Inhibited Angiogenesis in Aging: A Role for TIMP-2

Teruhiko Koike,1 Robert B. Vernon,2 Michel D. Gooden,2 Eman Sadoun,1 and May J. Reed1

1Division of Gerontology and Geriatric Medicine, Department of Medicine, University of Washington, Seattle.
2Department of Vascular Biology, The Hope Heart Institute, Seattle, Washington.

Factors responsible for age-associated impairment of angiogenesis are poorly understood. We observed that in aged mice, new fibrovascular tissue within subcutaneous polyvinyl alcohol sponge implanted in aged mice was impaired relative to vascular density, reduction in the deposition of collagen, an altered inflammatory response as well as a deficient expression of proangiogenic growth factors in aged animals relative to young animals (3,4). In contrast to animal models that measured the neovascular response during wound healing or ischemic injury, there was a decreased angiogenic growth response as well as a deficient expression of angiogenic growth factors in aged animals relative to young animals (3,4). In contrast to animal models based on closure of excisional cutaneous wounds or ischemic injury to tissues, the subcutaneous implantation of polyvinyl alcohol (PVA) sponges provides an inert, avascular scaffold that facilitates observation and measurement of neovascular invasion (5). In a recent study, neovascularization of PVA sponges implanted in aged mice was impaired relative to vascular growth into sponges implanted in young mice for similar periods of time (6). Elements of angiogenesis that were impaired in the aged mice included decreases in vessel period of time (6). Elements of angiogenesis that were impaired in the aged mice included decreases in vessel

ANGIOGENESIS, the generation of new vasculature from existing blood vessels, is impaired in aging (1–3). In both young and aged individuals, angiogenesis is accomplished by a series of coordinated steps that include penetration of endothelial cells through the basement membrane of the parent vessel, migration of the endothelial cells (in the form of a multicellular sprout) into the surrounding interstitial extracellular matrix (ECM), proliferation of endothelial cells to lengthen the sprout, and development of a patent lumen.

Although it has long been accepted that angiogenesis is impaired in aging, only recently have studies of aged animals defined factors that contribute to this impairment. In animal models that measured the neovascular response during wound healing or ischemic injury, there was a decreased inflammatory response as well as a deficient expression of angiogenic growth factors in aged animals relative to young animals (3,4). In contrast to animal models based on closure of excisional cutaneous wounds or ischemic injury to tissues, the subcutaneous implantation of polyvinyl alcohol (PVA) sponges provides an inert, avascular scaffold that facilitates observation and measurement of neovascular invasion (5). In a recent study, neovascularization of PVA sponges implanted in aged mice was impaired relative to vascular growth into sponges implanted in young mice for similar periods of time (6). Elements of angiogenesis that were impaired in the aged mice included decreases in vessel period of time (6). Elements of angiogenesis that were impaired in the aged mice included decreases in vessel density, reduction in the deposition of collagen, an altered inflammatory response, a reduced expression of proangiogenic factors, and an increased expression of the angiogenesis inhibitor thrombospondin-2.

As a complement to studies of angiogenesis in vivo, models of angiogenic morphogenesis in vitro can provide specific information about mechanisms that mediate or regulate vascular development. For example, human umbilical vein endothelial cells dispersed in three-dimensional (3D) collagen gels and cultured in serum-free (or low-serum) media supplemented with specific angiogenic factors organize into multicellular, thin-walled, tubelike structures that resemble capillaries (7). Such a “tubulogenic” culture system can be used as a readout assay to study the morphogenetic capacity of different endothelial cell populations or to measure the effects of agents that stimulate or inhibit specific pathways of morphogenesis.

Models of angiogenesis in vivo and in vitro have demonstrated that proteolysis of ECM, including type I collagen (the major structural protein of interstitial ECM), is necessary for vascular growth (8–13). A major group of proteases that regulate angiogenesis and that act on type I collagen are the matrix metalloproteinases (MMPs). In vertebrates, MMPs constitute a family of proteins that is divided into 6 groups on the basis of structural homology or substrate specificity (14). The regulation of MMPs is partially under the control of their endogenous inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) and also is affected by specific angiogenic growth factors. Although it is accepted that angiogenesis in aged individuals is suppressed by a variety of factors, data both in vitro and in vivo suggest that deficient activity of MMPs contributes to the inhibition of migration of endothelial cells and subsequent impairment of angiogenesis (15). Although the secreted MMPs (e.g., MMP-1) and their primary inhibitor TIMP-1 regulate the migration of endothelial cells on two-dimensional collagen substrates, we have recently found that it is cell surface-associated MMPs (e.g., MT1-MMP and cell-surface MMP-2) and their inhibitor TIMP-2 that modulate migration of endothelial cells in 3D collagen gels, which simulate interstitial ECM in vivo (16).

