T Lymphocytes Induce Endothelial Cell Matrix Metalloproteinase Expression by a CD40L-Dependent Mechanism

Implications for Tubule Formation

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Neovascularization frequently accompanies chronic immune responses characterized by T cell infiltration and activation. Angiogenesis requires endothelial cells (ECs) to penetrate extracellular matrix, a process that involves matrix metalloproteinases (MMPs). We report here that activated human T cells mediate contact-dependent expression of MMPs in ECs through CD40/CD40 ligand signaling. Ligation of CD40 on ECs induced *de novo* expression of gelatinase B (MMP-9), increased interstitial collagenase (MMP-1) and stromelysin (MMP-3), and activated gelatinase A (MMP-2). Recombinant human CD40L induced expression of MMPs by human vascular ECs to a greater extent than did maximally effective concentrations of interleukin-1 β or tumor necrosis factor- α . Moreover, activation of human vascular ECs through CD40 induced tube formation in a three-dimensional fibrin matrix gel assay, an effect antagonized by a MMP inhibitor. These results demonstrated that activation of ECs by interaction with T cells induced synthesis and release of MMPs and promoted an angiogenic function of ECs via CD40L-CD40 signaling. As vascular cells at the sites of chronic inflammation, such as atherosclerotic plaques, express CD40 and its ligand, our findings suggest that ligation of CD40 on ECs can mediate aspects of vascular remodeling and neovessel formation during atherogenesis and other chronic immune reactions. (*Am J Pathol* 1999, 154:229–238)

Neovascularization commonly accompanies chronic immune and inflammatory responses characterized by

prominent T cell infiltration and requires degradation of the vascular basement membrane and surrounding extracellular matrix to permit migration and proliferation of endothelial cells (ECs). Examples of conditions associated with neoangiogenesis include the lesions of rheumatoid synovitis, certain tumors, and atherosclerosis.^{1–6} Interactions between T cells and ECs may promote activation of the endothelium in such diseases.^{7–9} Critical mediators of neoangiogenesis include matrix metalloproteinases (MMPs), enzymes that can degrade extracellular matrix components, allowing cells to penetrate and reshape connective tissue.^{10,11} Substantial evidence supports crucial roles for MMPs in tumor growth and metastasis,^{12,13} tissue remodeling,^{14,15} and angiogenesis.^{13,16} Human vascular ECs *in vitro* express interstitial collagenase (MMP-1), gelatinases A and B (MMP-2 and MMP-9, respectively), and stromelysin (MMP-3) upon stimulation with soluble mediators such as interleukin (IL)-1 and tumor necrosis factor- α .¹⁷ Activated ECs also express various surface molecules, including vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, and E-selectin, that promote recruitment and activation of leukocytes to the sites of inflammation.^{18,19} However, it remains unknown whether contact-dependent interactions of T cells with ECs could promote MMP expression.

Recently, several reports have established that human vascular ECs express CD40.^{20–22} CD40 belongs to the nerve growth factor receptor family, which includes the TNF receptors and Fas (CD95).²³ These receptors participate in the regulation of cell proliferation, differentiation, or apoptosis.^{24,25} CD40 binds CD40 ligand (CD40L, also referred to as gp39 or TRAP), a cell surface molecule

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until recently considered restricted to activated CD4⁺ T cells.²⁶ CD40L belongs to the TNF family, which includes TNF- α , Fas ligand, and others.²³ Initial studies of CD40L-CD40 signaling focused on T-cell-dependent B cell differentiation and activation.^{23,24} Several recent reports have demonstrated that CD40L-CD40 interaction induces EC activation manifested by increased expression of adhesion molecules.^{20–22} Moreover, recent data implicates CD40 and CD40L in a variety of sites of human chronic immune and inflammatory conditions, including multiple sclerosis, various neoplasias, glomerulonephritis, and atherosclerosis.^{27–32}

