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A Deficit in Collagenase Activity Contributes to Impaired Migration of Aged Microvascular Endothelial Cells

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Abstract Angiogenesis is impaired in aging. Delayed neovascularization is due, in part, to slowed endothelial cell migration. Migration requires an optimal level of adhesion to matrix proteins, a process mediated by matrix-degrading metalloproteases (MMPs) such as MMP1. To determine whether impaired angiogenesis in aging is associated with altered synthesis and activity of MMP1, we examined the expression of collagenase and tissue inhibitor of metalloprotease 1 (TIMP1) by immunostain of angiogenic sponge implants from young and aged mice. To characterize the relevance of MMP activity during the movement of aged endothelial cells, the secretion of MMP1 and TIMP1 by late-passage human microvascular endothelial cells (hmEC aged in vitro) and their non-aged (young) counterparts was quantified. The migration of aged human microvascular endothelial cells and the effect of inhibition of TIMP1 on the migration of aged hmEC or collagen I was also measured. Relative to young mice, granulation tissue from aged mice showed less expression of collagenase and increased expression of TIMP1. In vitro, aged hmEC were deficient in MMP1 secretion (55 ± 13% relative to young cells) and activity but showed increased expression of TIMP1 (280 ± 109% relative to young cells). Aged hmEC migrated significantly less distance than did young hmEC over a 5-day period (59 ± 8% relative to young cells). In the presence of a blocking antibody to TIMP1, aged hmEC showed a significant increase in the distance migrated on collagen I over a 5 day period (142 ± 11% relative to untreated aged hmEC). We propose that deficient MMP1 activity contributes to impaired cellular movement in aged microvascular endothelial cells and that perturbations that enhance collagenase activity increase their migratory ability and angiogenic potential. J. Cell. Biochem. 77:116–126, 2000. © 2000 Wiley-Liss, Inc.

Key words: aging; aged; migration; collagen I; matrix metalloproteases; tissue inhibitors of matrix metalloproteases; human; angiogenesis

Angiogenesis is impaired in aging and contributes to delayed wound repair [Puolakkainen et al., 1995; Reed et al., 1996]. The formation of capillaries requires endothelial cell activation, proliferation, and migration; although each of these functions may be deficient with aging, we have shown that slowed migration of endothelial cells contributes to decreased angiogenesis in aged tissues [Arthur et al., 1998].

Studies in vitro show that cellular migration occurs maximally when an optimal level of flexibility exists between cells and their surrounding extracellular matrix [DiMilla et al., 1993]. During early wound repair, fibrin and fibronectin constitute the provisional matrix upon which cells migrate. Cellular proliferation, connective tissue synthesis, and remodeling in the later stages of healing occur within a structural matrix comprised primarily of collagen I [Singer and Clark, 1999]. As cells move, they have the potential to control their adhesion to the extracellular environment by modifying the structure of the proteins with which they come in contact. Such modifications are mediated, in part, by secretion of matrix metalloproteases (MMPs). MMPs are a family of zinc-dependent, matrix degrading enzymes which were originally classified according to specific degradation of a substrate: for example, collagenases, gelatinases, and stromelysins.

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Of the more than 20 MMPs known to date, the expression and activity of MMP1 (interstitial collagenase; collagenase-1) has been examined most extensively in endothelial cells and during endothelial migration [Hanemaaijar et al., 1993; Jackson and Nguyen, 1997; Cornelius et al., 1995]. MMP1 specifically degrades native fibrillar (interstitial) collagen, is induced in endothelial cells during movement within collagen I gels, and is required for the migration of keratinocytes on this substrate [Fisher et al., 1994; Pilcher et al., 1997]. During wound repair, MMP1 is highly expressed by basal keratinocytes moving across the dermis [Saarialho-Kere et al., 1993]. The net protease activity of MMPs is regulated by tissue inhibitors of matrix metalloproteases 1–4 (TIMPs 1–4), a family of secreted, low-molecular-weight proteins of which TIMP1 is the best characterized [Gomez et al., 1997]. Although complete inhibition of endogenous MMP1 activity by TIMP1 suppresses angiogenesis both in vitro and in vivo, evidence suggests that TIMPs regulate angiogenesis in vivo by keeping MMP-mediated proteolysis within parameters that are optimal for migration of endothelial cells within the extracellular matrix [Schnaper et al., 1993; Johnson et al., 1994].

Studies of the effect of age on the expression and activity of MMPs and TIMPs in human fibroblasts and during wound repair have led to the consensus that disregulation of protease activity occurs with cellular aging. For example, in comparison to non-aged cells, fibroblasts aged in vitro (by serial passage in culture) secrete increased levels of MMPs and decreased levels of TIMPs [Millis et al., 1992; Zeng and Millis, 1996]. Relative to young controls, aged donors have a propensity to express higher levels of all MMPs but are impaired in their expression of TIMPs during wound repair [Ashcroft et al., 1997a,b]. The implication from these studies is that aged cells and tissues have a net increase in protease activity that may compromise the repair of tissues. However, the expression of MMPs and TIMPs has not been examined in aged endothelial cells or during angiogenesis in aging.

In this study, we describe the expression of collagenase and TIMP1 during delayed angiogenesis in young and aged mice. In addition, we quantified the secretion of MMP1 and TIMP1 by non-aged human microvascular endothelial cells (hmEC) and hmEC aged in vitro by serial propagation in culture. Finally, we demonstrate that aged hmEC show impaired migration relative to non-aged hmEC and measure the effects of a blocking antibody to TIMP1 on the migration of aged hmEC on collagen I. We report that neovascular tissue from aged mice have increased expression of TIMP1 and decreased expression of collagenase. Moreover, aged hmEC are deficient in the synthesis of MMP1, but have increased secretion of TIMP1. Aged hmEC are less able than non-aged hmEC to migrate on collagen I, but show increased movement when treated with a blocking antibody to TIMP1. We propose that decreased MMP1 activity contributes to impaired migration in aged hmEC and that perturbations which enhance collagenase activity increase the migratory ability of aged hmEC.

