

Neovascularization in Aged Mice

Delayed Angiogenesis is Coincident with Decreased Levels of Transforming Growth Factor β 1 and Type I Collagen

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Angiogenesis, the growth of new vessels from existing microvasculature, is delayed in aged animals. In this study we asked whether this impairment might be due, in part, to changes in the expression of a growth factor, transforming growth factor- β 1 (TGF- β 1), and a matrix protein, type I collagen, which have been shown to regulate angiogenesis *in vivo*. We implanted polyvinyl alcohol sponges subcutaneously in the dorsa of young and aged mice and examined the sponges 7 to 21 days later for the presence of invasive fibrovascular bundles. Blood vessel ingrowth and proliferative activity were assessed by immunostain for von Willebrand factor and Ki-67, respectively. The fibrovascular bundles were also analyzed for TGF- β 1 and type I collagen. Relative to young mice, angiogenic invasion of sponges in aged mice was similar at 7 days, was diminished significantly (70%) at 14 days, but was again similar by 21 days after implantation. The expression of TGF- β 1 and type I collagen mRNA and protein in fibrovascular bundles was coincident but was also delayed (42 to 47%) at 14 days in the aged mice. Moreover, levels of active TGF- β 1 were decreased (48%) in the sera of aged relative to young mice. The delay in angiogenesis in aged mice was thus associated with decreased expression of TGF- β 1 and type I collagen by neovascular bundles. We conclude that changes in the levels of growth factors and proteins in the extracellular matrix contribute to impaired angiogenesis in aging. (*Am J Pathol* 1998, 152:113-123)

Aging is accompanied by decreased responses to physiological and pathological stresses, such as tissue repair after injury.¹ The observation that aged animals require a longer period of time to heal was made initially in humans^{2,3} but has subsequently been extended by numerous investigators to include aged rodents.⁴⁻⁶ In a young human, wound repair occurs rapidly in several charac-

teristic stages: an early phase of inflammation and coagulation; a middle stage of granulation tissue formation; and a final phase of cellular proliferation and biosynthesis of extracellular matrix proteins.⁷ These same stages are observed in rodents during wound healing. Moreover, aged rats and mice manifest impairments in wound repair similar to those of aged humans but have a decreased prevalence of concurrent diseases that can affect healing.^{6,8} The delay in wound repair with aging involves all of the above-mentioned stages,¹ including the growth of new vessels from existing vessels, termed angiogenesis, which occurs during the formation of granulation tissue.⁹

Endothelial cell proliferation, migration, biosynthesis, and contraction are requisite to angiogenesis.¹⁰ These activities are regulated by interactions between the cells and components of the extracellular environment, eg, growth factors and matrix proteins.¹¹ Holm-Pedersen et al¹² and Yamaura and Matsuzawa¹³ were the first to observe that the formation of capillaries is decreased in middle-aged rats relative to young rats. Although a delay in angiogenesis is detrimental to wound repair, a decrease in the angiogenic potential of aged tissues is believed to contribute to the slower proliferative rate of tumors in aged rodents and humans.¹⁴⁻¹⁶ It is unclear whether the defect in aged animals is due to endothelial cell, pericyte, fibroblast, and/or smooth muscle cell dysfunction, or to deficiencies in levels of growth factors and/or matrix proteins, as there have been no studies of these factors in the progression of angiogenesis in aged animals.

Growth factors that have been implicated in the response to injury include transforming growth factor- β 1 (TGF- β 1),¹⁷ platelet-derived growth factor,¹⁸ basic fibroblast growth factor (bFGF),¹⁹ epidermal growth factor,²⁰ and insulin-like growth factor-1.²¹ Several of these factors, eg, TGF- β 1, bFGF, and vascular endothelial growth

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factor/vascular permeability factor, enhance angiogenesis *in vivo*.²²⁻²⁵ The production of many of these proteins is decreased in aging and senescent cells.²⁶⁻²⁸ Compared with young cells, senescent fibroblasts proliferate to a lesser extent in response to stimulation by exogenous growth factors,²⁹⁻³² but they synthesize extracellular proteins and contract collagen gels to levels similar to those of young cells.³³ Few studies have examined the effect of growth factors on aged endothelial cells. Senescent human umbilical vein endothelial cells do not proliferate in response to bFGF, a growth factor that is nonetheless required for their survival.³⁴ However, bovine aortic endothelial cells aged *in vitro* respond to stimulation by exogenous bFGF in a manner comparable with that of young cells²⁸ with respect to proliferation and migration. Moreover, human microvascular endothelial cells aged *in vitro* are rescued from senescence by stimulation with vascular endothelial growth factor.³⁵ Phillips et al^{36,37} showed that microvascular endothelial cells derived from sponges implanted in aged rats exhibited a proliferative response to serum greater than that of endothelial cells derived from young rats. In contrast, cells from these aged rats were impaired in their chemotactic response to platelet-derived growth factor. The inconsistent response of aged cells to stimulation by exogenous cytokines is likely due, in part, to a retention of receptor number and signal transduction pathways for certain growth factors, but not others, in some of the relevant cell types.³⁸

Wound repair is also regulated by proteins of the extracellular matrix, such as fibronectin, tenascin-C, thrombospondin 1, type I collagen, and SPARC.³⁹⁻⁴³ Many of these proteins exhibit distinct temporal and spatial expression during the stages of wound healing. In general, experiments performed *in vitro* have shown that the matrix can enhance (type I collagen)^{44,45} or inhibit (thrombospondin 1)⁴⁶ the formation of blood vessels during wound repair. For example, wounds in mice that were treated with antisense oligonucleotides against thrombospondin 1 and transgenic mice that are deficient in thrombospondin 1 exhibited an enhanced angiogenic response during the healing process.^{41,47} The effect of matrix proteins on the formation of blood vessels is augmented additionally by growth factors, which increase angiogenesis through a direct effect on the cells or indirectly by the enhanced production of extracellular matrix proteins.^{11,48} TGF- β 1, for example, binds to endothelial cells⁴⁹ and increases the formation of type I collagen by aged cells.³³ If aging is associated with a decrease in the expression of growth factors²⁶⁻²⁸ and matrix proteins^{50,51} that have separate but complementary roles in the regulation of angiogenesis, the loss of the synergistic effect on neovascularization could be of greater significance in aged animals than in their younger counterparts.

