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Up-Regulation of Matrix Metalloproteinase-9 in T Lymphocytes of Mammary Tumor Bearers: Role of Vascular Endothelial Growth Factor

Jennifer L. Owen,* Vijaya Iragavarapu-Charyulu,§ Zeean Gunja-Smith,† Lynn M. Herbert,* Joseph F. Grosso,* and Diana M. Lopez2*‡

Matrix metalloproteinase-9 (MMP-9), a matrix-degrading enzyme, is crucial in tumor invasion and metastasis and is implicated in leukocyte extravasation. In this report, we demonstrate that during growth of the D1–7,12-dimethylbenzanthracene-3 mammary tumor in BALB/c mice, there is progressive up-regulation of MMP-9 in splenic T cells at both the transcriptional and translational levels. Our previous work has identified several factors produced by this tumor, including PGE2, GM-CSF, and phosphatidyl serine; however, none of these agents induces increased production of MMP-9 by normal splenic T cells. Although not produced by the tumor, TNF-α and IL-6 are up-regulated in both macrophages and B cells in tumor-bearing mice. Exposure of normal T cells to these two cytokines, however, also fails to up-regulate MMP-9 production. Vascular endothelial growth factor (VEGF) is produced by many tumors, and we determined that the mammary tumor used in our studies expresses high levels of this angiogenic growth factor. Importantly, splenic T cells from tumor bearers constitutively produce increased amounts of VEGF, and treatment of normal T cells with VEGF results in up-regulated MMP-9 production. Of crucial importance is the finding that tumor-infiltrating T cells also produce high levels of VEGF and MMP-9. Our studies indicate that VEGF can act directly on T lymphocytes and that elevated VEGF levels may contribute to the aberrant MMP-9 secretion by mammary tumor bearers’ T cells. The Journal of Immunology, 2003, 171: 4340–4351.

Matrix metalloproteinases (MMPs) are a family of more than 20 zinc-dependent endopeptidases that can degrade every component of the extracellular matrix (ECM), enabling cells to invade tissues. Because of their key roles in matrix degradation, activated members of the MMP family are involved in many physiological processes such as ovulation, embryogenesis, bone remodeling, tooth eruption, wound healing, and angiogenesis. In contrast, excessive or inappropriate expression of MMPs contributes to tissue-destructive diseases including arthritis, multiple sclerosis, atherosclerosis, gastric ulcers, fibrotic lung disease, and tumor progression (1). Consequenly, these proteases play a crucial role in the pathological processes of tumor invasion and metastasis (2–6) and contribute to chronic inflammatory diseases by aiding the migration of inflammatory cells into various tissues (7, 8).

The metalloproteinases have elevated expression in advanced tumors, and a number of studies outline roles for these enzymes during multiple stages of tumorigenesis, including establishment and growth, angiogenesis, invasion, migration, and metastasis (9). The pluripotent effects of MMPs are due to the ability of these proteases to regulate the tumor microenvironment by cleaving a variety of substrates, which include not only structural components of the ECM, but also growth factor-binding proteins, growth factor precursors, receptor tyrosine kinases, cell-adhesion molecules, and other proteinases (10, 11). Accordingly, the gene expression of MMPs is tightly regulated to maintain normal tissue function. At the protein level, regulation is achieved by the two-pronged action of proteolytic activation of the latent enzyme and inhibition of active enzymes by the tissue inhibitors of metalloproteinases (TIMPs) (12, 13).

Several MMPs, including the gelatinases, MMP-2 (gelatinase A) and MMP-9 (gelatinase B), are expressed at very high levels in tumors of various origins and in various species (14), and serum and tissue levels of both, especially MMP-9, correlate with malignant tumor grade (15–18). Although many studies had evaluated the production of proteases by tumor cells, immunohistochemical analyses revealed that MMPs are detected not only in the tumor cells themselves, but also in the stromal cells and, in some cases, preferentially in the latter (19, 20). Initially it was thought that fibroblast-like cells were the source of the stromal cell-derived MMPs. However, a series of reports from several laboratories have suggested that inflammatory cells invading the ECM are important producers of these enzymes. In a squamous cell carcinoma model, MMP-9 was primarily expressed in neutrophils, macrophages, and mast cells rather than in the neoplastic cells (21), whereas host macrophages were concluded to be the main source of MMP-9 in human ovarian carcinoma in mice (22). Additionally, it has been reported that, in epithelial cancer invasion, most MMPs are made by stromal cells of the host, and their expression is regulated by chemokines and cytokines produced by the tumor (23).
Due to their role in immunosurveillance, lymphocytes normally have an invasive phenotype, and findings support a role for MMP-9 in normal leukocyte migration from the vascular compartment to sites of inflammation (24). It has also been shown that T lymphocytes constitutively produce small amounts of MMP-9 that are up-regulated by phorbol esters and IL-2 stimulation (25) and that increased production of MMP-9 in human PBMCs enhances invasion through reconstituted basement membrane (26). Because lymphocyte invasion of a tumor mass is of crucial importance in the host’s defense against the developing neoplasm, we evaluated MMP production by the T cells from normal and mammary tumor-bearing mice. This study shows that although mammary tumor cells produce MMP-9, the T cells from tumor-bearing animals constitutively produce higher levels of this enzyme. The possible causes of enhanced production of MMP-9 in the T cell compartment of tumor bearers were also investigated. The findings presented in this study may have important implications in elucidating the effects of tumor-derived and/or -induced factors on T cells during tumor development.