METHODS AND MATERIALS

In Vivo Model

The expression of collagenase and TIMP1 was examined in an established model of aging and angiogenesis in vivo [Reed et al., 1998]. In this model, the fibrovascular invasion of polyvinyl alcohol (PVA) sponges implanted in the dorsal subcutaneous space of the mice can be quantified. Mice of the {C57BL/6 × DBA/2}F1 B6D2F1 and {C57BL/6 × C3H}F1 "B6C3F1" strains of age groups 4–8 months (young mice) and 30–33 months (aged mice) were used. Two PVA sponges (Ivalon, Unipoint Industries, High Point, NC) (1 × 1 × 2 cm) were implanted in the dorsum of each mouse. Three mice from the young group and 3–5 from the aged group were analyzed at each timepoint. Implants were harvested at 7, 14, and 21 days, fixed in neutral buffered formalin, and embedded in paraffin.

Propagation of Human Microvascular Endothelial Cells Aged In Vitro

Human dermal microvascular endothelial cells (hmEC) were obtained from Clonetics (San Diego, CA) and aged in vitro by serial propagation, a previously described model for the study of impaired function in aged endothelial cells [Maier et al., 1990; Garfinkel et al., 1996; Watanabe et al., 1997]. Experiments were restricted to one lot of pooled neonatal microvascular endothelial cells obtained from foreskins of newborn black males and white males. Cells were obtained at early passage (pdl 5) and propagated at 1:2 splits to ensure that each passage

results in approximately one doubling. Cells were defined as aged when they attained 75% of their maximum life span (pdl > 28) and showed phenotypic changes similar to those of cells obtained from aged donors (S. Kudravi and M.J. Reed, manuscript in preparation). Whereas aged hmEC required approximately 7 days to divide, non-aged (young) hmEC required only 3–4 days. Young and aged cells were cultured in an identical manner and were kept in culture postpassage for identical periods of time before experiments were performed.

Immunocytochemistry

In this study, 5-μm sections of sponge tissue were dewaxed in xylene and hydrated in a graded series (100–70%) of ethanol solutions. HmEC were plated on collagen I for 48 h and fixed in 10% neutral buffered formalin. Sections and cells were blocked overnight in phosphate-buffered saline (PBS) with 1% goat serum and immunostained with polyclonal antibodies against human MMP1 and TIMP1 at 5 μg/ml (Chemicon, Temecula, CA). After exposure to primary antibody, slides were incubated in rhodamine or biotinylated goat anti-rabbit IgG (5 μg/ml) (Vector, Burlingame, CA). After a rinse in PBS, tissues were incubated with avidin-biotin-complex (ABC, Vector) and visualized with DAB. In all studies, preimmune serum and secondary antibody alone served as negative controls. Immunostaining of sections was uniformly timed and assessed by inspection with microscopy.

Senescence-Associated β-Galactosidase Staining and ^{3}H-Thymidine Incorporation

Young and aged cells were grown to near-confluency, exposed to ^{3}H-thymidine in growth media for 4 h, washed with PBS, fixed for 3–5 min with 3% formaldehyde at room temperature, washed and incubated overnight in senescence-associated β-Gal (SAβ-Gal) stain solution at 37°C, without CO_2, at pH 6 [Dimri et al., 1995]. Slides were coated with NTB3 emulsion (Kodak, Rochester, NY) diluted to 40% with a solution of 4% Dreft detergent, exposed for 7 days at 4°C, developed in Kodak D-19 developer, and fixed in GBX fixer (Kodak). Samples were counterstained with toluidine blue, dehydrated with ethanol (solutions of 70–100%), cleared in xylene, and mounted with Histoclear.

Cell Culture

HmEC were grown in MCDB 131 (Life Technologies, Grand Island, NY) media supplemented with 10% fetal bovine serum (FBS) 2 mM L-glutamine, 90 mg/ml heparin, and 25 mg/ml ECGS (Biomedical Technologies, Stoughton, MA). For experiments, young and aged cells were plated at near-confluency on dishes coated with 1 μg/ml type I collagen (Vitrogen, Collagen Corporation, Palo Alto, CA) and allowed to adhere overnight. Cells were synchronized for 48 h in MCDB 131 with 1% FBS and no ECGS. Media was then changed and conditioned for 48 h in serum-free conditions. Media were collected and concentrated approximately fivefold with Centricon-10 (Amicon, Beverly, MA) for Western blots and collagenase assays. In all experiments, the volume of conditioned media among samples was adjusted for cell numbers as determined by hemocytometer.

Western Blotting

Conditioned media were collected, supplemented with proteinase inhibitors (2 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM N-ethylmaleimide), and concentrated as described above. Samples from the young and aged hmEC were corrected for cell number and quantified with the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA). Aliquots of media from equivalent numbers of cells were solubilized in Laemmli buffer, treated with 1 mM DTT, and boiled for 10 min. Samples were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on slab gels (stacking and resolving gels were 4% and a 4–15% gradient of acrylamide, respectively), transferred to nitrocellulose, and stained with amido black to assess the efficiency of transfer. Blots were blocked with 5% milk/Tween-20 buffer and probed with rabbit polyclonal antisera against human MMP1 (5 μg/ml) and TIMP1 (5 μg/ml) (Chemicon). The MMP1 antibody recognizes only collagenases on western blots and the TIMP1 antibody recognizes only its target antigen. Neither antibody recognizes bovine collagenase or TIMP1. Bound antibodies were visualized on x-ray film by use of peroxidase-conjugated antibody to rabbit Ig in conjunction with Enhanced Chemiluminescence (Amersham, Arlington Heights, IL). Relative levels of MMP1 and TIMP1 were determined by densitometric scans of the respective bands.