We have demonstrated that dermal fibroblasts from aged humans have a response comparable with those derived from young adults with respect to the biosynthesis of type I collagen and SPARC and the contraction of a collagen gel after stimulation by TGF- β 1.³³ In a model of impaired wound healing in aged rats, we and others

have shown that wound repair (including cell proliferation, biosynthesis, and angiogenesis) was accelerated by the application of intravenous⁵ and topical TGF- β 1.⁹ In our study of aged, calorically-restricted mice, we found that wound repair was enhanced markedly by a short-term return to an *ad libitum* diet. Accelerated wound healing was accompanied by increases in insulin-like growth factor-1, insulin-like growth factor-binding protein 3, type I collagen, and endothelial cell proliferation. These data support the hypothesis that aged animals can augment neovascularization if the extracellular environment provides an optimal source of the growth factors and extracellular matrix proteins that support wound repair.

In this study, we have determined the expression of TGF- β 1 and type I collagen during the fibrovascular invasion of a polyvinyl alcohol (PVA) sponge implant in aged and young mice. The mice are obtained from a pathogen-free strain of F₁ hybrids that represent an established model for research in aging.^{6,8,52} The PVA implant permits the study of the cellular and extracellular matrix components that are expressed during the sequential stages of angiogenesis *in vivo*⁵³⁻⁵⁵ and are relevant to wound repair. Our data show that the delay in angiogenesis in aged mice is associated with decreased expression of TGF- β 1 and type I collagen in the fibrovascular bundles that constituted the neovascular response. The expression of these proteins was localized primarily to fibroblast-like cells supporting the new vasculature. We therefore propose that changes in the levels of growth factors and matrix proteins in the extracellular matrix contribute to impaired angiogenesis and wound healing in aging.

Materials and Methods

Animal Model

We used mice of the [C57BL/6 $\times$ DBA/2]F₁ (B6D2F1) and [C57BL/6 $\times$ C3H]F₁ (B6C3F1) strains (all males to facilitate comparisons among groups) of age groups 4 to 8 months (young mice) and 30 to 33 months (aged mice). All animals were received from the National Institute on Aging aging and diet restriction, pathogen-free colony maintained by the National Center for Toxicological Research (Jefferson, AR). Two different strains were studied to ensure the data were applicable to other aged mice. Both strains are pathogen-free F₁ hybrids that represent an established model for research in aging.^{6,8,52} Mice were maintained in the specific pathogen-free and microbiologically-monitored environment of the Department of Comparative Medicine at the University of Washington School of Medicine. Three to five mice of both strains from each age group were studied in each of the analyses of the PVA sponges. Mice with tumors were excluded from all analyses.

Angiogenesis was studied by examination of the invasion of PVA implants.⁵³⁻⁵⁵ Two PVA sponges (Ivalon, Unipoint Industries, High Point, NC) (1 $\times$ 1 $\times$ 2 cm) were implanted in the dorsum of each mouse. Three mice from the young group and three to five from the aged group

were analyzed at each time point. Because of the large number of animals, two different series of experiments were performed. In the first series, sponges were recovered at 7, 14, and 21 days. In the second series, sponges were recovered at 10 and 17 days. Results from the two separate series were combined for data comparing the degree of expression between young and aged but were not combined for temporal analyses.

One implant was fixed in neutral-buffered formalin and was prepared for immunohistochemistry and *in situ* hybridization (see below). The other implant was placed in Tri-Reagent (Molecular Research Center, Cincinnati, OH) for protein extraction.

Histology and Invasion Index

Five- μ m sections of paraffin-embedded sponges were dewaxed in xylene, hydrated in a graded series (100 to 70%) of ethanol solutions, and counterstained with toluidine blue. The amount of invasion into the PVA sponge was calculated from micrometer measurements by two different investigators who were unaware of the identity of the samples. A scale from 0 to 4+ was based on the distance the cells had traveled from the edge of the sponge. Zero represented no invasion, and 4+ indicated invasion from both sides of the sponge, such that the entire sponge was filled with new tissue. The width of each sponge was approximately 2 fields at 25 $\times$; each field was analyzed separately. Fine invasion was defined as a reticular pattern consisting of fibroblast-like and mononuclear cells and an absence of cellular bundles and/or blood vessels. Dense invasion consisted of a network of numerous fibroblast-like cells containing vessels with lumens and components of the blood.

Immunohistochemistry

Slides were prepared for immunohistochemistry as previously described.³³ Endothelial cells in the PVA sponge implant were detected by immunostain for rabbit anti-human von Willebrand factor (10 μ g/ml) (Dako, Carpinteria, CA) followed by incubation in biotinylated goat anti-rabbit IgG (20 μ g/ml) (Vector Laboratories, Burlingame, CA). After a rinse in phosphate-buffered saline (PBS), skin samples were incubated with avidin-biotin-peroxidase complex (20 μ g/ml) (Vector Laboratories) followed by 3,3'-diaminobenzidine-4-HCl (DAB).

Proliferative activity in the PVA sponges was detected by immunostain for Ki-67 protein in the nuclei of cells in the fibrovascular bundles. Slides were incubated with a rabbit polyclonal antibody to Ki-67 (20 μ g/ml) (Dako), rinsed in PBS, exposed to biotinylated goat anti-rabbit IgG (20 μ g/ml), and developed as described above with DAB plus nickel enhancement.

Immunohistochemistry for TGF- β 1 protein was performed with a rabbit panspecific antibody reactive with the TGF- β family (R & D Systems, Minneapolis, MN). This antibody has a 10-fold greater reactivity to the active form of TGF- β . After incubation with anti-TGF- β (10 μ g/ml), slides were rinsed in PBS and were exposed to biotinylated

goat anti-rabbit IgG (5 μ g/ml) (Vector Laboratories). After a rinse in PBS, samples were incubated with avidin-biotin-peroxidase complex (20 μ g/ml) (Vector Laboratories) followed by DAB (Vector Laboratories). Preimmune serum and secondary antibody alone served as controls. The amount of immunostain was assessed by two investigators based on a 0 to 4+ scale in which 0 indicated no staining and 4+ was equivalent in intensity to a positive control of mouse skin. All slides were counterstained with 1% toluidine blue and were photographed with a Leitz DMRB photomicroscope.