In the present study, we have extended our previous investigations of TIMPs and cell-associated MMPs to
examine the involvement of these proteins in age-related impairment of vascular morphogenesis. Accordingly, we have examined the expression of MMP-2, MT1-MMP, and TIMP-2 during neovascularization of PVA sponges implanted into young and aged mice. In conjunction with this model in vivo, we have used a model of capillary tubulogenesis in vitro to show that a human dermal microvascular endothelial cell line aged in vivo (hmEC90) exhibited a decreased capacity for morphogenesis relative to an endothelial cell line of a considerably younger in vivo age (hmEC36). In additional experiments with the tubulogenesis model, we explored whether cell-associated MMPs and TIMP-2 might be involved in the impaired morphogenesis exhibited by the hmEC90 cell line.

**METHODS**

**Animal Model of Angiogenesis In Vivo**

For studies of expression of MMPs and TIMP-2 during angiogenesis in vivo, we utilized an established murine model of subcutaneous neovascularization (2,5,17). [C57BL/6 × DBA/2]F1 “B6D2F1” and [C57B1/6 × C3H]F1 “B6C3F1” strains of male mice aged 6–8 months (young) or 23–25 months (aged) were obtained from the National Institute on Aging mouse colony (Harlan Sprague Dawley, Indianapolis, IN) and were maintained in a pathogen-free environment. Two disk-shaped Clinicel polyvinyl alcohol (PVA) sponges (M-Pact, Eudora, KS) 10 mm in diameter and 2 mm thick were implanted subcutaneously 20 mm apart in the dorsum of each mouse. Sponges were removed from euthanized animals at 19 days postimplantation. Recovered sponges were fixed in neutral-buffered formalin, embedded in paraffin, and sectioned at 5 µm. Mounted sections were deparaffinized, blocked overnight in phosphate-buffered saline (PBS)/2% goat serum, and incubated for 1 hour with 37°C. Subsequently, each collagen gel was overlayed with 3% FBS to generate a solution of 2.7 mg/ml collagen and 1% FBS. The EGM-MV2 medium incorporated into the collagen gels was 1/9 volume of 10-strength Medium 199 (Life Technologies, Grand Island, NY), and sufficient EGM-MV2 medium and type I collagen stock (BD Biosciences, Bedford, MA) were added to generate a solution of 2.7 mg/ml collagen and 1% FBS. Expression of cell-associated MT1-MMP in samples was scored on the basis of the number of positive cells and the level of positivity per cell.

Expression of TIMP-2 in immunostained sections of sponges was quantified from digital images with Metamorph (Universal Image Corp., Westchester, PA) according to Kyrriakides and colleagues (18). Within Metamorph, a threshold value was set for each experiment that represented the maximum intensity of staining observed in control sections exposed to the second antibody, DAB, and toluidine blue counterstain only. For sections labeled with the TIMP-2 mAb, staining intensity above the threshold value was quantified in Intensity Units. Data for visual and digital quantitation of MMPs and TIMP-2 were expressed as the means ± standard error of measurement (SEM) (n = 6 and 14 sponges from young mice and aged mice, respectively).

**Cell Culture**

For studies in vitro, human microvascular endothelial cell lines hmEC36 and hmEC90 were established from cells isolated from subcutaneous reduction surgery of donors 36 and 90 years of age (BioWhittaker/Clonetics, Inc., Walkersville, MD). In culture, the hmEC90 line maintained an “aged” phenotype relative to the hmEC36 line with respect to decreased proliferation and migration on plastic substrata. HmECs at early passage stages ([i.e., less than 12 population doublings]) were selected for all experiments; however, we found the lines could be passaged to more than 20 population doublings without obvious changes in morphology. The hmEC lines were maintained in EGM-MV2 medium (BioWhittaker/Clonetics) supplemented with 5% fetal bovine serum (FBS). For propagation and for all experiments, cells were detached from culture dishes with Accutase (Innovative Cell Technologies, Inc., La Jolla, CA).