Collagenase Assay

Concentrated samples of conditioned media were corrected for cell number and placed in triplicate wells of a 96-well plate previously coated with 50 μg collagen I/well (Sircoll Assay, Accurate Chemical and Scientific, Westbury, NY). A standard curve was constructed. Samples were incubated in 100 mM NaCl, 50 mM Tris HCl, 10 mM $CaCl_2$, pH 7.45, for 24 h at 37°C as previously described [Nethery et al., 1986]. Wells were washed with PBS and counterstained with Coomassie blue. Plates were read on a Spectra Max 250 (Molecular Devices, San Francisco, CA) to quantify the relative amounts of collagen remaining in the wells after digestion with conditioned media.

Migration Assays

Measurements of cellular migration were performed on young hmEC, aged hmEC, and aged hmEC treated with a blocking antibody to TIMP1. To inhibit cellular proliferation, all hmEC were pretreated with mitomycin C (2.5 μg/ml) before the start of migration assays. Differences in the migration of young and aged hmEC on collagen I were first quantified by measuring the linear distance cells moved over a 5-day period after release from a silicone gasket. The radial migration of hmECs aged in vitro with and without treatment with a blocking antibody to TIMP1 was measured using a sedimentation manifold (Creative Scientific Methods, Mesa, AZ). Briefly, 10-well Teflon-printed microscope slides were coated with 1 μg/ml collagen I in PBS for 1 h at 37°C, blocked with 1% bovine serum albumin (BSA) in PBS for 1 h, and rinsed 3× with PBS. Wells were filled with 50 μl of MCDB131 media with 1% serum and the manifold placed on top of the slide; 1 μl of media was removed from each well through the manifold to create a standing column of media and ~2,000 cells were seeded into the wells. Cells attached overnight, the manifold was removed, and fresh media was applied. Cells migrated for 5 days with media changes at days 2 and 4. Cells were then fixed in 10% neutral buffered formalin for 30 min and counterstained with 2% Crystal Violet. Duplicate cultures of cells were stimulated with MCDB131 with 1% FBS and a blocking antibody to TIMP1 (2.5 μg/ml). The concentration of blocking antibody to TIMP1 was based on the maximal concentration that did not result in cellular detachment. Quantification of migration was performed on scanned images of the colonies with NIH Image software. The distance migrated was calculated by measuring the length between the center of the colony and each of the cells that moved past the radius of the initial colony size.

Statistical Analysis

Differences in response in each of the experiments were assessed for statistical significance by a two-tailed *t*-test.

RESULTS

Expression of Collagenase Is Decreased, but That of TIMP1 Is Increased, During Angiogenic Invasion of Sponge Implants in Aged Mice

The expression of collagenase and TIMP1 during angiogenesis in vivo was examined by immunolocalization in PVA implants (Fig. 1). Sponges were placed in young and aged mice and removed at 14 days postimplantation. We have previously reported that the greatest difference in fibrovascular invasion into the sponge between the young and aged mice was at 14 days; thus, we were most interested in immunostain for collagenase and TIMP1 at that timepoint [Reed et al., 1998].

Relative to young mice, the expression of collagenase was decreased in ingression areas and capillaries of the fibrovascular response in aged animals (Fig. 1A–D). Mice have not been shown to synthesize MMP1 but do express other interstitial collagenases in remodeling tissues [Balbin et al., 1998]. By contrast, the expression of TIMP1 was generally increased in aged mice relative to young mice in these same regions (Fig. 1E–H). Although fibroblasts constitute the majority of cells in areas of ingression, prominent differences in immunostain for collagenase and TIMP1 between the young and aged mice were observed in the endothelial cells and the matrix adjacent to the capillaries in the leading edges of the fibrovascular response. These data supported the focused examination of the effect of aging on collagenase and TIMP1 expression in human microvascular endothelial cells.

hmEC Aged In Vitro Demonstrate Minimal Uptake of ^{3}H-Thymidine and Positive Staining for β-Galactosidase at pH 6

High levels of incorporation of ^{3}H-thymidine and other markers of DNA synthesis are charac-