In Situ Hybridization

Riboprobes were transcribed with an RNA transcription kit (Promega, Madison, WI) in the presence of α -[³⁵S]dUTP (Amersham, Arlington Heights, IL). The following templates were used: a 974-bp *Sma*I/*Sma*I fragment of mouse TGF- β 1 (provided by Dr. Harold Moses, Vanderbilt University, Nashville, TN) in pBSK and a 312-bp *Hind*III/*Eco*RI fragment from the 3'UTR of mouse α (I) collagen in pBSK (provided by Dr. Paul Bornstein, University of Washington).⁵⁶ The specific activity of the probes was approximately 3 to 4 $\times 10^7$ cpm/ μ g. The respective riboprobes, transcribed in a sense orientation, were used as controls. The *in situ* hybridization protocol was a modification of a method described by Holland et al.⁵⁷ All slides were photographed with a Leitz DMRB photomicroscope equipped with a darkfield transilluminator.

Assay for TGF- β 1 in Serum

This assay uses the sensitivity of the mink lung cell fibroblast line CCL64 to the growth-inhibitory properties of TGF- β 1⁵⁸ and detects amounts as low as 10 pg of active TGF- β 1 per ml.⁵⁹ Mink cells (8000/well) were seeded into 96-well plates in the presence of Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) with 2% NuSerum (Collaborative Research, Bedford, MA) and test plasma or serum (diluted serially into Dulbecco's modified Eagle's medium with 2% NuSerum). Cells were allowed to grow for 48 hours that included [³H]thymidine (NEN Life Science Products, Boston, MA) for the last 12 hours. Standard inhibition curves with recombinant human TGF- β 1 were prepared to quantify TGF- β 1 in the samples. Neutralizing antibodies to mouse TGF- β (R & D Systems) were added to a separate series of dilutions (50 μ g/ml) of the test samples to quantify the fraction of inhibition caused by TGF- β . The fraction of latent TGF- β activity was determined by incubation of separate aliquots of serum for 5 minutes at 80°C before the assay.⁶⁰

Western Blotting

Tissues from the wound site were collected and snap-frozen at -70°C in Tri-Reagent. Proteins were extracted and were supplemented with proteinase inhibitors (2 mmol/L phenylmethylsulfonyl fluoride and 2.5 mmol/L ethylenediaminetetraacetic acid as final concentrations).

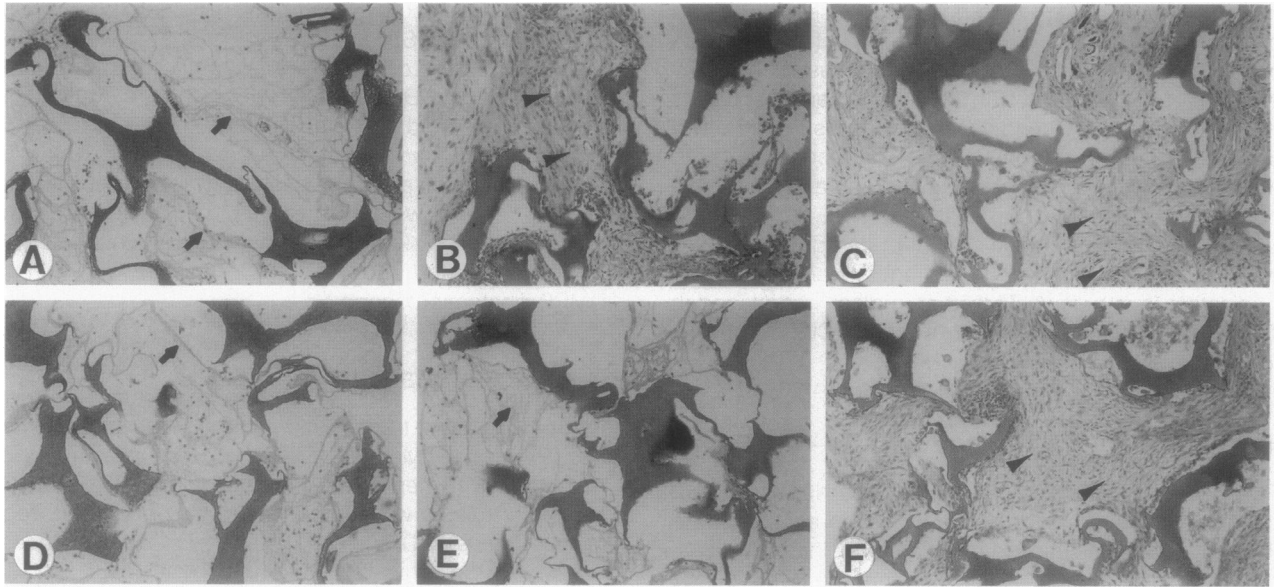


Figure 1. Fibrovascular invasion is delayed in aged mice. Shown are brightfield photographs of sections from the PVA sponge implant recovered at 7, 14, and 21 days in young (A through C) and aged (D through F) mice and stained with hematoxylin and eosin (H&E). Two types of invasion were noted: fine invasion (arrows) and dense invasion (arrowheads). Note the large amount of dense invasion at 14 days (B and E) in the young (B) relative to the aged mice (E) in the center region of the sponge. At 21 days (C and F), the degree of invasion was similar in the two groups of animals. Magnification, $\times 100$.

Samples from the young ($n = 3$) and aged ($n = 3$) mice were pooled at each of the time points and were quantified with the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA). Aliquots of tissue equivalent to 20 μg of total protein were solubilized in equal volumes of sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer,⁶¹ reduced with 50 mmol/L dithiothreitol, and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on slab gels (stacking and resolving gels were 4 and 7% acrylamide, respectively). Gels were transferred to nitrocellulose and were stained with amido black. Blots were blocked overnight in PBS containing 1% nonfat dry milk/0.05% Tween 20, exposed to 50 $\mu\text{g}/\text{ml}$ of a polyclonal antiserum against rat type I collagen in PBS with 1% nonfat dry milk,³³ and incubated in 5 μCi [^{125}I]-Protein A (NEN Life Science Products). Radioactive bands in blots were visualized by autoradiography and were quantified with National Institutes of Health Image on a MacIntosh Power PC.