The present study explored the hypothesis that ligation of CD40 on ECs could induce expression of MMPs and thus contribute to neovessel formation. We demonstrate here that activation of ECs through CD40 ligation, with either membranes from activated CD4⁺ T cells or human recombinant CD40L (rCD40L), activates MMP-2, induces expression of MMP-9, increases MMP-1 and MMP-3 expression, and stimulates capillary-like tube formation *in vitro*, a feature of neovascularization. Thus, T cells can stimulate endothelial production or activation of all three classes of MMP and in this manner probably promote neovascularization through a CD40-CD40L contact-dependent mechanism.

Materials and Methods

Reagents

Human recombinant CD40L (rCD40L) was obtained from Geneva Biomedical Research Institute (Geneva, Switzerland).³³ IL-1 β , TNF- α , and basic fibroblast growth factor was obtained from Endogen (Cambridge, MA). Gelatin was purchased from Bio-Rad (Hercules, CA). β -Casein, phorbol 12-myristate 13-acetate (PMA), and polymyxin B were obtained from Sigma (St. Louis, MO). Rabbit polyclonal anti-human MMP-1, MMP-3, and MMP-9 antibodies and the MMP inhibitor UK-231,890 were provided by Pfizer Central Research (Kent, UK). Mouse monoclonal anti-human tissue inhibitor of MMP-1 (TIMP-1) and TIMP-2 were obtained from Oncogene Science (San Diego, CA). Monoclonal anti-CD40L antibody and human fibrinogen were purchased from Calbiochem (San Diego, CA).

Cell Isolation and Culture

Human vascular ECs were isolated from saphenous veins by collagenase treatment and cultured in dishes coated with fibronectin (1.5 μ g/cm²; Upstate Biotechnology, Lake Placid, NY) as described elsewhere.³⁴ Cells were maintained in medium 199 (M199; BioWhittaker, Walkersville, MD), supplemented with 1% penicillin/streptomycin (BioWhittaker), 5% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), 100 μ g/ml heparin (Sigma), and 50 μ g/ml endothelial cell growth factor (Pel-Freez Biological, Rogers, AK) and used throughout passages two to four. Culture media and FBS contained less than 40 pg of endotoxin/ml as determined by chromogenic *Limulus* amebocyte assay (QLC-1000; BioWhittaker). ECs were

characterized by immunostaining with anti-von Willebrand factor monoclonal antibody (Dako, Carpinteria, CA). Vascular ECs were cultured 24 hours before the experiments in M199 supplemented with 0.1% human serum albumin (Immuno-US, Rochester, MI) and in fresh M199 (serum-free) for the experiments.

Freshly isolated human CD4⁺ T cells were a gift from Dr. Andrew Lichtman (Brigham and Women's Hospital, Boston, MA). Purity of CD4⁺ T cells was $\geq 98\%$, as determined by FACS analysis (anti-CD4 monoclonal antibody (Mab) FITC; Calbiochem). Human CD4⁺ T cells were activated with PMA (50 ng/ml for 12 hours), and CD40L cell-surface expression was confirmed by FACS analysis using anti-CD40L Mab FITC (Calbiochem). Activated CD4⁺ T cell membranes were prepared as described previously,³⁵ and membrane fractions were added for stimulation at a ratio equivalent to 10 T cells to 1 EC. All experiments presented in this paper were performed in the presence of Polymyxin B (1 μ g/ml).