**Capillary Morphogenesis Assay In Vitro**

HmEC lines were cultured at 1 × 10⁶/ml in 3D collagen gels by the method of Davis and Camarillo (7). Collagen gels were prepared by addition of cells to 1 volume of rat tail type I collagen stock (BD Biosciences, Bedford, MA), 1/9 volume of 10-strength Medium 199 (Life Technologies, Grand Island, NY), and sufficient EGM-MV2 medium and FBS to generate a solution of 2.7 mg/ml collagen and 1% FBS. The EGM-MV2 medium incorporated into the collagen gels and used to overlay the polymerized collagen (see below) was prepared with omission of the proprietary basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) supplied by the manufacturer. The collagen solution, with suspended cells, was added to wells (100 µl/well) of a 96-well tissue culture plate (Corning Costar Corp., Cambridge, MA) and polymerized for 90 minutes at 37°C. Subsequently, each collagen gel was overlaid with 100 µl of EGM-MV2 culture medium supplemented with 1% FBS, 30 ng/ml of recombinant human bFGF (R&D Systems, Inc., Minneapolis, MN), 30 ng/ml of VEGF165 (R&D Systems), and 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma). All cultures were maintained at 37°C in a CO₂ incubator for 4 days with a change of medium on the second day. Subsequently, cultures were fixed with 1% neutral-buffered formalin and stained with...
1% crystal violet. Brightfield images of stained gels were acquired with a Leitz-Wild MZ FLIII stereomicroscope fitted with a CCD camera (SPOT, Diagnostic Instruments, Inc., Sterling Heights, MI). Images were recorded in RGB color (8 bits/channel) with Adobe Photoshop (Adobe Systems, Inc., San Jose, CA) and converted to 8-bit grayscale. In certain experiments, the genesis of capillary-like structures in 3D collagen was quantified by division of the total luminal area (in $\mu m^2$) of cellular tubes located within microscope fields of standard size by the total number of cells within each field to generate a value of luminal area ($\mu m^2$) per cell. Quantification of luminal areas (as two-dimensional projections) was accomplished from digital images by use of NIH Image (U.S. National Institutes of Health, http://rsb.info.nih.gov/nih-image/).

**Western Blot Assays**

HmECs were suspended at $1 \times 10^6$/ml in 3D gels of 2.7 mg/ml collagen in the presence of bFGF, VEGF, and PMA as described previously for the capillary morphogenesis assay, but with 0.1% FBS. After 48 hours, conditioned media were collected from the 3D gels as described previously (16). For cell lysates, extracts of hmECs cultured in the gels were prepared in the following manner: The gels with embedded cells were pelleted by centrifugation at 12,000 $\times$ g and the pellets were solubilized in lysis buffer composed of PBS.

Figure 1. Expression of matrix metalloproteinase (MMP)-2 and MT1-MMP during subcutaneous neovascularization in young and aged mice is similar. Polyvinyl alcohol sponges implanted into young and aged mice were removed after 19 days in vivo, sectioned, and evaluated by immunohistochemistry for expression of MMP-2 and MT1-MMP in the fibrovascular stroma that had invaded each sponge. Stromal fibroblasts of young (A) and aged (B) mice express similar levels of MMP-2, as indicated by the presence of brown 3,3'-diaminobenzidine reaction product (arrows). Expression of cell-associated MT1-MMP in the stroma of both young (C) and aged (D) mice is low (arrows). Quantification of MMP-2 (E) and MT1-MMP (F) in immunostained sections indicates no significant differences in expression of these MMPs in young versus aged mice. Error bars indicate SEM (standard error of measurement) ($n = 6$ and 14 sponges from young mice and aged mice, respectively). Bars in A–D = 140 $\mu m$. 

KOIKE ET AL.
supplemented with 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin, pH 7.4. The samples in lysis buffer were supplemented with 1/5 volume of 6-strength SDS-polyacrylamide gel electrophoresis (PAGE) buffer (19) and heated to 100°C for 5 minutes in the presence of 100 mM dithiothreitol for reduction of disulfide bonds. Samples of conditioned media representing equivalent numbers of cells were resolved by SDS-PAGE (through separating gels of 10%–12% acrylamide), transferred to nitrocellulose membranes, and blocked with 2% bovine serum albumin (BSA) in 50 mM Tris-buffered saline (TBS) for 12 hours at 4°C. Samples of cell lysates representing equivalent numbers of cells were resolved by SDS-PAGE (in 10% acrylamide gels), transferred to nitrocellulose, and blocked with 5% nonfat dry milk in TBS for 12 hours at 4°C. All blots were rinsed in TBS and probed with rabbit polyclonal antibodies (at 1–5 μg/ml in TBS/2% BSA) to the following human proteins: TIMP-1 (AB800, Chemicon), TIMP-2 (T8062, Sigma), and MT1-MMP (MMP-14) (M3927, Sigma). Antibodies bound to the nitrocellulose were visualized on X-ray film by use of peroxidase-conjugated Protein A or anti-Ig in conjunction with an Enhanced Chemiluminescence kit (Amersham, Arlington Heights, IL). Autoradiographs were recorded digitally with a flat-bed scanner and quantified with NIH Image.