Statistical Analysis

Significant differences between sample means were determined by a Student's two-tailed t -test.

Results

Fibrovascular Invasion

The degree of fibrovascular invasion was assessed by micrometer measurements of the amount of fine and dense invasion per high power field. These two types of invasion are shown in the brightfield photographs of sections from the implant harvested at 7, 14, and 21 days (Figure 1) from young (Figure 1, A to C) and aged mice

(Figure 1, D to F). At 7 days, neither group of animals exhibited significant invasion (Figure 1, A and D). There was an increased amount of dense invasion at the center of the implant at 14 days in young mice (Figure 1B) in comparison with aged mice (Figure 1E). At 21 days, the degree of invasion was similar in the two groups of animals (Figure 1, C and F).

Characterization of Dense Invasion

Additional examination of the two types of invasion by immunohistochemistry showed that fine invasion, as represented by Figure 2A showing a 14-day sponge in an aged mouse, was composed of fibroblastic and endothelial cells; however, no distinct vessels were noted. Figure 2B, showing a 14-day sponge in a young mouse, illustrates dense invasion that was characterized by numerous areas of vascular invasion that contained capillaries. Vascularization was confirmed by the presence of von Willebrand factor-positive cells lining channels that contained blood components (Figure 2C). Proliferation was assessed by immunostain for Ki-67 in the cells of the fibrovascular networks, as shown by the dark nuclei in Figure 2D. Only a small percentage of the cells ($<20\%$) were positive for Ki-67 in the sponges of both young and aged mice at any time point.

Dense Invasion Is Delayed in Aged Mice

The degree of dense invasion was quantified at 7, 14, and 21 days by two investigators. At least three mice of each age group were studied at each time point. A graphical representation of the amount of invasion showed no difference between the two age groups at 7 days, a significant delay of 70% in the aged mice ($1.67 \pm$

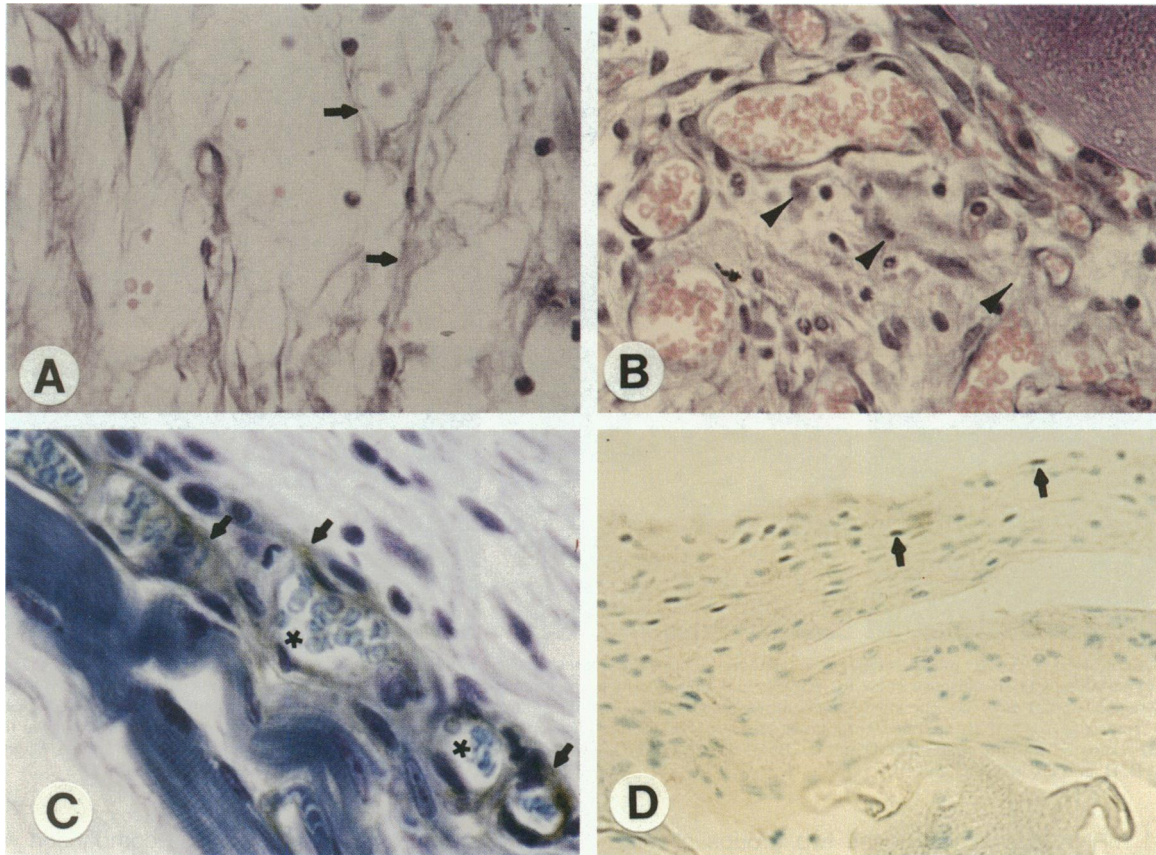


Figure 2. Dense invasion is vascular. Shown are representative areas of fine (arrows, A) and dense invasion (arrowheads, B) in sections of 14-day implants counterstained with H&E. Areas of dense invasion were noted to have capillaries that were positive for von Willebrand factor (brown stain noted with arrows, C) containing blood components (asterisks, C). D shows that a small percentage of the cells in the fibrovascular areas of dense invasion are proliferating (detected by a black nuclear stain for Ki-67, arrows). Magnification, $\times 600$ (A and B); $\times 800$ (C); and $\times 400$ (D).

0.33 in young mice versus 0.5 ± 0.22 in aged mice) at 14 days ($P < 0.05$) and again a similar degree of invasion between the two age groups at 21 days (Figure 3). There were no differences in the amount of fine invasion between the aged and young mice at any of the time points examined. Analyses of the dense invasion of the sponges in the 17-day series showed that the difference between the young and aged mice was decreased relative to that observed at 14 days. However, invasion did not become equivalent between the two groups until 21 days after implantation (data not shown).