Zymography

Supernatants of cultured ECs were centrifuged (500 $\times g$ for 10 minutes at 4°C), concentrated 10X (Ultrafree centrifugal filter-4; Millipore, Bedford, MA), and separated under nonreducing conditions by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) containing 1 mg/ml gelatin or casein.³⁶ After electrophoresis, the proteins were renatured by soaking gels (two times for 15 minutes each) in 2.5% Triton X-100 (VWR Scientific, West Chester, PA). Subsequently, gels were incubated (18 hours at 37°C) in 50 mmol/L Tris/HCl (pH 7.4), containing 10 mmol/L CaCl₂ and 0.05% Brij 35 (Sigma). To verify the metalloproteinase activity detected by zymography, identical gels were incubated in the above buffer containing either 20 mmol/L EDTA, an inhibitor of MMPs, or 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), an inhibitor of serine proteases. Finally, gels were stained with Coomassie brilliant blue R250 (Sigma; 30 mmol/L in 45% methanol/10% acetic acid) and then destained in 25% methanol/20% acetic acid.

Western Blotting and Immunoprecipitation Analysis

Supernatants of cultured ECs were centrifuged (500 $\times g$ for 10 minutes at 4°C), concentrated 10X, separated by standard SDS-PAGE under reducing conditions, and blotted onto polyvinylidene difluoride (PVDF) membranes (Millipore) using a semi-dry blotting apparatus (3.0 mA/cm² for 30 minutes; Bio-Rad). Blots were preincubated (2 hours) in 5% defatted dry milk/PBS/0.1% Tween 20 to limit nonspecific binding, and then antibodies were applied in the same buffer. After 1 hour of incubation with the respective primary antibody (1:10,000 anti-MMP-1 or 1:2,000 anti-MMP-3 or anti-MMP-9), blots were washed four times (PBS/0.1% Tween 20), and the secondary peroxidase-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) was added (1:20,000) for another hour. Finally, after washing, detection

of the antigen was carried out using the enhanced chemiluminescent detection method according to the manufacturer's recommendations (Dupont-NEN, Boston, MA) and subsequent exposure to the membranes to x-ray film.

For immunoprecipitation, cells were washed with methionine/cysteine-free M199 medium (Sigma). Subsequently, the same medium containing 100 $\mu\text{Ci/ml}$ [^{35}S]methionine/cysteine was added to the cells for 24 hours. Supernatants of cultured ECs were centrifuged ($500 \times g$ for 10 minutes at 4°C), concentrated 10X, and incubated 24 hours (4°C) with nonimmune rabbit serum to reduce nonspecific binding (Vector, Burlingame, CA). After centrifugation ($10,000 \times g$ for 10 minutes at 4°C), supernatants were incubated (2 hours at 4°C) with the respective rabbit polyclonal anti-human MMP or TIMP antibody. Then, protein A agarose beads (Gibco, Gaithersburg, MD) were added (2 hours at 4°C). The samples were washed four times in a mixture of 50 mmol/L Tris/HCl, 0.02% SDS, 0.1% Nonidet P-40, 0.5 mol/L NaCl, 5 mmol/L EDTA, 0.1 mmol/L PMSF, 20 $\mu\text{g/ml}$ soybean trypsin inhibitor, 5 $\mu\text{mol/L}$ leupeptin, and 5 $\mu\text{mol/L}$ aprotinin, and thereafter, immunoprecipitated proteins were eluted by heating (10 minutes at 95°C) in reducing SDS-PAGE buffer (65 mmol/L Tris (pH 6.8), 10% glycerol, 2% SDS, 5% β -mercaptoethanol, and 0.0025% bromophenol blue). After centrifugation ($500 \times g$ for 10 minutes at 4°C), supernatants of these samples were separated on SDS-PAGE and transferred to PVDF membranes. Membranes were dried and exposed to x-ray film for detection of immunoprecipitated proteins.

To analyze the same supernatants by Western blotting and immunoprecipitation as well as by zymography, we used serum-free conditions for the experiments presented. Serum-free conditions are necessary for zymographic analysis, as serum is known to contain inhibitors of MMP activities. However, we also analyzed MMP expression under serum-containing conditions in Western blot or radioimmunoprecipitation analysis, achieving identical results.