Materials and Methods

Mice and cell lines

BALB/c mice used in these studies were 8–12 wk of age and were bred in our animal facility at the University of Miami. Animal care and use was according to the guidelines of the National Institutes of Health. The D1–7,12-dimethylbenzanthracene-3 (D1-DMBA-3) tumor, syngeneic to BALB/c mice, is a transplantable mammary adenocarcinoma derived from a nonviral, noncarcinogen-induced prneoplastic nodule after treatment with DMBA (27). The D1-DMBA-3 tumor is immunogenic to the host of origin and nonmetastatic to the spleen or other organs, but minute metastases to the lung do occasionally occur. The DA-3 mammary tumor cell line was derived in our laboratory from the D1-DMBA-3 tumor and maintained in DMEM/high glucose, 10% characterized heat-inactivates FCS (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin with OPI medium supplement (Sigma-Aldrich, St. Louis, MO). The renal carcinoma (Renca) cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% glutamine, 1% sodium pyruvate, 1% nonessential amino acids, and 50 mM 2-ME. The K-7 osteosarcoma cells were grown in DMEM/high glucose with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Both the Renca and K-7 cell lines were a gift from Eduardo Sotomayor (Moffit Cancer Center, Tampa, FL). Tumors were implanted in BALB/c mice by s.c. injection of 1 × 10⁶ tumor cells, resulting in a measurable tumor 7–10 days postimplantation.

Purification of splenic T cells

Spleens were compressed in Teflon tissue homogenizers, and the resulting single cell suspension was pelleted at 300 × g, subjected to hypotonic shock for red cell removal, washed, and counted. Macrophages were removed from the cell suspension by plastic adherence in prewarmed RPMI 1640, 5% FCS, 37°C for 1 h in CO₂ incubator. The nonadherent T lymphocytes were purified on nylon wool columns according to the method of Julius et al. (28) and by positive selection using the MACS magnetic separation system (Miltenyi Biotec, Auburn, CA), according to the manufacturer’s instructions. Briefly, single-cell suspensions in cold PEB buffer (PBS supplemented with 2 mM EDTA and 0.5% BSA) were incubated with superparamagnetic microbeads conjugated to anti-mouse CD90 (Thy1.2), anti-mouse CD4, or anti-mouse CD8 mAb at 4°C for 15 min. Cells were washed twice and loaded onto the magnetic separation columns. The columns were washed three times with cold PEB buffer, and the positively selected Thy1.2⁺, CD4⁺, or CD8⁺ T cells were then eluted. After purification, the cells were routinely ≥95% viable, as assessed by trypan blue exclusion. FACS analysis using an LSR analyzer and anti-mouse FITC-CD90, anti-mouse FITC-CD4, and anti-mouse PE-CD8 Abs (BD Pharmingen, San Diego, CA) confirmed the populations to be ≥93% Thy1.2⁺, ≥94% CD4⁺, and ≥90% CD8⁺ T lymphocytes.

Cell culture

Purified splenic T cells used in zymography assays were first washed three times with RPMI 1640 to remove all residual serum before overnight culture (2 × 10⁶ cells/ml) in DMEM/F12 medium with 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM L-glutamine, 1 mM sodium pyruvate, and 1× nonessential amino acids (all from Life Technologies, Grand Island, NY). At the end of the incubation period, the supernatants were removed and stored at −80°C. After purification, splenic T cells cultured for ELISAs were resuspended in complete medium consisting of RPMI 1640, 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 mM 2-ME and were cultured overnight. Supernatants were removed and stored at −80°C until use. The murine DA-3 and D1-DMBA-3 adenocarcinomas were cultured in complete medium as described above. After 1, 2, 3, and 4 days in culture, supernatants were removed and stored at −80°C until analysis in the vascular endothelial growth factor (VEGF) ELISA. The following reagents were used in experiments as noted: Con A, PMA, and PGE₂ (Sigma-Aldrich); recombiant murine TNF-α (mTNF-α), GM-CSF, and IL-6, (PeproTech, Rocky Hill, NJ); murine VEGF and anti-mouse VEGF Ab (R&D Systems, Minneapolis, MN); and phosphatidyliner (Avanti Polar Lipids, Alabaster, AL).

Zymography

Gelatin zymography followed a modified procedure of Heussen and Dordle (29) for detecting picogram amounts of MMP-2 and MMP-9. Identical amounts of supernatants were electrophoresed under nonreducing conditions using 10% SDS polyacrylamide gels containing 0.33 mg/ml gelatin. After electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 15 min to remove SDS. After overnight incubation at 37°C in assay/incubation buffer (50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 μM ZnCl₂, 0.02% NaN₃, and 0.005% NaN₃), for 2 h with Coomassie blue R 250 and destained with 7% acetic acid. Both latent and active forms of gelatinases produce clear areas in the gel.

Western blot analysis

Equal amounts of T cell supernatants were separated on 10% SDS polyacrylamide gels under reducing conditions and then were transferred onto Protran nitrocellulose membranes (0.45-μm pore size; Schleicher & Schuell, Keene, NH) using a Trans-Blot electrophoretic cell (Bio-Rad, Hercules, CA). Membranes were blocked for 1 h at room temperature in 5% nonfat dry milk in 1× TBS-0.1% Tween 20 followed by 1-h incubation with an anti-MMP-9 Ab (Chemicon International, Temecula, CA) at room temperature. Blots were washed for 50 min with five changes of 1× TBS-0.1% Tween 20 solution followed by 1-h incubation at room temperature with the HRP-conjugated anti-rabbit IgG Ab (Chemicon International). Blots were washed again for 50 min and incubated for 3 min with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). The results were visualized by exposing blots to BioMax autoradiographic film (Kodak, Rochester, NY).

Reverse zymography

TIMPs secreted into the culture medium were detected using a modification of the method of Herron et al. (30). Briefly, conditioned medium from cultured D1-3 or splenic T cells was electrophoresed under nonreducing conditions using 12% SDS polyacrylamide gels containing 0.75 mg/ml gelatin and 4-aminophenylmercuric acetate-2-acivated MMP-2 (1–2 U/ml, purified from human skin fibroblasts). The gel was rinsed twice in 50 mM Tris-HCl, 2.5% Triton X-100 solution for 20 min and incubated overnight with zymography assay/incubation buffer at 37°C. Undigested gelatin in the gel was visualized by staining with 0.25% Coomassie blue R 250 and destaining in 7% acetic acid. Areas of the gel containing TIMPs appeared as dark blue bands against a paler blue background.