Expression of TGF- $\beta 1$ mRNA

To determine whether the levels of TGF- $\beta 1$ mRNA were correlated with the delay in angiogenesis seen in the aged mice, we performed *in situ* hybridization, as shown in Figure 4. Transcripts were localized to areas of dense fibrovascular invasion, and there was increased expression at 14 days in the young mice (Figure 4, A and B) relative to the aged mice (Figure 4, D and E). Differences between young and aged mice persisted when levels of transcripts were corrected for the amount of dense invasion determined by the distance the dense tissue had invaded into the sponge. By 21 days, expression of

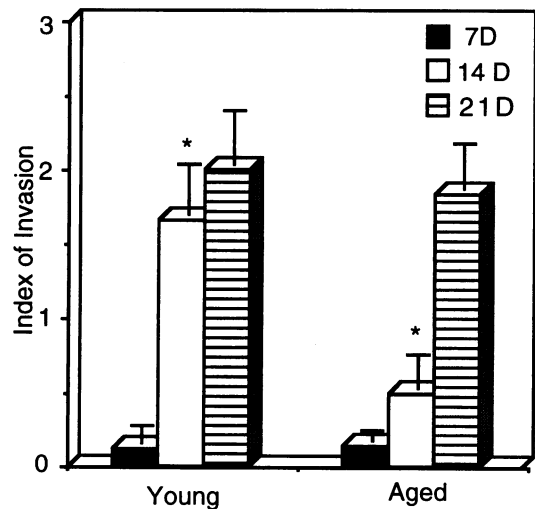


Figure 3. Dense invasion is delayed in aged mice. The degree of dense invasion was measured at 7, 14, and 21 days by quantification with a micrometer. At least three mice of each age group were studied at each time point. A graphical representation of the amount of invasion is shown, with the y axis representing the amount of invasion into the sponge on a 0 to 4 scale. A score of 4 would be equivalent to invasion across the entire span of the sponge. Both groups of animals showed minimal invasion at 7 days. There was a 70% delay in invasion at 14 days (1.67 ± 0.33 in young mice versus 0.5 ± 0.22 in aged mice; *, $P < 0.05$), but a comparable degree of invasion by 21 days, in the aged mice in comparison with the young mice.

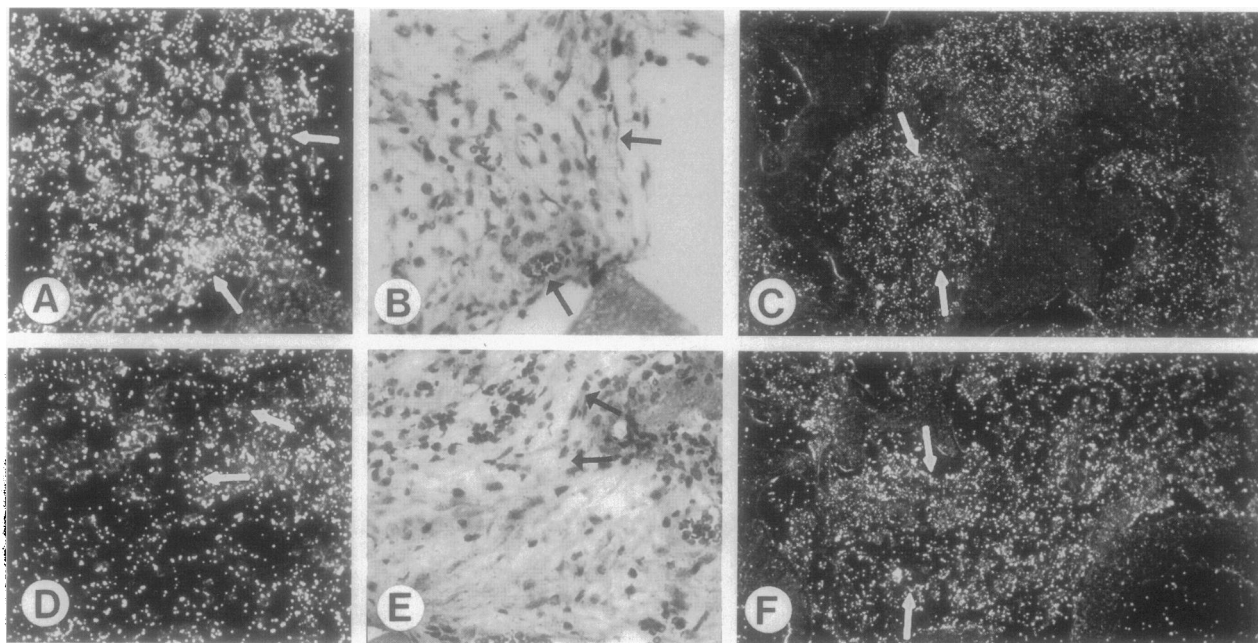


Figure 4. Expression of TGF- β 1 mRNA is delayed in aged mice. Shown are representative brightfield (B and E) and darkfield photographs (A, C, D, and F) of *in situ* hybridization with an antisense probe for TGF- β 1. In the upper panels are sponge implants from young mice at 14 (A is darkfield and B is brightfield of the same region) and 21 days (C). The lower panels show sponge implants from aged mice at 14 (D is darkfield and E is brightfield of the same region) and 21 days (F). Transcripts for TGF- β 1 are noted throughout areas of fibrovascular invasion (arrows, A to F). Note the increased expression at 14 days in the young (A and B) compared with the aged mice (D and E). By 21 days (C and F), levels of transcripts for TGF- β 1 were similar in the two age groups. Magnification, $\times 300$ (A, B, D, and E), $\times 200$ (C and F).

TGF- β 1 mRNA was similar in the two groups of mice (Figure 4, C and F).

TGF- β 1 Protein in the Sponges

Immunohistochemistry was performed with a panspecific antibody against the TGF- β family of growth factors. The amount of immunostain per high power field was assessed by two investigators and was corrected for the degree of fine and dense invasion as quantified previously. Fibroblasts, endothelial cells, and the extracellular matrix were reactive with the antibody. Figure 5A is a representative photograph of a sponge at 14 days in young mice. In contrast, there was a lack of immunostain at all time points in the aged mice (Figure 5B). The amount of TGF- β protein was expressed as a ratio between the young and aged mice at each of the time points (Figure 5C). The difference in TGF- β 1 immunoreactivity between the two age groups was maximal ($42 \pm 9.2\%$) at 14 days ($P < 0.05$) as determined by three separate rounds of immunohistochemistry.