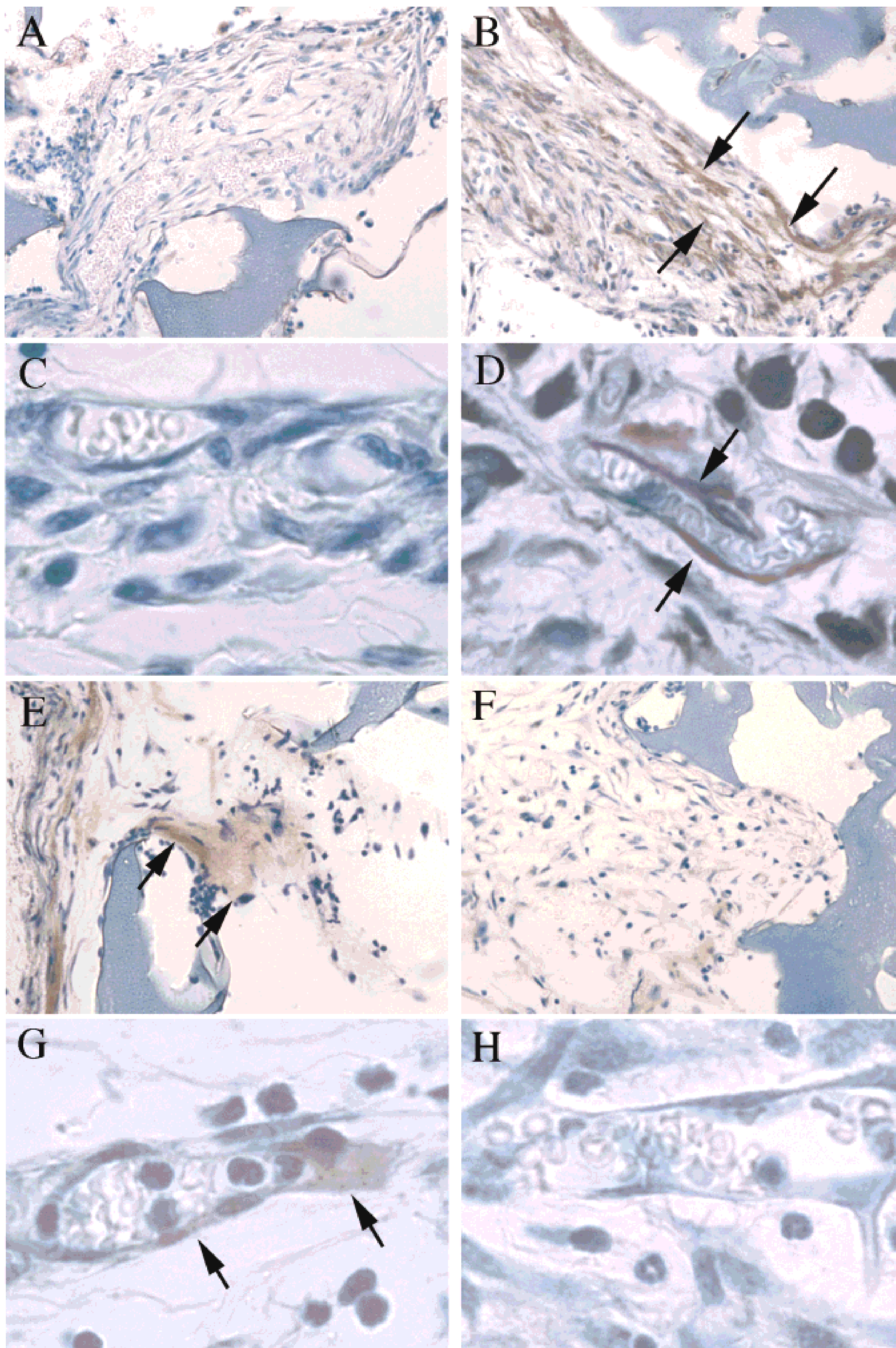

Fig. 1. Expression of collagenase is decreased, but that of TIMP1 is increased, in the tissues and endothelial cells of aged mice during angiogenesis. Sections from sponge implants were immunostained with antibodies against MMP1 and TIMP1 and visualized with DAB. Relative to young mice (B), the expression of collagenase was decreased in ingression areas of the fibrovascular response in aged animals (**A**). Arrows point to staining in the perivascular areas. **C,D:** Oil immersion photographs of capillaries in the tissues of the aged and young mice, respectively. Note the immunoreactivity for collagenase that is present in the endothelial cells (arrows) of the young, but not in aged mice. By contrast, the expression of TIMP1 was generally increased in the sponges of aged mice (**E**) in comparison to young mice (**F**). **G,H:** Oil immersion photographs of capillaries in the tissues of the aged and young mice, respectively. Immunoreactivity for TIMP1 is shown in the endothelial cells (arrows) of the aged, but not in young mice. **A,B,E,F:** ×300; **C,D,G,H:** ×1,000.

teristic of cells that proliferate rapidly (e.g., non-aged hmEC) and cells found in the tissues of young, but not aged, animals [Reed et al., 1996]. By contrast, staining for β-galactosidase at pH 6 is characteristic of cells that are aged both in vitro and in vivo [Dimri et al., 1995; Campisi 1996]. At pH 4, both young and aged cells are equally reactive, whereas neither stains at pH 7.5. Thus, the presence of an aged phenotype in late passage hmEC was established by expression of β-galactosidase at pH 6 and minimal uptake of ^{3}H-thymidine. As shown in Figure 2A, non-aged hmEC have no detectable stain for β-galactosidase in the cytoplasm and high incorporation of ^{3}H-thymidine in the nucleus. By contrast, hmEC aged in vitro demonstrated strong staining for β-galactosidase activity and minimal detectable uptake of ^{3}H-thymidine (Fig. 2B). These differences between early- and late-passage hmEC established that they were non-aged (young) and aged in vitro, respectively, as described by previous studies that have established this model as a method of examining impaired endothelial cell function with aging [Maier et al., 1990; Garfinkel et al., 1996; Watanabe et al., 1997]. We then proceeded to analyze the expression of MMP1 and TIMP1 by young and aged hmEC.

HmEC Aged In Vitro Have Decreased Expression of MMP1

Differences in levels of MMP1 in conditioned media from young and aged hmEC plated on collagen I were examined by Western blotting. Aged hmEC demonstrated a decrease (55% ± 13%, relative to young hmEC, $P < 0.05$) in the expression of MMP1 (Fig. 3A) relative to an equivalent number of young hmEC. Similar results were obtained from whole cell lysates, and from conditioned media containing both low (1%) and high (10%) serum (data not shown). Immunofluorescent detection of intracellular MMP1 showed that this protease is synthesized to a greater extent in young hmEC (Fig. 3B) than in aged hmEC (Fig. 3C).