RNA analyses

Three types of RNA analyses were performed on 2- to 4-h splenic T cell cultures in this study: RT-PCR, Northern blotting, and RNA gene arrays. For the RT-PCR analysis, total RNA was isolated using TriReagent (Molecular Research Center, Cincinnati, OH). One microgram of total RNA from each sample was reverse-transcribed using a Primus thermocycler (MWG Biotech, High Point, NC) and the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA) with oligo d(T)₁₆ primers, according to the manufacturer’s instructions. The reaction was incubated for 60 min at 42°C, followed by inactivation of the murine leukemia virus reverse transcriptase at 99°C for 5 min. The cDNA products obtained were subjected to PCR for 35 cycles, after which 15 μl of the amplified DNA fragments were electrophoresed on a 1.6% agarose gel stained with ethidium bromide and visualized by UV transillumination. The gels were analyzed with Scion Image B 4.02 densitometry software (Scion, Frederick, MD) and normalized with β-actin band intensity. Percentages were calculated relative to the maximum for each experiment. The PCR for MMP-9 amplification was conducted by incubating the samples at 94°C for 10 min, followed by 35
cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension for 10 min at 72°C. For fms-like tyrosine kinase-1 (Flk-1) and fetal liver kinase-1 (Flt-1) amplification, the samples were incubated at 94°C for 10 min, followed by 35 cycles of 90°C for 30 s, 65°C for 45 s, and 72°C for 15 s, with a final extension for 10 min at 72°C. The primers were as follows: murine MMP-9 sense, 5′-CGAGACCACTGGAACTTTCA-3′; antisense, 5′-TCGGTGCTTCTGACATCAAGGTT-3′ (31); murine Flt-1 sense, 5′-CTCAGATCTTCTACAGTTC-3′; antisense, 5′-CTTGCAGGGTTCTCAGGCAT-3′ (32); murine Flk-1 sense, 5′-CTTAGGTTGCTTCCACTCACCCTG-3′; antisense, 5′-TCGCCGCTTCTGACATCAAGGTT-3′ (33); murine VEGF sense, 5′-CGAGACCCTGTTCCATCTCTTCTC-3′; antisense, 5′-CACCGCTCATGCCGTTCGAC-3′ (34); murine β-actin sense, 5′-TCTGGCACCACACCTCTAC-3′; and antisense, 5′-GAAGGAAAGCTGGGAAGAGATG-3′ (35).

Standard Northern blot technique was followed using the Northern Max formaldehyde-based system (Ambion, Austin, TX). Briefly, total RNA (10 μg) was electrophoresed through agarose-formaldehyde gels, blotted onto nylon membranes by capillary electrophoresis, followed by prehybridization of the membrane, and hybridized with the appropriate radiolabeled probe. Blots were preincubated in the ULTRAhyb hybridization solution for 3 h at 42°C and then hybridized at 42°C overnight in ULTRAhyb with an [α-32P]dATP-radiolabeled DNA probe. Probes were prepared by random priming with either β-actin cDNA or a PCR fragment of MMP-9 (Decaprime II kit; Ambion). Blots were exposed to BioMax autoradiography film overnight.

The relative mRNA expression of mouse ECM and adhesion molecules was analyzed with the chemiluminescent GEAArray Q Series (SuperArray, Bethesda, MD) according to the manufacturer's protocol. Briefly, total RNA was isolated and 5 μg from each sample was reverse transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) in the presence of Biotin-16-dUTP (Roche, Indianapolis, IN). The resulting cDNA probes were hybridized to cDNA fragments that were spotted on the GEAArray nylon membranes. The relative expression level of each gene was analyzed using GEAArray Analyzer and Scanalyze software.

Cytokine ELISA

The amounts of VEGF present in the supernatants of tumor cells and T lymphocytes were measured by Quantikine M murine VEGF ELISA (R&D Systems) according to the manufacturer's instructions. Absorbance at 450 nm was read on a Tecan SLT Rainbow Reader (Labinstruments, Research Triangle Park, NC), and OD values of samples were converted to picograms against a standard curve of known quantities of mVEGF.

Immunohistochemistry

Tissue from 3-wk D1-DMBA-3 tumors was formalin-fixed, embedded in paraffin, cut, and processed according to standard procedures. Serial sections were stained with goat IgG anti-CD3ε (Santa Cruz Biotechnology, Santa Cruz, CA) and detected with a biotinylated HRP complex as a detection reagent (Vector Laboratories, Burlingame, CA) per the manufacturer's directions. Omission of the primary Ab was used as a negative control.

Isolation of Thy1.2+ tumor-infiltrating lymphocytes

Thy1.2+ tumor-infiltrating lymphocytes were isolated based on the method of Rosenberg and coworkers (36). Briefly, 3–4 wk after implantation, tumors were aseptically removed, minced into 1- to 2-mm3 pieces, and enzymatically digested in 1% collagenase and 325 U/ml DNAse (both from Roche) at 37°C for 2 h. The resulting single cell suspension was washed twice and resuspended in RPMI 1640 medium, 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 3000 U/ml rIL-1 (Protech). After overnight incubation at 37°C and 5% CO2 in plastic culture dishes, the nonadherent population was removed and counted. Nonadherent cells were stained with anti-Thy1.2 (BD PharMingen) and sorted on a FACSVantage SE (BD Biosciences, Mountain View, CA). Population purity was analyzed on a BD PharMingen LSR Analyzer. The positive population was washed three times to remove any serum and then was cultured overnight.