Tubule Formation Assay

Formation of capillary-like tubes by vascular human ECs from saphenous veins were analyzed using a three-dimensional fibrin matrix gel assay as described.³⁷ Briefly, endotoxin- and plasminogen-free human fibrinogen (10 mg/ml; Calbiochem) was dissolved in serum-free medium (M199; BioWhittaker) and filtered through 0.22- μm filters (Millex-GS, Millipore). Fibrin matrices were prepared in six-well plates by polymerizing the fibrinogen solution with α -thrombin (2.0 U/ml; Sigma). After polymerization, gels were soaked in culture medium (M199; BioWhittaker) containing 10% FBS (Atlanta Biologicals) for 2 hours at 37°C to inactivate the thrombin. Human vascular saphenous veins ECs were seeded on the surface of the fibrin gel (50,000 cells/ cm^2) and overlaid with serum-free medium M199 (BioWhittaker) for the periods up to 72 hours in the presence or absence of stimulus described above. Photomicrographs of the matrix-gel surface as well as of cross sections were taken using a Nikon VFX-

IIA microscope ($\times 40$ and $\times 200$). For this purpose, fibrin matrices were washed twice and supernatants removed before photomicrographs of the gel surface were obtained. Subsequently, the matrices were carefully removed from the six-well plates, fixed on clean glass fiber filter strips, and sliced with a fine surgical blade (Becton Dickinson, Franklin Town, NJ). The slices were washed with PBS onto microscopic slides, and tubule formation was determined by the point-hit counting method.

Quantification of Endothelial Capillary-Like Tubule Formation

Photomicrographs of the matrix-gel surface and of the cross sections described above were analyzed using a computer-assisted morphometric analysis system (Optimas 5.2, Optimas Corp., Bothell, WA) by blinded observers. Tube-like structures ($>3 \mu\text{m}$) were identified on surface photomicrographs, and total tube length was determined for each of four randomly chosen fields. On cross section photomicrographs, the percentage area of capillary-like tubule formation was measured for each of four randomly chosen sections.

Results

Interaction of T Cells with Vascular ECs through CD40L-CD40 Activates Gelatinase A, Induces Synthesis and Secretion of Gelatinase B, and Increases Expression of Interstitial Collagenase and Stromelysin

We tested the expression of MMPs by human vascular ECs exposed to membranes isolated from CD40L-bearing CD4^+ T cells. Analysis of serum-free conditioned media of ECs incubated with membranes of PMA-activated CD4^+ T cells revealed induction and secretion of MMPs by ECs. Gelatin zymography (Figure 1A) showed constitutive expression of latent 72-kd gelatinase (MMP-2) by ECs as previously reported¹⁷ not appreciably affected by either membranes of activated CD4^+ T cell or by PMA. Membranes of activated CD4^+ T cells induced activation of MMP-2 by ECs revealed by the appearance of a 66-kd gelatinolytic band. This treatment also caused release of the 92-kd gelatinase (MMP-9) by ECs. Western blotting demonstrated that the small amounts of MMP-1 and MMP-3 secreted by unstimulated human vascular ECs in culture increased after stimulation with membranes of activated CD4^+ T cells (Figure 1, B and C). For MMP-9, Western blot analysis supported the results obtained by zymography (data not shown). Studies using an anti-CD40L MAb established the specificity of modulation of MMPs through CD40 ligation. The slight residual MMP-9 expression even after anti-CD40L pretreatment likely reflects the effect of non-CD40L-dependent T cell-EC interactions or could result from incomplete blocking by the antibody used, which was raised against a recombinant CD40L protein. Preincubation of T cell membranes with IgG control antibody did not affect

Figure 1. Activation of endothelial cells by interactions with T cells induces production of matrix metalloproteinases through CD40L-CD40 signals. Human vascular ECs cultured in serum-free M199 medium were co-incubated for 24 hours with membranes of activated CD4⁺ T cells (see Materials and Methods) in the presence or absence of an anti-CD40L MAb (α-CD40L; 1 μg/ml). Supernatants of culture media were analyzed by gelatin zymography (A) to detect gelatinase A and B (MMP-2 and -9) and by Western blotting to detect interstitial collagenase (MMP-1; B) and stromelysin (MMP-3; C), respectively. Medium of ECs cultured without stimulation (ECs alone) served as control. The positions of the molecular weight markers are indicated (kDa). Similar data were obtained in independent experiments with ECs from three different donors.