Basal Levels of TGF- β 1 Protein in the Serum

Although TGF- β 1 is synthesized *de novo* during angiogenesis, the amount of TGF- β 1 in the sera of animals could determine, in part, the availability of this growth factor to tissues. Basal levels of active TGF- β 1 were measured by the mink lung cell inhibition assay in the sera of mice from the same age groups and genetic strains as those with the implants. The bioassay showed that levels of TGF- β 1 were decreased 48% in the aged

mice (6.6 ± 1.7 ng/ml; $n = 11$) in comparison with the young mice (12.6 ± 2.3 ng/ml; $n = 9$) ($P = 0.05$, old relative to young) (Figure 6). Levels of TGF- β 1 were also measured in a matched group of mice that had undergone excisional wounds 3 and 7 days before sacrifice. During wound healing, aged mice showed increased levels of TGF- β 1 in their sera but had decreased levels at all time points relative to values derived from young mice (data not shown).

Expression of Type I Collagen mRNA

Type I collagen is thought to facilitate invasion of blood vessels.⁴⁴ During aging, the synthesis of type I collagen by fibroblasts is impaired in skin and other tissues.^{50–51} *In situ* hybridization with an antisense probe for type I collagen in the sponge implants indicated a delay in the synthesis of type I collagen in the aged mice (Figure 7, D to F) relative to that observed for young mice (Figure 7, A to C). In this study, the highest levels of transcripts were localized to areas of dense fibrovascular invasion. In comparison with young mice (Figure 7, A and B), there was decreased expression at 14 days in aged mice (Figure 7, D and E). By 21 days, levels of transcripts were similar in the two groups (Figure 7, C and F).

Type I Collagen Protein is Decreased in Aged Mice

Immunoblotting was performed to determine whether the synthesis of type I collagen protein was decreased in

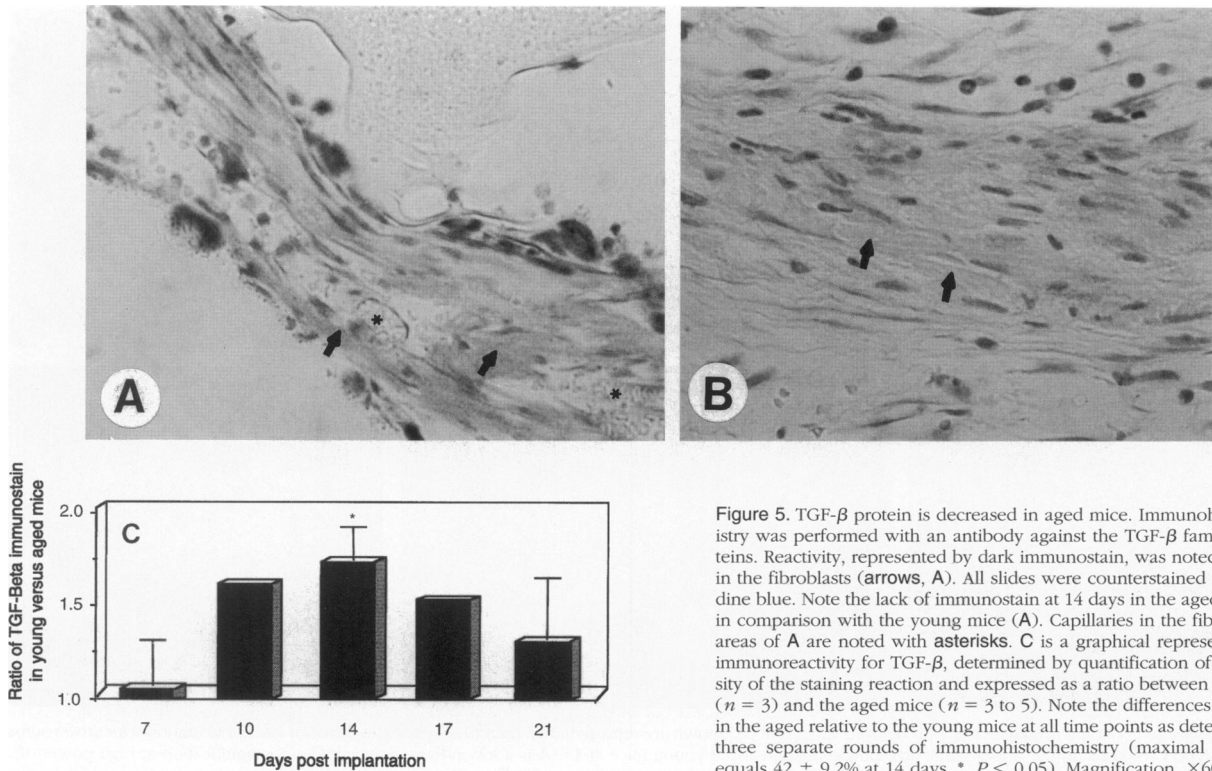


Figure 5. TGF- β protein is decreased in aged mice. Immunohistochemistry was performed with an antibody against the TGF- β family of proteins. Reactivity, represented by dark immunostain, was noted primarily in the fibroblasts (arrows, A). All slides were counterstained with toluidine blue. Note the lack of immunostain at 14 days in the aged mice (B) in comparison with the young mice (A). Capillaries in the fibrovascular areas of A are noted with asterisks. C is a graphical representation of immunoreactivity for TGF- β , determined by quantification of the intensity of the staining reaction and expressed as a ratio between the young ($n = 3$) and the aged mice ($n = 3$ to 5). Note the differences in protein in the aged relative to the young mice at all time points as determined by three separate rounds of immunohistochemistry (maximal difference equals $42 \pm 9.2\%$ at 14 days, *, $P < 0.05$). Magnification, $\times 600$.

aged mice relative to young mice. The antibody recognized bands representing the $\alpha 1(I)$ and $\alpha 2(I)$ chains of type I collagen. As shown by the representative Western blot performed on protein from pooled samples of 7-, 10-, 14-, 17-, and 21-day-old implants (Figure 8A), there was less $\alpha 1(I)$ chain in aged mice ($n = 3$ at each of the time points) relative to young mice ($n = 3$ at each of the time points) during the phase of maximal neovascularization

(14 days in young mice). Figure 8B shows results from densitometric scans of the $\alpha 1(I)$ chain identified from the various samples. At 7 and 10 days, the amount of protein was low in both age groups, and the differences were not statistically significant. The difference between the groups was maximal (47%, nearly twofold) at 14 days, was decreased at 17 days, and was not significant at 21 days.