Collagenase Activity Is Decreased in Aged hmEC

MMP1 initiates the process of collagen degradation through cleavage of intact collagen I chains. We employed a colorimetric assay to evaluate the ability of collagenase in the conditioned media from young and aged hmEC to degrade and release collagen I fragments from an insoluble collagen I substrate. Consistent with the levels of MMP1 found by Western blotting (Fig. 3), conditioned media from an equivalent number of aged hmEC had approximately 35% less collagenase activity (64% ± 12%) than that of young hmEC ($P < 0.03$) (Fig. 4).

HmEC Aged In Vitro Have Increased Expression of TIMP1

Levels of TIMP1 in conditioned media from aged and young hmEC cultures were also quantified by Western blotting. Conditioned media from aged hmEC showed greater expression of TIMP1 (280% ± 109%) than young hmEC ($P < 0.05$) (Fig. 5A). Similar results were obtained with cell lysates from young and aged hmEC and in the presence of low (1%) and high (10%) serum (data not shown). In contrast to MMP1, the immunofluorescent detection of TIMP1 showed that synthesis by aged hmEC (Fig. 5C) is greater than that of young hmEC (Fig. 5B). The observations presented in Figures 3–5 supported our hypothesis that aged hmEC have a deficit in the degradation of type I collagen.

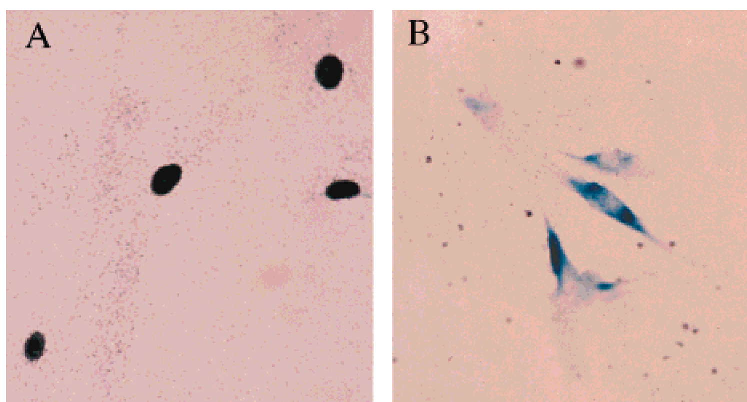

Fig. 2. HmEC aged in vitro show minimal incorporation of ^{3}H-thymidine and positive staining for β-galactosidase. Young (early-passage) and aged (late-passage) hmEC were examined for β-galactosidase staining at pH 6 and incorporation of tritiated thymidine. **A:** Young hmEC exhibit undetectable β-galactosidase staining and intense localization of ^{3}H-thymidine in their nuclei. **B:** By contrast, hmEC aged in vitro demonstrate strong staining for β-galactosidase and minimal uptake of ^{3}H-thymidine. ×800.

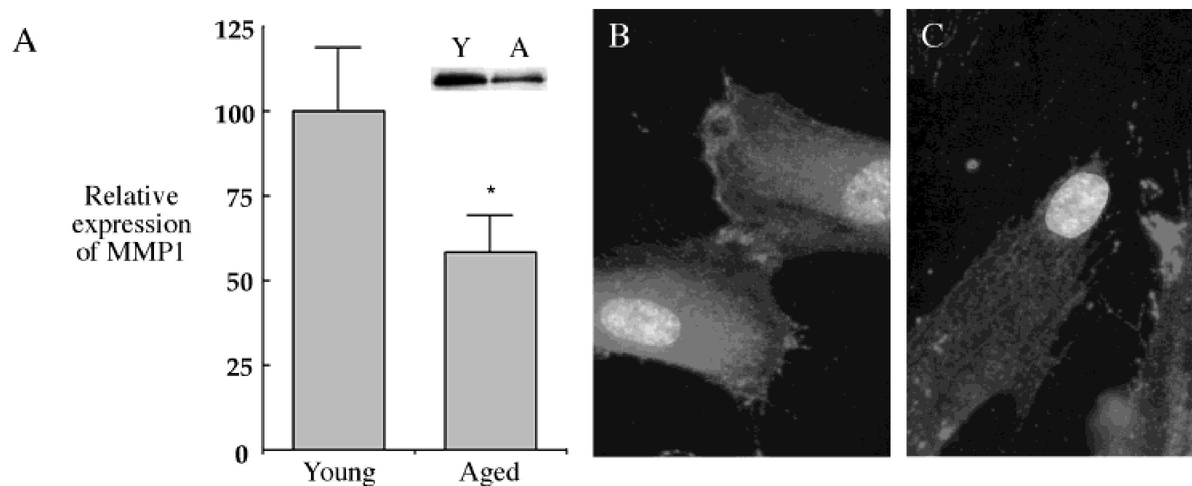

Fig. 3. HmEC aged in vitro show decreased secretion and expression of MMP1. **A:** MMP1 protein levels in conditioned media from young and aged hmEC as determined by scanning densitometry of Western blots. Inset, representative autoradiogram. Data are expressed relative to levels from young hmEC (normalized to 100%) ±SEM and represent the average of six separate experiments. Note that the expression of MMP1 by aged hmEC is 55% ± 13% of the expression of MMP1 by young hmEC (*$P < 0.05$). **B:** Immunofluorescence for MMP1 in young hmEC with DAPI-counterstained nuclei. ×4,000. **C:** Immunofluorescence for MMP1 in aged hmEC with DAPI-counterstained nuclei. ×4,000. Thus, relative to young hmEC, aged hmEC show decreased levels of MMP1 in conditioned media and less intracellular expression of MMP1.