MMP expression. Stimulation of ECs with extracts of membranes from unstimulated CD4⁺ T lymphocytes as well as activated CD4⁺ T cell membranes alone did not induce MMP expression (data not shown). The expression of MMPs elicited by CD40 ligation on ECs exceeded that produced by maximal IL-1β or TNF-α stimulation (data not shown).

Exposure of Human Vascular ECs to Recombinant Human CD40L Activates Gelatinase A, Induces Synthesis and Secretion of Gelatinase B, and Increases Interstitial Collagenase and Stromelysin

Additional studies used rCD40L to analyze in more detail the effect of CD40 ligation on ECs and the release of

MMPs. Analysis of serum-free conditioned media showed constitutive expression of latent 72-kd gelatinase (MMP-2) by ECs unaffected by either rCD40L or PMA (Figure 2A, left). Supernatants from ECs stimulated with PMA, an agonist known to induce synthesis and secretion of MMPs,¹⁷ served as a positive control. Recombinant human CD40L induced activation of MMP-2 (as demonstrated by the appearance of a 66-kd gelatinolytic band) and caused release of MMP-9 by ECs. Casein zymography, a method that detects MMP-3 but not MMP-1, showed that unstimulated human vascular ECs in culture constitutively secrete small amounts of MMP-3 and that exposure to rCD40L increased the MMP-3 secretion (Figure 2B, left). The gelatinolytic and caseinolytic activities were metal dependent as they were completely inhibited by EDTA (Figure 2, A and B, center panels) but not by the serine protease inhibitor PMSF (Figure 2, A and B, right). Treatment with rCD40L increased the release of immunoreactive MMP-1 (Figure 2C, left), MMP-3 (Figure 2C, center), and MMP-9 (Figure 2C, right) by human ECs. Addition of an anti-CD40L MAb blocked the release of MMPs by ECs in response to rCD40L.

Release of MMPs by Human Vascular ECs Depends on Concentration of CD40L and Time

To characterize further the induction of MMPs by ECs through CD40, we investigated the release of MMPs as a function of CD40L concentration and time. Exposure of ECs to rCD40L activated MMP-2, induced MMP-9 secretion, and increased MMP-3 and MMP-1 expression in a concentration- and time-dependent manner (Figure 3). The induction of MMP-9 through CD40 activation required at least 200 ng/ml rCD40L for 24 hours. For MMP-2 activation and MMP-3 release, EC activation required 600 ng/ml rCD40L for 24 hours, and 10 μg/ml rCD40L induced maximal MMP release. Elaboration of immunoreactive MMP-3 and MMP-9 detected by Western blot analysis had similar concentration and time dependence (data not shown). Gelatinolytic or caseinolytic activity due to MMP-9, MMP-2, and MMP-3, respectively, occurred after 6 and 12 hours, with maximal levels after 24 hours of stimulation with rCD40L. Forty-eight hours of stimulation with rCD40L produced no further increase (data not shown). For MMP-1, Western blot analysis confirmed low constitutive expression of the protein, which increased after 12 hours of exposure to rCD40L and peaked after 24 hours. Release of MMPs from ECs induced by CD40 ligation and PMA showed similar time dependence (data not shown). Radioimmunoprecipitation experiments established that rCD40L induced *de novo* synthesis of MMP-9 and increased *de novo* synthesis of MMP-1 and MMP-3 by ECs (Figure 4).