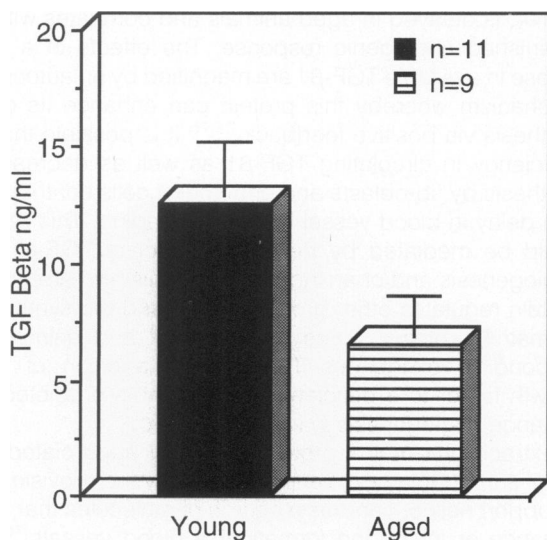


Figure 6. Serum levels of active TGF- $\beta 1$ are decreased in aged mice. Shown is a graphical representation of the amount of TGF- $\beta 1$ in the serum of the young and aged mice as determined by a growth-inhibition assay. Note the lower levels of TGF- $\beta 1$ in the aged mice (6.6 ± 1.7 ng/ml; $n = 11$) in comparison with the young mice (12.6 ± 2.3 ng/ml; $n = 9$) ($P = 0.05$).

Discussion

The formation of blood vessels requires complex interactions between cells and components of the extracellular environment, such as growth factors and matrix proteins.^{62,63} In this and prior studies we have found that the delay in angiogenesis associated with aging is characterized by normal vessels that appear later than those in the young animal.^{6,9} At late time points, the aged animals show the same amount of angiogenesis as their younger counterparts. We examined the expression of mRNA and protein for the growth factor TGF- $\beta 1$ and the extracellular matrix protein type I collagen in the PVA sponge model of angiogenesis in young and aged mice. The PVA sponge provides an inert substrate for the slow invasion of fibrovascular bundles into an isolated field with minimal inflammation. This model permits the study of the temporal and spatial expression of extracellular matrix proteins and growth factors associated with the angiogenic response during wound repair.⁵⁵ We have found that neovascularization in the sponges occurs in areas of dense invasion that consist of fibroblasts with numerous capil-

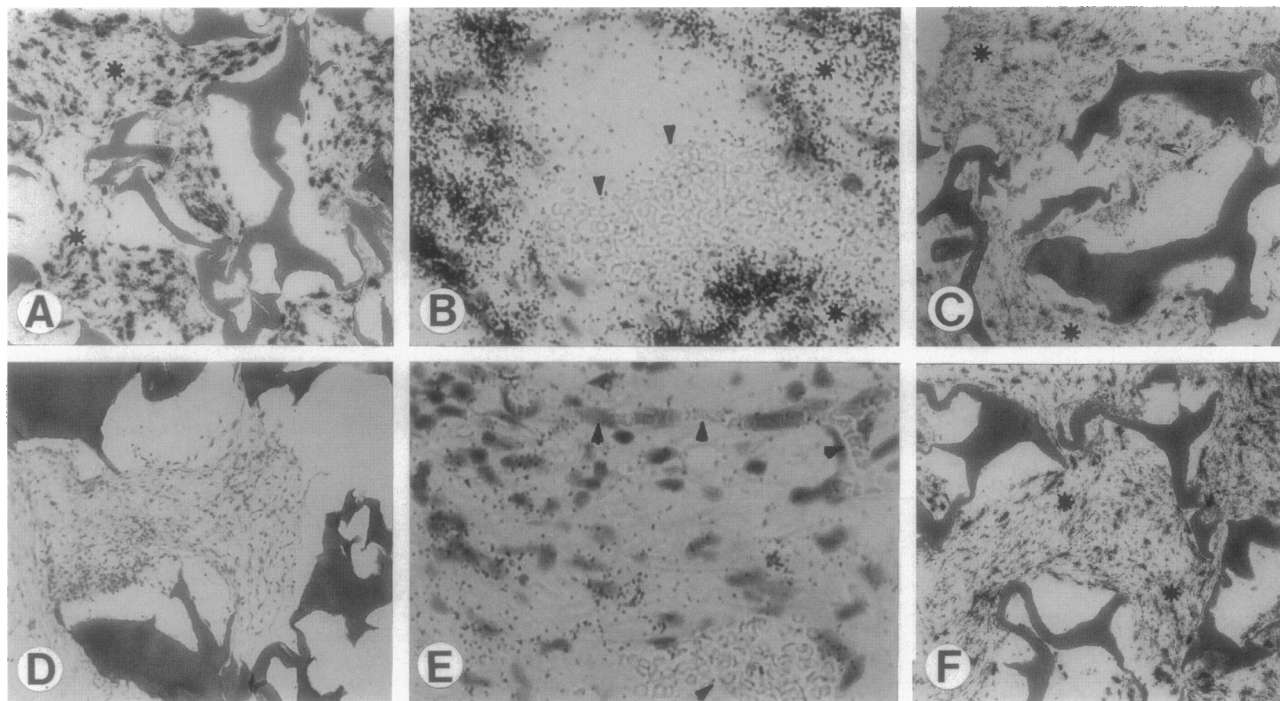


Figure 7. Expression of type I collagen mRNA is delayed in aged mice. Shown are representative brightfield photographs of *in situ* hybridization with an antisense probe for type I collagen. In the upper panel are sponge implants from young mice at 14 (A is a low power view and B is a magnification at high power of a similar region) and 21 days (C). The lower panels show sponge implants from aged mice at 14 (D is a low power view and E is a magnification at high power of a similar region) and 21 days (F). Transcripts for type I collagen are apparent throughout areas of fibrovascular invasion (asterisks, A through C, F). There was increased expression at 14 days in the young (A and B) compared with the aged mice (D and E). By 21 days (C and F), levels of transcripts for type I collagen were similar in the two age groups. Magnification, $\times 100$ (A, C, D, and F); $\times 600$ (B and E).

laries. In these regions, there is a delay in the expression of TGF- $\beta 1$ and type I collagen, primarily by fibroblasts, that was correlated with decreased neovascularization in the aged mice. Moreover, we have shown that the level of circulating TGF- $\beta 1$ was decreased in aged mice relative to young mice.