Results

Evaluation of gelatinase production in splenic T cells from normal and tumor-bearing mice

The gelatinase activity produced by splenic T cells from normal mice and from animals bearing the D1-DMBA-3 mammary tumor was evaluated by zymography using gelatin as a substrate (Fig. 1A). Splenic T cells from normal and mice bearing tumors for 1, 2, and 3 wk were cultured in serum-free medium for 18 h, and the supernatants were collected to assay by zymography for enzyme activity. No detectable MMP-2 (72 kDa) was observed in the T cell cultures, but MMP-9 activity increased in parallel with tumor burden from the basal levels seen in normal T cells. Importantly, these enhanced MMP-9 levels were not only of the latent pro-enzyme form observed as a single band at 105 kDa, but they were also of the active form, as indicated by the bands of activation at 95 kDa. Proteolytic cleavage of the proenzyme domain of MMP-9 results in the active enzyme that is ~10 kDa less in molecular mass. It is
Because the level of enzymatic activity of MMP-9 is determined only in part by the amount of MMP-9 secreted, we next assessed the protein levels of this metalloproteinase. Duplicate aliquots of the cell culture supernatants tested for enzymatic activity were subjected to SDS-PAGE and immunoblotting analysis for protein expression (Fig. 1B). The results indicated that the increased MMP-9 enzymatic activity paralleling tumor burden correlates with increased MMP-9 protein synthesis. To determine whether CD4+ and CD8+ T lymphocytes vary in their secretion of MMP-9, these populations were positively selected using Miltenyi Biotec magnetic beads conjugated to anti-CD4 and anti-CD8 Abs. Thy1.2+, CD4+, and CD8+ splenic T cells from normal and tumor-bearing mice were cultured overnight, and the supernatants were assayed by zymography. CD4+ and CD8+ splenic T cells demonstrated similar levels of MMP-9 activity (Fig. 1C).

This metalloproteinase up-regulation is not solely a phenomenon of the D1-DMBA-3 tumor. The zymogram in Fig. 2A shows that T cells from animals bearing both D1-DMBA-3 (lanes 4, 5, and 6) or DA-3 tumors (lanes 7, 8, and 9), an in vitro-derived cell line from the D1-DMBA-3 in vivo tumor, produced enhanced levels of MMP-9 compared with T cells from normal mice (lanes 10, 11, and 12). It was also determined that the protein kinase C activator PMA (25 ng/ml) failed to elevate MMP-9 in the BALB/c T lymphocytes, in contrast with its reported induction capabilities for this metalloproteinase in various T cell lines and human peripheral blood cells (25, 38). Likewise, Con A did not increase the levels of MMP-9 in normal or tumor-bearing mouse lymphocytes. Mouse MMP-9 is 105 kDa because of an additional 24-aa insert (39, 40) and is located higher in the gel than is the human MMP-9 (92 kDa) positive control. To ascertain whether this up-regulation was only seen in a mammary adenocarcinoma animal model, T cells from BALB/c mice bearing either the K-7 osteosarcoma or the Renca tumors were also tested for MMP-9 activity (Fig. 2B). Splenic T cells isolated from these animals also demonstrated increased MMP-9 activity with tumor growth.

**MMP-9 mRNA expression parallels enzyme production**

To determine whether the increased levels of MMP-9 secretion in the T cells from tumor bearers were due to altered transcription, MMP-9 gene expression in normal and tumor-bearing mice was analyzed by RT-PCR and Northern blots. A progressive increase in the levels of MMP-9 mRNA was seen in 4-h cultures of unstimulated splenic T cells from mice bearing 1-, 2-, or 3-wk tumors, as detected by RT-PCR (Fig. 3A). Additionally, Northern blot analysis using a murine-specific cDNA probe confirmed that steady state levels of MMP-9 mRNA increase with tumor burden (Fig. 3B). Splenic T cells from mice bearing 3-wk tumors expressed higher levels of each of the two different murine MMP-9 mRNAs (2.5 and 3.2 kb), in comparison with T cells from normal mice.

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**FIGURE 2.** Splenic T cells from various solid tumors secrete elevated levels of MMP-9. A, MMP-9 activity is increased in T cells from both D1-DMBA-3 and DA-3 tumor-bearing mice. Equal amounts of supernatant from splenic T cell cultures from animals bearing either D1-DMBA-3 (lanes 4, 5, and 6) or DA-3 tumors for 4 wk (lanes 7, 8, and 9) and from normal mice (lanes 10, 11, and 12) were concentrated 10× and assayed by gelatin zymography. Some T cell cultures were incubated with Con A (10 μg/ml) or PMA (25 ng/ml). Lane 2 contains a molecular mass marker, and lane 3 contains the 92-kDa human MMP-9 standard. Data are representative of three independent experiments using three mice per group. B, MMP-9 activity is increased in T cells from mice bearing Renca and K-7 tumors for 4 wk. Equal amounts of supernatants from T cell cultures were assayed by gelatin zymography for enzyme activity. Data are representative of three independent experiments using two mice per group.

**FIGURE 3.** Increased MMP-9 mRNA expression in splenic T cells from tumor-bearing mice. A, Splenic T cells from normal and tumor-bearing mice were cultured without stimulation for 4 h. Semi-quantitative RT-PCR was performed on total RNA (1 μg) isolated from the cells. A 100-bp DNA ladder was used as molecular mass marker. β-Actin band intensity was used for normalization, and integration intensity percentages were calculated relative to the maximum for each experiment. The data are representative of four independent experiments. B, Total RNA (10 μg) from unstimulated splenic T cells from normal and 3-wk tumor-bearing mice was subjected to Northern blot analysis by hybridization with a specific cDNA probe for MMP-9. The two different murine MMP-9 mRNAs (2.5 and 3.2 kb) were detected. Equivalence of RNA loading was confirmed by rehybridization to a β-actin probe. Data are representative of three independent experiments.
Equivalence of RNA loading in different lanes was ascertained by rehybridization to a β-actin probe. Results indicated that splenic T cells from tumor-bearing animals express high levels of MMP-9 mRNA, compared with normal animals. These results corroborate that the transcriptional activity of MMP-9 correlates with its secreted enzyme activity.

