growing tumors and slower disease progression. The protective role of MMP-3 may be related to its role in wound repair. MMP-3 is rapidly induced on wounding, and MMP-3 null mice have a delay in excisional reepithelialization (24, 25). Thus, MMP-3 expression may reflect a physiologically normal “wound healing” response of the associated tumor stroma as it attempts to reconcile the newly formed tumor back into normal tissue architecture.

In contrast to the findings in the present study, Sternlicht et al. (14) have shown overexpression of MMP-3 to be protumorigenic in mammary epithelium. MMP-3 targeted to murine mammary epithelium (WAP–MMP-3) in a CD-1 background acts as a tumor promoter, and a small percentage of these mice develop invasive carcinoma (14). However, work from our laboratory previously has shown a protective
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role for MMP-3 in breast tumorigenesis. In this system, MMP-3 was targeted to breast epithelium of C57Bl6xDBA mice using the mouse mammary tumor virus promoter and tumors induced by DMBA treatment (26). Mouse mammary tumor virus–MMP-3 mice did not develop spontaneous mammary tumors and had reduced tumor incidence compared with nontransgenic controls when treated with DMBA, which correlated with enhanced apoptosis in response to loss of cell to matrix attachment (26). The difference in tumorigenic response between the two mammary-targeted MMP-3 transgenic models seems to be related to a difference in the genetic background of the mice because backcrossing the mouse mammary tumor virus–MMP-3 mice from a C57Bl6xDBA background into a CD-1 genetic background also resulted in mice that spontaneously developed mammary tumors (4). Thus, the major difference in the two models, which correlates with either an overall antitumorigenic or a protumorigenic MMP-3–dependent effect, is the method of oncogenic activation: whether the means of generating tumors relies on chemically induced protocols or the genetic background of the mice.

MMP-3 expression correlated with slower-growing tumors and reduced nuclear presence of PCNA, which suggests that lack of MMP-3 expression conferred a selective proliferative advantage. One molecule of singular importance to tumor growth early in tumorigenesis is transforming growth factor β (TGF-β; ref. 27). MMP-3 can modulate TGF-β1 signaling pathways either by enhancing bioavailability or activity (28–31). TGF-β engagement with its receptor, TGF-βR, at the cell surface results in activation of this receptor tyrosine kinase and phosphorylation of receptor-associated transcription factors, the Smads (27). Activation of Smads through phosphorylation results in the translocation of these molecules to the nucleus, where they participate in regulating gene expression. To determine whether TGF-β signaling was altered in MMP-3 null mice, we examined papillomas for the presence of nuclear phospho-Smad2. Overall, papillomas from MMP-3 null and wild-type animals were similarly positive for nuclear phospho-Smad2 (5). Skin from MMP-3 null mice also treated with TPA, a potent inducer of MMP-3 expression, did not show a reduction in active TGF-β (6). Thus, TGF-β signaling seems to be intact whether in the presence or absence of MMP-3.

Tumors originating on MMP-3 null animals had a reduction in the number of infiltrating neutrophils and macrophages. Neutrophils and macrophages are important effectors of innate host defense and also regulate adaptive immune functions through the release of cytokines and chemokines. Leukocyte infiltration has long been recognized as a host defense response during tumorigenesis, although tumor-associated neutrophils and monocytes provide a rich source of growth factors and angiogenic factors, which could benefit tumors that have developed resistance to host immune surveillance properties (32–34). Immune cell infiltration is thought to precede and influence the “angiogenic switch” necessary to produce a new blood supply (35). MMP-3 null tumors did have a reduction in blood vessel density. Thus, one protumorigenic effect associated with MMP-3 expression, as well as possibly associated with immune cell presence, is angiogenesis. Interestingly, male mice null for MMP-8, an MMP mainly expressed by neutrophils, also are more sensitive to skin carcinomas, and this effect is correlative with an early deficiency of neutrophil influx (36). The association between MMP-3 levels and leukocyte infiltration and/or function is evident in a number of model systems. We have shown previously in a murine herniated disk model that chondrocytes derived from MMP-3 mice had a reduced ability to chemoattract macrophages (23). Neutrophil infiltration also was se-

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4 L. Matrisian and M. Henderson, unpublished results.

5 L. McCawley, unpublished observations.

6 H. Crawford and M. E. Barcellos-Hoff, unpublished observations.
An influence of MMP-3 over immune cells is not surprising based on known proteolytic targets. A number of substrates known to modulate inflammation have been identified, including matrix, cytokines, chemokines, and adhesion factors such as osteopontin, tumor necrosis factor α (TNF-α), IL-1β, l-selectin, monocyte chemoattractant protein-1 (MCP-1), MCP-2, MCP-3, MCP-4, and stromal cell-derived factor-1/CXC ligand 12 (37–42). Although the molecular target of MMP-3 is not yet identified for the MMP-3–dependent protective effect in SCC, two potential substrates have been examined in SCC progression. TNF-α is membrane attached and requires proteolysis for liberation before engagement with its receptor. Although TNF-α converting enzyme is thought to be the primary enzyme responsible for this event, MMP-3 is one of a number of metallocproteinases that can target TNF-α for cleavage (38, 43). However, TNF-α null animals challenged with chemically induced SCC have a delay in onset and a large reduction in tumor incidence, which is not reflective of the MMP-3 null phenotype (44). We also have identified osteopontin as an MMP-3 substrate and have shown that the cleaved form of osteopontin acts as a more potent macrophage chemoattractant (37). Furthermore, we have shown that osteopontin null animals bear a strikingly similar phenotype to MMP-3 null animals when examined for chemically induced SCC tumorigenesis, namely, the reduction in tumor growth rates, the reduction in macrophage infiltration, and the enhanced progression to metastatic SpSCC (22). Thus, osteopontin is a potential substrate for MMP-3 that explains the antitumorigenic effect of MMP-3 null mice. However, the effect of MMP-3 is likely to represent the overall balance of conflicting or complementary responses generated by targeting a variety of divergent substrates.

Recent studies have expanded our current thinking about MMPs from submissive tools for bulk matrix clearance to them being major signaling players impacting every step of carcinogenesis (4, 5). Because of the wide substrate variability and complexity of carcinogenesis, MMP expression can have antitumorigenic and protumorigenic effects based on the cellular context. Our data support an antiangiogenic role for MMP-3 in SCC. Despite substantial preclinical data substantiating a proangiogenic role of MMPs in tumorigenesis, recent clinical studies broadly targeting all of the MMP members have not proven fruitful (45). The current study showing a protective role for MMP-3 would suggest that inhibiting MMP-3 activity could adversely affect the disease progression during SCC. MMP-3 presence is likely to be tumor promoting and tumor protective within the same tumor, and the consequence of inhibiting MMP-3 will depend on its relative contribution to these two opposing processes during each stage of tumor progression. Furthermore, there is substantial cross-talk between MMP family members; thus, regulation of one enzyme could impact other family members. Certain MPPs can regulate the activation of other family members, and the expression levels of MMP family members have been shown to increase as a possible compensatory mechanism on complete absence of an individual MMP member (4, 5, 46). Thus, understanding the relative contributions of MPPs to antitumorigenic and protumorigenic processes, and in particular understanding the molecular mechanisms of the antitumorigenic MMP-dependent effects, could prove invaluable to the future application of MMP inhibitors to cancer therapy.

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