Stimulation of Human Vascular ECs through CD40 Increases TIMP-1 but Not TIMP-2

Western blot analysis showed that serum-free conditioned medium of unstimulated human vascular ECs con-

Figure 2. Stimulation of vascular ECs with recombinant human CD40L induces activation of gelatinase A (MMP-2) and secretion of gelatinase B (MMP-9) and increases production of interstitial collagenase (MMP-1) and stromelysin (MMP-3). Human vascular ECs cultured in serum-free M199 medium were stimulated for 24 hours with media alone (None), PMA (10 nmol/L), recombinant human CD40L (rCD40L; 5 μ g/ml), or rCD40L (5 μ g/ml) preincubated (10 minutes) with anti-CD40L MAb (rCD40L/ α -CD40L; 1 μ g/ml). Supernatants of culture media were analyzed by gelatin (A) or casein (B) zymography with (+) or without EDTA (20 mmol/L) or PMSF (1 mmol/L). Aliquots of the same supernatants were analyzed by Western blot (C) for MMP-1, MMP-3, and MMP-9 expression. The positions of the molecular weight markers are indicated (kDa). Similar data were obtained in independent experiments with ECs from four different donors.

tained both TIMP-1 and TIMP-2. Exposure to rCD40L (24 hours) enhanced the low basal levels of TIMP-1, as did PMA used as positive control (Figure 5). Treatment with either rCD40L or PMA did not alter TIMP-2 expression. As found for MMPs, TIMP-1 release was rCD40L time and concentration dependent, rising after 12 hours and requiring at least 200 ng/ml rCD40L (data not shown).

Exposure of Human Vascular ECs to Recombinant CD40L Induces Capillary-Like Tubule Formation

To test the potential functional significance of CD40-induced MMP by vascular ECs, we examined the forma-

Figure 3. Matrix metalloproteinase release by vascular ECs stimulated with recombinant CD40L depends on concentration and time. Human vascular ECs cultured in serum-free M199 medium were stimulated for 24 hours with different concentrations of recombinant CD40L (rCD40L) (left panels) or with rCD40L (5 μ g/ml) for different periods of time (right panels). Supernatants of culture media were analyzed by gelatin (A) or casein (B) zymography and by Western blotting for MMP-1 (C). The positions of the molecular weight markers are indicated (kDa). Similar data were obtained in independent experiments with ECs from four different donors.

tion of capillary-like structures in an *in vitro* tube formation assay. Human vascular ECs cultured without stimulation showed neither morphological change nor tubule formation (Figure 6, left panels). In contrast, vascular ECs stimulated with basic fibroblast growth factor (bFGF), a known angiogenic factor,³⁸ or with rCD40L yielded identical morphological changes and formation of capillary-

like tubules (Figure 6, center and right panels, respectively). Anti-CD40L antibody completely blocked this effect. Furthermore, the MMP inhibitor BB-94 markedly reduced capillary-like tubule formation in response to CD40 ligation, consistent with a functional role of the induced MMP in this angiogenic behavior. In addition, using computer-assisted image analysis we quantified

Figure 4. Immunoprecipitation of metabolically labeled matrix metalloproteinases synthesized *de novo* by vascular ECs stimulated with recombinant human CD40L. Human vascular ECs cultured in serum-free M199 medium were stimulated for 24 hours alone (None) or with PMA (10 nmol/L) or recombinant human CD40L (rCD40L; 5 μ g/ml) in media containing 35 S-labeled methionine/cysteine. Proteins were immunoprecipitated from supernatants of culture media with antibodies raised against interstitial collagenase (MMP-1), stromelysin (MMP-3), and gelatinase B (MMP-9) and analyzed by Western blotting. The positions of the molecular weight markers are indicated (kDa). Similar data were obtained in independent experiments with ECs from four different donors.