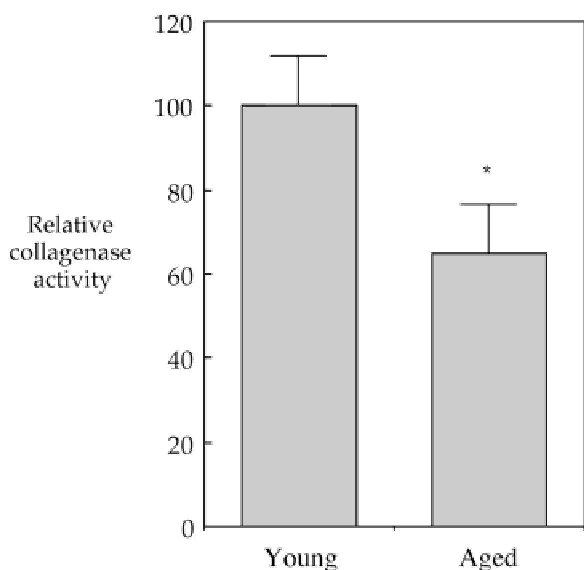

Fig. 4. Collagenase activity is decreased in aged hmEC. Collagenase activity was measured by quantification of undigested collagen by spectrophotometry. Data are expressed as percentage undigested collagen relative to young hmEC (normalized to 100%) ±SEM. Graph represents the average of 7 separate experiments and shows less collagenase activity in conditioned media from aged hmEC (64% ± 12% of the activity of media from young hmEC; *$P < 0.03$).

Migration Is Impaired in hmEC Aged In Vitro

The migration of aged and young hmEC on collagen I was determined by measuring the linear movement of these cells after release from a silicone gasket. As shown in Figure 6, aged hmEC moved only 59% ± 8% of the distance migrated by young hmEC over a 5-day period ($P < 0.003$). All cells were pretreated with mitomycin C to prevent the contribution of proliferation to differences in the size of the colony.

Inhibition of TIMP1 Activity Increases the Migration of Aged hmEC

To determine if increasing collagenase activity improved the migration of aged hmEC on collagen I, a blocking antibody to TIMP1 was added to duplicate colonies of cells in a radial migration assay. As shown in Figure 7A, aged hmEC treated with the antibody showed a significant increase (142% ± 11% relative to untreated aged hmEC, $P < 0.0005$) in the radial distance migrated over 5 days. Figure 7B,C shows representative colonies of aged (Fig. 7B) and aged cells treated with a blocking antibody to TIMP1 (Fig. 7C) hmEC during cellular migration. Microscopic examination of aged hmEC treated with the antibody to TIMP1 demonstrated a more elongate morphology than cells not exposed to the antibody.

DISCUSSION

Impaired migration of endothelial cells contributes to delayed angiogenesis in aged tissues

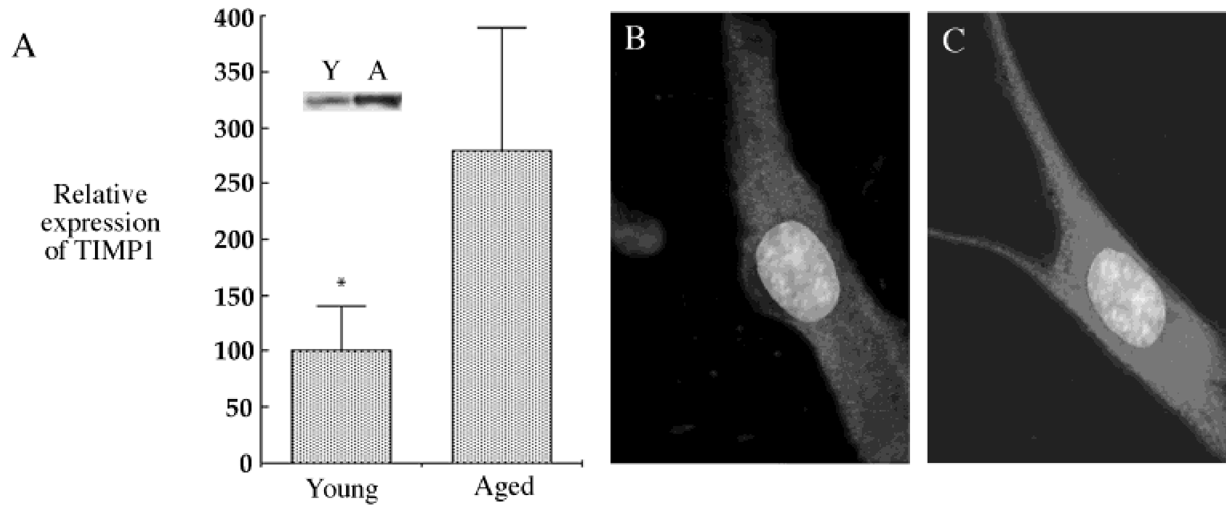

Fig. 5. Aged hmEC have increased expression of TIMP1 on type I collagen. **A:** TIMP1 protein levels in conditioned media from young and aged hmEC as determined by scanning densitometry of Western blots. Inset, representative autoradiogram. Data are expressed relative to levels from young hmEC (normalized to 100%) ±SEM and represent the average of six separate experiments. Note the expression of TIMP1 by aged hmEC is 280% ± 109% of the expression of TIMP1 by young hmEC (*$P < 0.05$). **B:** Immunofluorescence for TIMP1 in young hmEC with DAPI-counterstained nuclei. ×4,000. **C:** Immunofluorescence for TIMP1 in aged hmEC with DAPI-counterstained nuclei. ×4,000. Thus, relative to young hmEC, aged hmECs show increased levels of TIMP1 in conditioned media and greater intracellular expression of TIMP1.