TIMP secretion by mammary tumor cells and T lymphocytes

The enzymatic activity of MMPs is dependent upon equilibrium among the production of the proenzyme, activation of the latent enzyme, and production of the naturally occurring inhibitors, especially TIMPs (41). Currently, the TIMP family consists of four members: TIMP-1, -2, -3, and -4. The gelatinase activities of both human and mouse MMP-2 and -9 are regulated by TIMP-2 and TIMP-1, respectively, which inhibit protease activity and can block cell invasion in vitro and metastasis in vivo (42, 43). These inhibitors are often produced by the same cells that produce the gelatinases and are usually found complexed noncovalently with the enzymes at the carboxy-terminal domain (44). Therefore, TIMP activity was assayed by reverse zymography using the same cell cultures tested for MMPs. As seen in Fig. 4A, DA-3 tumor cells secreted very low levels of TIMP-2 (21 kDa), and their levels of secreted TIMP-1 (29 kDa), the natural inhibitor of MMP-9, were barely detectable. No TIMP-3 (24 kDa) or TIMP-4 (23 kDa) was detected. Although these inhibitors are often produced by the same cells that secrete MMPs, no TIMPs could be detected in the supernatants of cultured splenic T cells from either normal or tumor-bearing mice (Fig. 4B). Furthermore, no TIMP-1 mRNA could be detected in cultured splenic T cells, and minimal levels of TIMP-2 mRNA expression were seen only in normal T cells using gene expression array analysis (Fig. 4C). Results were confirmed by RT-PCR (data not shown). The high levels of MMP-9 mRNA seen only in RNA isolated from tumor bearers’ splenic T cells also confirmed the results of Fig. 3.

Assessment of the role of tumor-derived factors and tumor-induced cytokines in the elevation of MMP-9 in T cells from tumor bearers

The mammary tumors used in our studies, D1-DMBA-3 and its cloned cell line DA-3, have been shown in previous work to constitutively produce several factors, such as PGE2 (45), GM-CSF (46), phosphatidyl serine (PS) (47), and noncharacterized angiogenic molecules (45). To investigate whether one or more of these tumor-derived factors was causing the enhanced levels of active MMP-9 observed in T cells from D1-DMBA-3 mammary tumor bearers, we treated normal lymphocytes with PGE2, PS, and GM-CSF at concentrations known to be in circulation in tumor-bearing mice (Fig. 5A). The cell supernatants of normal T cells cultured...
overnight with 50 U/ml GM-CSF, 30 and 60 μg/ml phosphatidyl serine, or 10^{-6} M PGE2, were analyzed by zymography. Titrations of these tumor-derived factors, either alone or in combination (data not shown), failed to increase the levels of proenzyme or to activate MMP-9 from normal T lymphocytes. Supernatants from untreated tumor bearers’ T cells were included in the gel as a comparison of MMP-9 activity.

After eliminating these characterized tumor-derived factors as mediators of MMP-9 up-regulation, we looked at known tumor-induced factors in this model system. The proinflammatory cytokines TNF-α and IL-6 are up-regulated in the B cells (48, 49) and macrophages (50, 51) of tumor bearers, leading to elevated levels of these cytokines in their sera compared with normal BALB/c mice. Therefore, these two molecules were possible candidates for soluble factor(s) regulating MMP-9 expression. Splenic T cells from normal mice were cultured overnight with 60 U/ml IL-6 or 50 U/ml TNF-α, and MMP-9 enzyme activity in the cell supernatants was assessed by zymography. Again, treatment of normal T cells with the proinflammatory cytokines had no effect on MMP-9 secretion (Fig. 5B). Identical MMP-9 secretion was seen in control and cytokine-treated normal T cells with titrations of both of the recombinant cytokines, either alone or in combination (data not shown). Supernatants from untreated tumor bearers’ T cells were included in the gel as a comparison of MMP-9 activity. It should be noted that the efficacy of the recombinant cytokines and other factors was ascertained by their ability to modulate cytokine/chemokine production in parallel cultures of T cells and macrophages (data not shown).

**VEGF production in the mammary tumor model and its effects on MMP-9**

Because none of the previously described tumor-derived factors or cytokines in our mammary tumor model was capable of modulating MMP-9 in the T cells, we looked for other tumor-derived and/or -induced factors that may contribute to elevated MMP-9 production. Many solid tumors express VEGF, and this expression directly correlates with regions of angiogenesis and high vascular density (52). Furthermore, the newly formed blood vessels are inherently leaky, enhancing the likelihood of cell migration and metastasis (53). Based on the knowledge that most solid tumors express VEGF and that the supernatants of DA-3 cells have angiogenic activity, we measured DA-3 tumor cell production of VEGF by ELISA. The results depicted in Fig. 6A confirm that cultured DA-3 cells express physiologically relevant concentrations of VEGF, because this growth factor is known to affect biologic activity at concentrations below 1 pM (54). D1-DMBA-3 cells cultured under identical conditions secreted 2364 ± 37 pg/ml VEGF after 4 days in culture.

Of the few published reports of VEGF production by T cells, most cite studies of human PBLs (55, 56). We sought to investigate whether murine T lymphocytes produce detectable levels of VEGF and whether such a production is affected by tumor progression. Splenic T cells from normal and 4-wk tumor-bearing mice were cultured overnight in the absence of any stimulus and were tested by ELISA for the production of VEGF (Fig. 6B). Low levels of VEGF were constitutively expressed by normal BALB/c T lymphocytes, with higher concentrations of this growth factor detected in the culture supernatants of T lymphocytes from 4-wk tumor-bearing mice. Stimulation with PHA (2 μg/ml) or IL-2 (50 U/ml) did not increase the levels of VEGF in T cells from normal or tumor-bearing mice. Interestingly, PMA (10 ng/ml) enhanced the VEGF production by T cells from tumor bearers but not from normal mice.

The physiological levels of VEGF in our system were determined by ELISA testing of sera from normal mice and animals bearing 2- and 4-wk D1-DMBA-3 tumors. The mean serum level of VEGF nearly doubles after 4 wk of tumor burden (Fig. 6C). Because macrophages are also known producers of VEGF, peritoneal elicited macrophages were analyzed for their production of VEGF, but no significant differences were seen between the levels produced by cells from normal and tumor-bearing mice (data not shown).