the vascular endothelial capillary-like tubule formation (Figure 7). Activation of human vascular ECs through CD40 resulted in morphological changes and formation of capillary-like structures, similar in response to the an-

giogenic factor bFGF. Compared with unstimulated conditions, CD40 ligation induced percentage area and total length of capillary-tubule formation by 4.5 ± 0.7 and 3.1 ± 0.6 fold, respectively. These effects were blocked

Figure 5. Stimulation of vascular ECs with recombinant human CD40L induces production of tissue inhibitor of matrix metalloproteinase 1 (TIMP-1). Human vascular ECs cultured in serum-free M199 medium were stimulated for 24 hours with media alone (None), PMA (10 nmol/L), recombinant human CD40L (rCD40L; 5 μ g/ml), or rCD40L (5 μ g/ml) preincubated (10 minutes) with anti-CD40L MAbs (rCD40L/ α -CD40L; 1 μ g/ml). Supernatants of culture media were analyzed by Western blotting for TIMP-1 and TIMP-2. The positions of the molecular weight markers are indicated (kDa). Similar data were obtained in independent experiments with ECs from four different donors.

Figure 6. Activation of vascular ECs through CD40 induces capillary-like tubule formation. Human vascular ECs were cultured serum-free (M199) on a fibrin gel for 72 hours in the absence of angiogenic factor (None) or in the presence of basic fibroblast growth factor (bFGF; 100 ng/ml), recombinant CD40L (rCD40L; 5 μ g/ml), or recombinant CD40L and the MMP inhibitor BB-94 (1 μ g/ml) (rCD40L + BB-94). The upper panels show photomicrographs of the surface of the fibrin matrix gel ($\times 40$), the lower panels show photomicrographs of a cross section of the fibrin matrix gel ($\times 200$). The asterisks indicate the top of the fibrin gels. Similar data were obtained in independent experiments with ECs from three different donors.

substantially ($P < 0.03$) by addition of either an anti-CD40L antibody or the MMP inhibitor BB-94, in accord with the data mentioned above.

Discussion

Interactions between T lymphocytes and ECs may influence the development of vascular diseases and participate in a variety of immune and inflammatory responses. Neovessel formation commonly occurs at the sites of T

cell infiltration and activation in tissues. Arterial allografts of hypercholesterolemic rabbits develop exuberant neovascularization of intimal lesions in association with increased numbers of T lymphocytes,³⁹ whereas native arteries in the same animals exposed to the equivalent degree of hyperlipidemia display few microvessels in their atherosclerotic lesions. These findings provide direct experimental demonstration of a link between T cell activation (during the allogenic response) and angiogenesis in atheromatous plaques. Activated ECs and T cells are present early in vascular immune responses and inflammatory conditions, such as atherosclerosis.^{40,41} However, the mechanisms that may link interactions between T lymphocyte and endothelium in relation to neo-angiogenesis remain poorly understood.

Angiogenesis required ECs to penetrate the ECM, a process that probably involves the secretion of MMPs. In atheroma, the abundant neovessels may provide a portal for leukocyte trafficking as well as a source of intraplaque hemorrhage, which may contribute to the expansion or evolution of these lesions.^{42,43} The activation of vascular ECs to secrete MMPs may contribute importantly to angiogenesis in atheroma and during tumor growth as well as at other sites of chronic inflammation.

T cells can produce vascular endothelial growth factor,⁴⁴ which induces MMP expression by ECs.⁴⁵ In addition to soluble mediators, T cells often signal via contact-dependent pathways. Interactions between T cells and vascular ECs via CD40L-CD40 signals induce expression of adhesion molecules such as VCAM-1, ICAM-1, and E-selectin on ECs,²⁰⁻²² which may mediate leukocyte recruitment and provide co-stimulation to T cells. We have recently reported that vascular human ECs, smooth muscle cells (SMCs), and human macrophages express