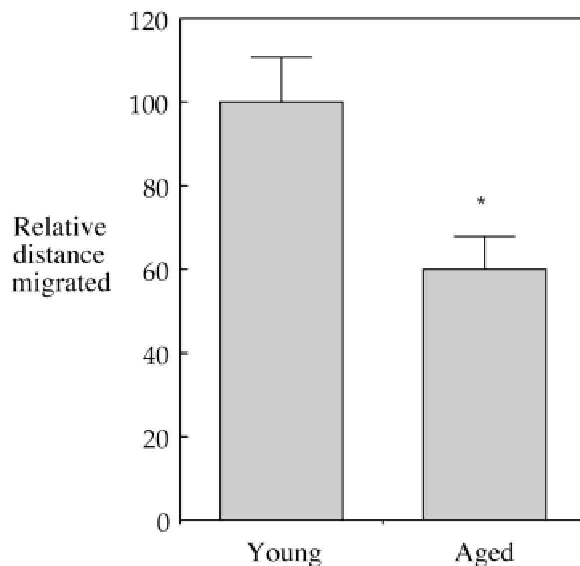

Fig. 6. Aged hmECs show impaired cellular migration on collagen I. The migration of young and aged hmEC on collagen I was quantified in a linear migration assay. Shown is a graphic representation of distance migrated by aged hmEC relative to young hmEC over 5 days. Note that aged hmEC moved only 59% ± 8% of the distance moved by young hmEC (*‡$P < 0.003$). Data represent the average of three separate experiments.

[Arthur et al., 1998]. Efficient movement of cells is regulated, in part, by proteolytic degradation of the matrix by MMPs. Thus, deficient MMP activity impedes the detachment of cells from the substratum and prevents the exposure of cryptic binding sites that are necessary for proper cellular attachment to the matrix. The predominant extracellular matrix protein present during the angiogenic phase of wound repair and the major structural component upon which microvascular endothelial cells migrate is collagen I. Since collagen I is initially degraded by MMP1 (interstitial collagenase), it is likely that the activity of this metalloprotease directly affects the migratory ability of cells during angiogenesis.

In this study, we noted decreased collagenase and increased TIMP1 expression in the leading edges of fibrovascular invasion into a PVA sponge in aged mice relative to young mice during angiogenesis in vivo. The tissue of the aged mice were deficient in immunostain for collagenase in the newly formed capillaries, as well as their surrounding matrix. Although vascular ingressions are comprised of fibroblasts, endothelial cells, and extracellular matrix these data supported our hypothesis that deficits in collagenase activity may contribute to impaired neovascularization in aging. To examine protease secretion specifically in endothelial cells, studies were performed on hmEC aged in vitro by serial propagation. This model is based on the finite number of population doublings that most cells have when placed in culture. As cells approach the end of their replicative life span

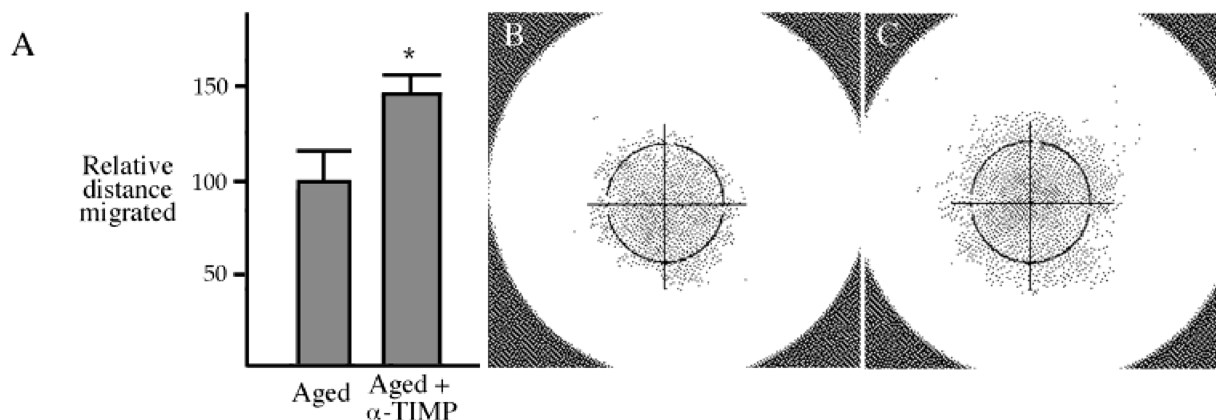

Fig. 7. Inhibition of TIMP1 improves the migration of aged hmEC on collagen I. The radial migration of aged and aged hmEC treated with a blocking antibody to TIMP 1 on collagen I was quantified in a cell sedimentation manifold assay. **A:** Graphic representation of the increase in the distance migrated by aged hmEC exposed to the blocking antibody in comparison to their untreated counterparts (142% ± 11% relative to untreated aged hmEC, $*P < 0.0005$). Data represent the average of four separate experiments. **B,C:** Representative colonies of aged hmEC and aged hmEC exposed to the blocking antibody to TIMP1, respectively, migrating on collagen I.

they exhibit features common to cells from aged donors (aged in vivo) with respect to increased cell size, lengthening of the cell cycle, and delayed cellular migration [Hayflick, 1965; Schneider and Mitsui, 1976; Matsuda et al., 1992] (S. Kudravi and M.J. Reed, manuscript in preparation]. Although aging in vitro is not equal to aging in vivo, the use of this model is supported by a significant body of literature ranging from discovery of cell cycle regulators, oncogenes, biomarkers and, more recently, as a relevant model for studies of endothelial cell function in aging [Maier et al., 1990; Campisi, 1996; Garfinkel et al., 1996]. Aging in vitro has also been established as a useful method for examination of impaired function in human microvascular endothelial cells [Watanabe et al., 1997]. Unlike in vivo aging, aging in vitro avoids the complication of diseases and medication exposures, which pose a significant problem in the study of microvascular endothelial cell function.