To determine whether VEGF could act directly on the T lymphocytes, it was important to demonstrate the expression of VEGF receptors by splenic T cells. There are no reports in the literature demonstrating the expression of such receptors in primary murine T cells. The two tyrosine kinases of the VEGF receptor family,
Flt-1 (VEGFR-1) and Flk-1 (kinase domain region/VEGFR-2), were the likely candidates for the putative receptors of this growth factor because they are expressed on endothelium, skeletal muscle, hematopoietic stem cells, megakaryocytes, macrophages, and human CD34 cells (53). The expression of both receptors has also been detected in some leukemia cell lines (57). RT-PCR was performed on total RNA isolated from 2-h cultures of T cells from normal and tumor-bearing animals. The expression of Flt-1 and Flk-1 mRNA was seen in both normal and tumor bearers’ splenic T cells. Actin mRNA levels served as controls to normalize RNA quantity. Interestingly, both Flt-1 and Flk-1 mRNA levels were greater in the T cells from mice bearing mammary tumors than in the normal BALB/c animals (Fig. 7A).

In other cells expressing VEGF receptors, VEGF has been shown to up-regulate its production in an autocrine manner (57). To determine whether VEGF up-regulates its own production in murine splenic T cells, T lymphocytes from normal and tumor-bearing mice were cultured with rVEGF, and total RNA was isolated and analyzed by RT-PCR (Fig. 7B). In contrast with other cell types, VEGF failed to up-regulate its expression in splenic T cells from either normal or tumor-bearing mice. Two different mRNAs were detected in both normal and tumor-bearing animals, corresponding with VEGF165 and VEGF120. VEGF165 is the predominant isoform and has the optimal characteristics of bioavailability and biological potency (57). No difference in intensity of bands corresponding with the two isoforms VEGF165 and VEGF120 was detected in those cells cultured with rVEGF, compared with untreated cells. There was increased expression of VEGF165 in tumor bearers’ T cells compared with T cells from normal animals, corroborating data at the protein level (Fig. 6B).

The up-regulation of the expression of MMP-9 in the tumor bearers’ T cells appears to be induced by the developing cancer, possibly via one or more of the tumor-derived factors. Therefore, we investigated whether VEGF plays a role in the elevated secretion of MMP-9 by the T cells of tumor bearers. Normal splenic T cells were cultured overnight with a titration of rmVEGF. Fig. 8A shows an up-regulation of MMP-9 in T cells treated with rVEGF. Because VEGF is known to be a potent mitogen of endothelial cells, 5-bromo-2-deoxyuridine incorporation assays were performed to confirm that this increased production of MMP-9 was not due to proliferation of the T cells cultured with VEGF (data not shown). We also see increased levels of MMP-9 mRNA in T cells cultured with rVEGF compared with normal T cells cultured without treatment (Fig. 8B). Furthermore, an anti-VEGF neutralizing Ab reduced the up-regulated levels of MMP-9 in normal T cells cultured with rVEGF (Fig. 8C). These results strongly suggest that although there may be other yet to be identified modulatory factors, VEGF certainly plays a role in the up-regulation of MMP-9 in T lymphocytes of mammary tumor bearers.

Production of MMP-9 in the tumor

The mammary tumor cells used in our studies were assessed for their production of MMP-9. In Fig. 9A, it can be seen that the DA-3 tumor cells produce measurable levels of MMP-9, as detected by zymography. Inflammatory cells have been shown to produce MMP-9 in the tumor model. Inflammatory cells have been shown to produce MMP-9 in the tumor.
secrete several MMPs, including MMP-9, -12, and -14, potentially promoting cancer progression by the release of these proteases (21). To assess the contributions of T cells to the levels of proteases secreted within the tumor microenvironment, immunohistochemistry was first performed using an anti-CD3 Ab to evaluate the presence of T lymphocytes in sections of tumors obtained 3–4

FIGURE 7. Expression of VEGF receptors by splenic T cells. A, Splenic T cells from normal mice (N) and mice bearing tumors (T) were cultured without stimulation for 2 h. Total RNA was isolated for analysis of Flt-1 and Flik-1 expression by RT-PCR. Amplified products were subjected to electrophoresis on 1.6% agarose gels and visualized by ethidium bromide staining. A 100-bp DNA ladder was used as molecular mass marker. β-Actin band intensity was used for normalization, and integration intensity percentages were calculated relative to the maximum for each experiment. Gels shown are representative of four experiments. B, Purified splenic T cells from normal (N) and tumor-bearing animals (T) were cultured untreated or with 1 ng/ml rmVEGF for 4 h. Total RNA was isolated for analysis of VEGF expression by semiquantitative RT-PCR. Gel shown is representative of three experiments.

FIGURE 8. VEGF up-regulates the production of MMP-9 in splenic T cells. A, Normal splenic T cells were either untreated (lane 3) or treated with 0.05 ng/ml VEGF (lane 4), 0.1 ng/ml VEGF (lane 5), 1 ng/ml VEGF (lane 6), 5 ng/ml VEGF (lane 7), 10 ng/ml VEGF (lane 8), 20 ng/ml VEGF (lane 9), 50 ng/ml VEGF (lane 10), or 100 ng/ml VEGF (lane 11). Lane 1 contains a molecular mass marker. Equal amounts of supernatant were assayed by gelatin zymography. Data are representative of at least four independent experiments. B, Normal splenic T cells were cultured untreated or with 1 ng/ml rmVEGF for 4 h. Total RNA was isolated for analysis of MMP-9 expression by semiquantitative RT-PCR. A 100-bp DNA ladder was used as molecular mass marker. Amplified products were subjected to electrophoresis on 1.6% agarose gels and visualized by ethidium bromide staining. β-Actin band intensity was used for normalization, and integration intensity percentages were calculated relative to the maximum for each experiment. The gel shown is representative of four experiments. C, Anti-VEGF neutralizing Ab reduces the up-regulation of MMP-9 by recombinant VEGF. Recombinant murine VEGF (1 ng/ml) was incubated with 0.1 μg/ml of the Ab (R&D Systems) for 1 h. After this preincubation period, normal splenic T cells were added and incubated overnight. Equal amounts of supernatant were assayed by gelatin zymography. Data are representative of three independent experiments.
wk postimplantation. As seen in Fig. 9B, tumor-infiltrating T lymphocytes could be detected within the tumor bed. In further studies, T cells were isolated from the tumors after enzymatic digestion, as described in Materials and Methods. After overnight culture, the nonadherent cells were sorted based on their expression of Thy1.2. As seen in Fig. 9C (left panel), the preparations were greatly enriched for Thy1.2 after sorting, from 10.8% to 97.8%. The purified tumor-infiltrating T cells were then analyzed for their production of MMP-9 by zymography. The Thy1.2+ cells produced very high levels of this proenzyme and its active form (Fig. 9C, right panel). As was the case with the splenic T lymphocytes from tumor bearers, when duplicate cultures of the tumor-infiltrating Thy1.2+ cells were assayed for VEGF production, a high constitutive level of this angiogenic factor was also observed (72 pg/ml).