Figure 7. Activation of vascular ECs through CD40 induces capillary-like tubule formation. Human vascular ECs were cultured in serum-free medium (M199) on a fibrin gel for 72 hours in the absence of angiogenic factor (None) or in the presence of basic fibroblast growth factor (bFGF; 100 ng/ml), recombinant CD40L (rCD40L; 5 μ g/ml), or recombinant CD40L and the MMP inhibitor BB-94 (1 μ g/ml) (rCD40L + BB-94). Percentage area measured on cross section (gray bars) and total length determined on surface section (black bars) of capillary-like tubule formation are shown. Similar data were obtained in independent experiments with ECs from three different donors.

functional CD40 and CD40L *in vitro* and that within atherosclerotic lesions these cells can co-express CD40 and CD40L.³² We and others^{31,32} also identified CD40L⁺/CD4⁺ T cells in human atheroma, suggesting CD40L-CD40 interactions during atherogenesis.⁴⁶ Moreover, CD40 ligation can augment MMP expression in human vascular SMCs and monocytes/macrophages *in vitro*.^{47,48} Therefore, this study investigated the possibility that interactions between T cells and vascular endothelium may induce expression of MMPs by ECs through CD40 ligation and thus favor angiogenesis.

We report here a novel aspect of T cell-endothelial interaction: induction of MMP expression and capillary-like tube formation via CD40L-CD40 signaling. Exposure of human vascular ECs to CD40L, by either membranes of activated CD4⁺ T cells or rCD40L, augments expression of members of all three families of MMPs. Both sources of CD40L promoted formation of the active form of MMP-2 and induced the expression of MMP-9. Moreover, ligation of CD40 on ECs increased levels of MMP-1 and MMP-3. Besides MMP expression, vascular ECs constitutively express TIMP-1 and TIMP-2 and increase TIMP-1 levels after CD40 ligation. However, within the human atherosclerotic plaque, the balance between MMP and TIMP appears shifted toward predominance of the enzymatic activity.⁴⁹ Furthermore, we demonstrate here that basal expression of MMPs is not adequate to promote capillary-like tubule formation, whereas CD40 ligation on vascular ECs shifts the balance toward angiogenic functions. The MMPs/TIMP (im)balance at sites of angiogenesis might further be affected by the reversed induction of MMPs during T cell-endothelial interaction. Romanic and Madri demonstrated that T lymphocyte binding to ECs results in increased T cell MMP synthesis,⁵⁰ thus providing an additional source of matrix-degrading enzymes.

Although cytokines such as IL-1 β or TNF- α can induce expression of MMPs by ECs,¹⁷ these cytokines probably do not mediate expression of MMPs secondarily via CD40L-CD40 signals. Endothelial cells do not produce TNF- α after stimulation with rCD40L (our unpublished observations). Ligation of CD40 on human ECs does induce release of functional mature IL-1 β .⁵¹ Indeed, the expression of MMPs by ECs after CD40 ligation exceeded that produced by maximally effective concentrations of recombinant IL-1 β . These results suggest that induction of MMPs through CD40 ligation on ECs occurs independent of soluble cytokine production.

Matrix metalloproteinases degrade the ECM, allowing cells to penetrate and remodel connective tissue. The production of MMPs by ECs contributes to neovascularization, a process crucial in tumor growth, especially in the early phase of metastasis, in formation of granulation tissue, and considered important in atherogenesis. CD40L expressed on CD4⁺ T cells delivers contact-dependent activating signals to vascular ECs, yielding induction of adhesion molecules and cytokines.²⁰⁻²² This report provides new evidence that CD40L on T cells can induce expression and activation of MMPs allowing neovascularization via engagement of CD40 on human vascular ECs. The present observations furnish a novel

mechanistic link between T cell activation and angiogenesis observed in atherosclerotic lesions as well as at other sites of chronic inflammation. The induction of MMPs in ECs via CD40 ligation may accelerate the digestion of the ECM and facilitate the migration of cells as well as the formation of neovessels, which serve many functions in pathophysiology.

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