Measurements of MMP1 and TIMP1 in conditioned media from young and aged hmEC plated on collagen I were consistent with data from the sponge model of angiogenesis; aged hmEC demonstrated deficient expression and activity of MMP1 and increased secretion of TIMP1 relative to young hmEC. The decreased synthesis of MMP1 in aged hmEC contrasts with studies that found increased MMP1 and decreased TIMP1 expression in aged fibroblasts and in the wounds of aged donors [Millis et al., 1992; Zeng and Millis, 1996; Ashcroft et al., 1997a,b]. This difference could result from the fact that the microvascular endothelial cell constitutes only a small percentage of the cells in the wound bed; thus, age-related differences in the expression of MMPs and TIMPs by endothelium may not be reflected in tissues in the process of wound repair. Alternatively, differences among aged cells could reflect tissue-specific activity. For example, small and large vessel endothelial cells differ in MMP and TIMP expression. Studies comparing the expression of MMP-1, -2, -9, and TIMP 1 and 2 in macrovascular and microvascular endothelial cells have shown that whereas both types of endothelium secrete MMP and TIMP, macrovascular endothelial cells exhibit greater basal secretion of MMPs 1 and 2 and less induction of MMP9 and TIMP1 in response to phorbol myristate acetate (PMA) relative to microvascular endothelial cells [Hanemaaijer et al., 1993; Jackson and Nguyen, 1997]. In addition, a recent study by Kräling et al. (1999) found that endothelial cells stimulated to undergo a switch to a maturation phenotype decrease their synthesis of MMP and increase their synthesis of TIMP1, thereby decreasing their potential to exhibit an angiogenic (invasive) phenotype. These findings indicate that endothelial cells can markedly change their MMP activity in response to external signals. A premature change to this noninvasive phenotype may underlie the defect in MMP synthesis/activity in aged hmEC and contribute to impaired angiogenesis in aging.

MMP1 is required for the movement of keratinocytes on collagen I and for angiogenesis in vitro [Fisher et al., 1994; Pilcher et al., 1997]. Thus, we propose that the deficit in MMP1 activity in the aged hmEC contributes to impaired migratory ability of aged cells. Moreover, comparisons of MMP1 synthesis in this study were conducted on a "per-cell" basis, and since aged hmEC are larger than their young counterparts, it is probable that an even greater amount of collagenase activity is necessary to move aged cells in an efficient manner. Putative mechanism(s) for deficient MMP1 synthesis in aged hmEC remain a matter of conjecture. Others have shown that ligand activation of $\alpha_2\beta_1$ integrin induces the synthesis of MMP1 in certain cells [Riikonen et al., 1995]. We have found that aged hmEC demonstrate a deficit in cell surface expression of $\alpha_2\beta_1$ and a delay in adhesion to collagen I relative to young hmEC (M.J. Reed, A.C. Corsa, and R.S. McCormick, unpublished observations). The link between integrins and regulation of protease activity was recently established [Brooks et al., 1996, 1998] and an age-related defect in integrin function, as reported by others [Hu et al., 1996], is likely to inhibit matrix metalloprotease activity. As such, it is possible that impaired integrin expression/function and adhesion contribute to the lack of MMP1 synthesis in aged hmEC.

In contrast to MMP1, there was increased expression of TIMP1 in the conditioned media of aged relative to young hmEC. An increase in TIMP1 would further decrease the available MMP1 activity in the environment of the aged hmEC. Although TIMPs serve to keep MMP-mediated proteolysis within parameters that are optimal for endothelial cell adhesion to and migration through the extracellular matrix, excessive activity of this inhibitor slows cellular movement and inhibits angiogenesis in vitro and in vivo [Gomez et al., 1997]. In addition, TIMP1 independently stimulates the synthesis and secretion of MMP1 by dermal fibroblasts [Clark et al., 1994]. It is unknown whether increased TIMP1 is an inherent feature of aged hmEC or a response to decreased MMP1 synthesis by these cells.

As expected from prior studies in vitro and in vivo, cellular movement was significantly impaired in aged hmEC relative to young hmEC. It is worth noting that the 40% decrease in migration is similar to the delay in the angiogenic and wound repair response in aged mice and rats relative to young controls [Puolakkainen et al., 1995; Reed et al., 1998]. Although multiple mechanisms contribute to the impaired mobility of aged cells, we predicted that enhancing the collagenase activity of aged hmEC, by blocking TIMP1, would significantly increase their radial migration on collagen I. Of note, treatment of aged hmEC did not confer the same migratory ability as their young counterparts, a result that underscores the complexity of cellular locomotion. These data do, however, support our hypothesis that deficient MMP1 activity contributes to impaired movement of aged hmEC and that perturbations that increase collagenase activity augment the migration of these cells. Future studies will establish whether an enhanced ability to degrade collagen I improves the angiogenic potential of aged hmEC and of aged tissues in vivo.

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