Discussion
Progressive tumor growth is associated with multiple changes in the immune compartment of the host. In this study, it was found that MMP-9 expression and VEGF production are up-regulated in lymphocytes of animals with developing neoplasia. The up-regulated expression of MMP-9 is found not only in splenic T cells but, more importantly, in the T cells infiltrating the tumor microenvironment, and this increased expression correlates with tumor growth.
growth. Our previous studies (58) and those of many other investigators (19, 59) have shown the infiltration of the tumor microenvironment by lymphocytes through a process involving adhesion molecules (60). The presence of MMP-9 in tumor bearers’ lymphocytes may also play a role, because this metalloproteinase has the potential to degrade matrix protein and to promote leukocyte traffic.

Although there are many reports about the regulation of metalloproteinases in tumor cells, less attention has been focused on their expression in lymphocytes. The production of MMP-9 has been studied by several investigators using cultured macrophages and macrophage and T cell lines (61, 62), and most studies have used stimuli to induce secretion of MMPs (37). Conversely, the higher levels of MMP-9 observed in our mammary tumor bearers’ T cells is a constitutive event, in that it occurs in the absence of added stimuli.

Another aspect of this study investigated the production of TIMPs by the mammary tumor and the splenic T cells. An important mechanism for the regulation of MMP proteolysis is via the actions of the TIMPs that bind to the active site of both latent and active MMPs, forming stable, but inactive, enzyme-inhibitor complexes (63). TIMPs are considered the key inhibitors of MMPs in tissue and, like MMPs, the expression of these inhibitors is controlled to maintain a balance in the integrity of the ECM (64). The TIMPs differ in their inhibitory properties. Thus, TIMP-2 binds to the proMMP-2, whereas TIMP-1 forms a complex with proMMP-9 (44). Investigators have seen coregulation of MMP-9 and its specific inhibitor, TIMP-1, in a malignant form of non-Hodgkin’s lymphoma, and it has been suggested that this coregulation of gelatinase and inhibitor plays an important role in controlling the aggressiveness of this cancer (65). We observed very low levels of TIMP-1 secretion by the mammary tumor cells and no expression of this molecule by the splenic T cells of either normal or tumor-bearing mice. Therefore, the up-regulation of TIMP-9 does not seem to correlate with increased TIMP production in our system.

The up-regulation of the expression of MMP-9 in the tumor bearers’ T cells appears to be induced by the developing cancer, possibly via one or more of the tumor-derived or tumor-induced factors. Experiments in which normal T cells were cultured with PGE2, GM-CSF, and PS were performed to evaluate the potential contribution(s) of tumor-derived factors to the induction of MMP-9. We have previously shown that these factors are produced by the mammary tumors used in our studies and that they can exert potent regulatory actions on the cells of the immune system during tumor growth (46, 66, 67). Moreover, Leppert et al. (68) have shown that 10^-6 M PGE2 increased the secretion of MMP-2, -3, and -9 by the human lymphoblastoma cell line Tsup-1. In addition, TNF-α and IL-6, although not produced by the tumor cells, are up-regulated in the macrophages (50, 51) and the B cells (48, 49) of mammary tumor bearers. Therefore, the effects of these cytokines were also evaluated in these experiments. The autocrine regulation of MMP-9 expression by TNF-α has been described in the human promyelocytic cell line, HL-60 (69), and IL-6 has been shown to up-regulate MMP-9 in malignant non-Hodgkin’s lymphomas (70). Thus, it was of special interest to investigate the effects of these cytokines on T cells. However, our results indicated that treatment with these cytokines and tumor products had no effect on the levels of MMP-9 secreted by murine splenic T cells. This is not surprising, because MMPs are stimulated by different factors, and different cell types have varying responses to a range of stimuli in the induction of these proteases.

Previously we demonstrated that supernatants of the DA-3 mammary tumor cells had angiogenic activity (45). VEGF is produced by a variety of tumors and tissues, and in fact we have found that our tumors are high producers of this growth factor. Thus, the mammary tumor cell-derived VEGF could be responsible for the angiogenic activity detected in the supernatants of these cells. VEGF plays a central role in neovascularization, because it is an endothelial cell mitogen in vitro and a potent inducer of angiogenesis in vivo (52). The tumor cells were found to produce substantial quantities of VEGF, and increased circulating levels of this growth factor were measured in the sera of tumor bearers.

Interestingly, splenic T lymphocytes from mice bearing tumors also secrete VEGF. These constitutive levels were not significantly altered by incubation of the T cells with either PHA or IL-2, but PMA did up-regulate production in tumor bearers’ T cells. Tumor-infiltrating Thy1.2+ cells were also found to secrete VEGF. Human lymphocytes are known to produce VEGF; negligible VEGF mRNA expression was detected in resting human peripheral T cells, whereas PMA activation markedly increased the expression of this growth factor (55). More relevant for our studies is the observation that immunohistochemically identifiable T lymphocytes infiltrating human prostate and bladder cancers were identified by in situ hybridization as expressing VEGF mRNA (56). This finding, as well as our observations, suggests that T cell infiltrations, regarded as evidence of a protective immune response against tumors, may be promoting vasculogenesis and microvascular remodeling. To our knowledge, the only other demonstration of VEGF production by murine T lymphocytes is during the process of collateral vessel growth in an ApoE-/- mouse model (71).