Digital images of zymograms were quantified with NIH Image.

**Zymography**

Conditioned media prepared as described previously were resolved by SDS-PAGE in the absence of disulfide bond reduction in 10% polyacrylamide gels supplemented with 1 mg/ml of gelatin. Following electrophoresis, the gels were washed twice for 30 minutes with 2.5% Triton X-100, incubated for 16 hours at 37°C in 50 mM Tris-HCl/5 mM CaCl₂ (pH 7.5), and stained with Coomassie Brilliant Blue.
TIMP-2 (mostly extracellular) was localized in stroma from aged mice (Figure 2B). Quantification of immunostained sections by computer-assisted morphometry revealed a 2.4-fold (average) increase in TIMP-2 expression in the fibrovascular stroma of aged mice relative to the stroma of young mice (Figure 2C).

In conjunction with our studies in mice in vivo, we examined the morphogenetic capacity of young and aged hmEC cultured within 3D collagen gels in vitro. We observed that hmEC36 cells cultured under conditions favoring tubulogenesis underwent a robust morphogenetic response, i.e., within 4 days they organized into thin-walled multicellular tubelike structures with wide lumens (Figure 3A). In contrast, hmEC90 cells remained dispersed within the collagen and showed little inclination to undergo tubulogenesis (Figure 3B), even after culture periods of 1 week or greater.

Previous studies have reported that tubulogenesis by endothelial cells cultured in 3D fibrin or collagen gels could be influenced by modulation of proteolytic activity (21,22). Accordingly, in the present study, we asked whether MMP activity might be a component of the different tubulogenic capacities of hmEC36 versus hmEC90. HmEC36 were cultured in the presence or absence of GM6001, a broad-spectrum inhibitor of MMPs. In contrast to the control cultures that lacked GM6001, tubulogenesis was effectively inhibited by the presence of the compound (Figure 4).

Our results indicated that a general inhibition of MMP activity could cause hmEC36 cultures to resemble hmEC90 cultures in terms of tubulogenic capacity. Correspondingly, Western blot and zymographic analyses revealed differences in expression of MMPs and TIMPs between hmEC36 and hmEC90 cultured in 3D collagen gels (Figure 5). By Western blot assay, hmEC36 and hmEC90 expressed similar levels of TIMP-1. In contrast, hmEC90 consistently expressed higher quantities of TIMP-2 than did hmEC36. HmEC90 expressed similar or lesser amounts of the active (60 kD) form of MT1-MMP relative to hmEC36. Zymography indicated that proMMP-2 was synthesized by both hmEC36 and hmEC90; however, the active form of this enzyme was not produced by hmEC90.

To examine the functional consequences of elevated TIMP-2 expression by hmEC90, we tested whether hmEC36 could acquire the tubulogenesis-deficient phenotype of hmEC90 by exposure to exogenous, active TIMP-2. HmEC36 were cultured in 3D collagen gels for 4 days either in control media or in media supplemented with 200 nM of human recombinant TIMP-2. Relative to controls, exposure of the cultures to TIMP-2 inhibited tubulogenesis significantly (Figure 6). In contrast, exogenous TIMP-1 at a concentration of 200 nM did not inhibit tubulogenesis by hmEC36 cultures (data not shown).

**DISCUSSION**

Angiogenesis is a critical component of wound repair and revascularization of ischemic tissues. Accordingly, the reduced capacity of aged tissues to support angiogenesis is generally detrimental to health (3,4), although impaired angiogenesis might limit the growth of tumors in the aged (23–25). Despite the clinical relevance of deficient angiogenesis to geriatric disease, many of the proposed interventions to enhance vascular growth are designed with an incomplete understanding of parameters that are altered in aged tissue.