Importantly, we also present the novel finding of the expression of the VEGF receptors Flt-1 and Flk-1/kinase domain region in murine splenic T cells and the increased expression of these receptors paralleling that of VEGF and tumor burden. Previous investigations have shown that Flt-1 and Flk-1 are normally expressed at low levels in endothelial cells and in inflamed joint tissue, but that they are up-regulated at sites where there is a concomitant up-regulation of VEGF (72, 73). Our mammary tumor cells produce VEGF, and there are increased circulating levels of this growth factor post-tumor implantation. In this study, we demonstrate that VEGF can up-regulate MMP-9 expression in the murine T lymphocytes. Because splenic T cells from tumor-bearing mice produce higher levels of endogenous VEGF, we examined the possibility that there may be an autocrine effect on T cell-derived MMP-9. To this end, we blocked the in vitro VEGF secretion by tumor bearers’ T cells and measured their MMP-9 production (data not shown). There were no differences between the anti-VEGF-treated and -untreated cells’ MMP-9 levels. Because the T lymphocytes are exposed to high serum levels of VEGF for several weeks in vivo, it appears that it may be too late to revert the exaggerated production of MMP-9 in tumor bearers’ T cells.

VEGF signaling is mediated through two transmembrane tyrosine kinase receptors, Flt-1/VEGFR-1 and Flk-1/VEGFR-2. VEGFR-2 is the critical receptor for the proliferation and differentiation of endothelial cells, whereas VEGFR-1 may be more important for vascular remodeling (74). Preliminary data using anti-VEGF treated and -untreated cells’ MMP-9 levels. Because the T lymphocytes are exposed to high serum levels of VEGF for several weeks in vivo, it appears that it may be too late to revert the exaggerated production of MMP-9 in tumor bearers’ T cells.

In the literature, there are only a few reports of MMP-9 up-regulation by this growth factor in human aortic smooth muscle cells (75), human brain tumor cells (76), and human myelo-monocytic leukemia cell lines (74). However, there are no reports demonstrating the up-regulation of MMP-9 in T cells by this growth factor. Of all the tumor-derived and tumor-induced factors tested, VEGF was the only one that exerted an effect in the splenic T cells. However, it should be emphasized that we do not believe that this is the only factor present either in the tumor cells or in the host, which is responsible for the observed up-regulation of MMP-9. Indeed, we continue our investigations for different molecules.
present in our model system that may be contributing, as is VEGF, to the modulation of this metalloproteinase in the T lymphocyte compartment of tumor bearers.

Our work further supports the previous observations that inflammatory cells produce significant amounts of proteases within the tumor bed. Coussens et al. have implicated monocytes, neutrophils, and mast cells as predominant producers of MMP-9 in skin carcinogenesis in K14-HPV16 transgenic mice (21). Recently, this group reported that CD4+ T cells enhance skin carcinogenesis by recruiting MMP-secreting Gr-1+/Mac-1+ cells into the neoplastic lesions, but that they do not themselves produce MMP-9 (77). In this analysis, we have demonstrated that T cells appear to be key contributors of MMP-9 in a mammary tumor model, both in the periphery and within the tumor microenvironment. MMPs are positive regulators of angiogenesis, in that both endogenous and synthetic MMP inhibitors reduce tumor angiogenesis in tumor models (10). Angiogenesis is crucial for expansive tumor growth and dissemination. Tumors do not grow beyond a few mm³ in size without vascularization for proper nourishment and removal of metabolic wastes from the site of the tumor (78, 79). MMPs are important modulators of these events because they are responsible for the proteolysis of connective tissue barriers necessary for new vessel formation. Furthermore, there is evidence that MMP-9 renders the angiogenic factor, VEGF, more available to its receptors on endothelial cells during pancreatic islet carcinogenesis (80), adding another dimension to the proangiogenic potential of MMP-9.

Based on our findings that MMP-9 and VEGF are up-regulated in the T lymphocytes from tumor bearers and the role of VEGF in the elevated MMP-9 production, it is tempting to speculate that these two factors may be interacting to promote neoplastic progression in this mammary tumor model. Within the tumor microenvironment of our mammary adenocarcinoma model, T cells are supplying MMP-9 and VEGF. In addition, several investigations have shown that, via cellular contact, T cells can induce MMP-9 expression in a variety of cell lines, including monocytes and fibroblastic cells in coculture (81, 82), suggesting that the T cells’ secretion of MMP-9 could further potentiate protease production by other inflammatory cells within the tumor bed. In light of recent experiments, Coussens et al. have suggested that inflammatory cell types may become an important class of tumor-associated cells targeted by anticancer drugs (21). Our studies indicate that VEGF acts directly on T lymphocytes and that elevated VEGF levels may be associated with aberrant MMP-9 secretion, suggesting that modulation of MMP-9 expression by anti-VEGF therapeutics might be useful as a strategy to attenuate protease activity in developing cancers.

VEGF has also been shown to inhibit the functional maturation of dendritic cells, which could result in a decreased ability of the immune system to generate effective antitumor responses (83). In addition, VEGF has been recently shown to cause thymic atrophy and the inhibition of T cell development (84). In light of the demonstration of VEGF receptor expression in murine splenic T cells, it should be determined whether VEGF is capable of mediating T cell function, including cytokine secretion, proliferation, and/or cytotoxicity, in addition to its induction of MMP-9. Indeed, in our model tumor system, we have observed thymic atrophy accompanying tumor progression (85), as well as a decrease in IFN-γ production by splenic T cells (67). Perhaps VEGF has additional effects on the T cells of our mammary tumor bearers beyond that of protease induction.

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References