In the present study, expression of TIMP-2 was significantly increased in neovascularized stromal tissue of aged mice relative to young mice. The increased expression of TIMP-2 supports our hypothesis that a dysregulation of MMP activity impairs angiogenesis in aging. The contribution of TIMP-2 to age-associated inhibition of angiogenesis is underscored by our observation that inhibition of TIMP-1...
with a function-blocking antibody) enhanced angiogenesis in young, but not aged, mice (26).

By use of a model of capillary tubulogenesis in 3D collagen gels (27,28), we demonstrated that a microvascular endothelial cell line of advanced age in vivo (hmEC90) exhibited markedly impaired tubulogenesis relative to a young cell line (hmEC36). Moreover, we observed that a blockage of general MMP activity with the broad-spectrum MMP inhibitor GM6001 effectively converted the tubulogenic phenotype of hmEC36 to the nontubulogenic phenotype of hmEC90—a result that implies an absolute requirement for MMP activity in tubulogenesis. In a previous study in vitro, we presented evidence that neither secretory-type MMPs (e.g., MMP-1, -2, -9, and -13) nor their inhibitor TIMP-1 influenced the migration of newborn hmECs within 3D collagen gels. In contrast, MT1-MMP and its inhibitor TIMP-2 were implicated as stimulatory (MT1-MMP) and inhibitory (TIMP-2) modulators of newborn hmEC migration (16). Our present results in vitro demonstrate the importance of TIMP-2 in vascular morphogenesis in the context of age-associated impairment of function. Unlike TIMP-1, which was expressed equivalently in both hmEC36 and hmEC90 lines in 3D collagen gels, nontubulogenic hmEC90 exhibited a significant upregulation of TIMP-2 relative to tubulogenic hmEC36. The upregulation of TIMP-2 in hmEC90 was correlated with suppression of MMP-2 activation—a finding consonant with...
with reports that proteolytic activation of MMP-2 by MT1-MMP (29–31) is blocked by TIMP-2 (32).

MT1-MMP is a broad-spectrum proteinase of ECM components that include gelatin, fibronectin, laminin, vitronectin, tenascin, nidogen, aggrecan, and perlecan (33–35). MT1-MMP can also cleave native fibrillar collagens I, II, and III to produce 3/4- and 1/4-length fragments like those generated by the neutral collagenases, MMP-1, MMP-8, and MMP-13 (35). Primary substrates for MMP-2 include type IV collagen and denatured collagens (gelatins). In the present study, we found that exogenous, purified TIMP-2 (but not TIMP-1) acted like GM6001 to convert the tubulogenic phenotype of hmEC36 to the nontubulogenic phenotype of hmEC90. TIMP-2 inhibits the collagenolytic activity of MT1-MMP and regulates the activity of MMP-2 that complexes with MT1-MMP at the cell surface (33,36).

The MT1-MMP/TIMP-2 complex is believed to mediate a controlled, focal proteolysis of pericellular ECM that facilitates the penetration of ECM by migratory cells, including endothelial cells (37). Accordingly, enhanced activity of MT1-MMP/MMP-2 by hmEC36 would facilitate tubulogenesis by improvement of cell motility that leads to cell–cell contact. It is possible, however, that proteolytic modification of pericellular ECM by MT1-MMP/MMP-2 influences tubular morphogenesis directly by altering interactions between the ECM and cell surface integrins that promote tubulogenesis (7,38,39).

In contrast to the contribution of decreased MMP activity to impairment of migration and tubulogenesis of aged microvascular cells, elevated MMP activity (i.e., MMP-2) is associated with intimal thickening and remodeling of large vessels of aging rats (40,41)—findings that indicate that enhanced degradation of vascular ECM contributes to the dysfunction of aged macrovessels. Thus, although it has been proposed that the activity of MMPs in aged cells and tissues is typically high and is coincident with a lowered expression of TIMPs (42–44), it appears that the activity of MMPs of aging vasculature can be either increased or decreased, depending on the type of vessel. Collectively, the present study and earlier reports indicate that a dysregulation of MMP-mediated proteolysis of ECM (i.e., to levels either higher or lower than normal) contributes to impaired angiogenesis and wound healing in aging. Although much work remains to be done, continued study of the regulation of vascular growth and morphogenesis by MMPs and their inhibitors promise new therapeutic approaches to improve revascularization and healing of ischemic and injured tissues in the aged.

References

TIMP-2 AND ANGIOGENESIS IN AGING

805