Growth factors modulate the proliferation, migration, and invasion of endothelial cells, pericytes, and fibroblasts during the formation of new blood vessels. TGF- $\beta 1$ is a multifunctional growth factor that inhibits endothelial cell proliferation *in vitro*^{64–66} but enhances angiogenesis *in vitro*^{49,67} and *in vivo*.^{22,68} The disparate effect of this growth factor on endothelial cells has been attributed to differences in the relative concentration of TGF- $\beta 1$ and the cytokines induced by TGF- $\beta 1$ (eg, bFGF) during angiogenesis *in vivo*.⁶⁸ Thus, the initial effect of TGF- $\beta 1$ would be the induction of other cytokines that increase endothelial cell proliferation.⁶⁹ In the later stages of vessel formation, TGF- $\beta 1$ is thought to enhance cellular migration and differentiation.⁶⁷ Moreover, TGF- $\beta 1$ modulates the synthesis and breakdown of the extracellular matrix through its stimulation of the synthesis of type I collagen, SPARC, and fibronectin, concomitant with its inhibition of interstitial collagenase production.^{33,70,71} In an earlier study, we showed that the addition of TGF- $\beta 1$ enhanced wound repair in aged animals by the stimulation of the growth of new vessels, cell proliferation, and biosynthesis of matrix components.⁹ The source of this essentially ubiquitous growth factor is the extracellular environment, as well as synthesis *de novo* by platelets,

endothelial cells, macrophages, lymphocytes, and fibroblast-like cells (for reviews see Ref. 72 and 73). The present study indicates that the amount of active TGF- $\beta 1$ in the circulation of aged animals is decreased. Therefore, conceivably less protein might accumulate in tissues undergoing neovascularization. In addition, synthesis of TGF- $\beta 1$, measured by the expression of TGF- $\beta 1$ mRNA, is delayed in aged animals and correlates with a diminished angiogenic response. The effects of a decrease in available TGF- $\beta 1$ are magnified by an autocrine mechanism whereby this protein can enhance its own synthesis via positive feedback.^{74,75} It is possible that a deficiency in circulating TGF- $\beta 1$ as well as decreased synthesis by fibroblasts and endothelial cells contributes to a delay in blood vessel formation in aging. This result could be mediated by the direct effect of TGF- $\beta 1$ on angiogenesis and/or an indirect mechanism whereby the protein regulates other growth factors and the synthesis of matrix proteins. Since aged cells³³ and animals^{5,9} respond to exogenous TGF- $\beta 1$, the addition of this growth factor to a deficient environment is predicted to enhance angiogenesis *in vitro* and *in vivo*.

Extracellular matrix proteins are now appreciated as significant regulators of angiogenesis by the provision of a support network and as a source of molecules that can enhance or inhibit the formation of blood vessels.^{76–78} Type I collagen has been shown to provide a lattice that enhances branching and proliferation of endothelial cells.⁴⁴ The production of type I collagen by fibroblasts is decreased with aging.^{50–51} This deficit was noted in our

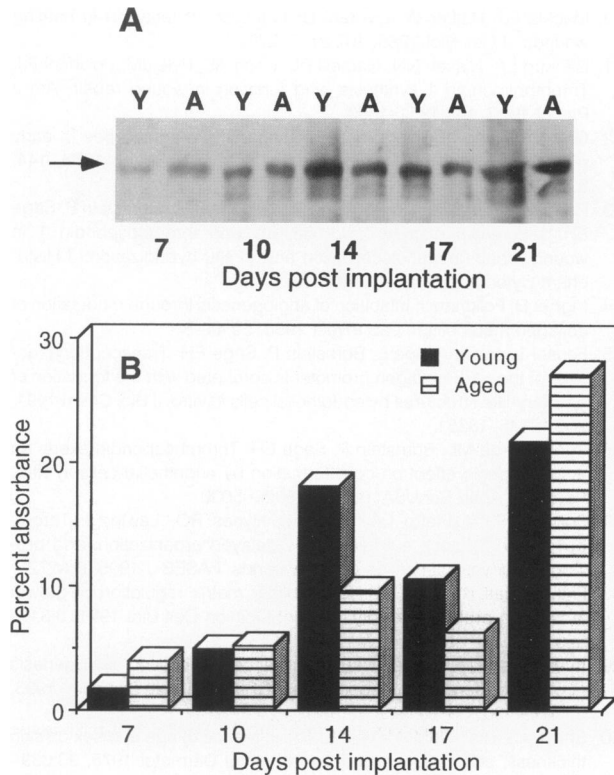


Figure 8. Type I collagen protein is decreased in aged mice. In **A** is shown a representative Western blot performed with protein from pooled samples of 7-day- (lanes 1 and 2), 10-day- (lanes 3 and 4), 14-day- (lanes 5 and 6), 17-day-old (lanes 7 and 8), 21-day- (lanes 9 and 10)-old implants in young (lanes 1, 3, 5, 7, and 9) ($n = 3$ at each time point) and aged mice (lanes 2, 4, 6, 8, and 10) ($n = 3$ at each time point). Each lane represents 20 μ g of the total protein recovered from the implant. A band representing the $\alpha 1(I)$ chain of type I collagen is denoted by the arrow. **B** is a graphical representation of densitometric scanning of 2 separate autoradiograms on which the chains of type I collagen were resolved. Differences in values from separate scans did not exceed 10%. At 7 and 10 days, the amount of protein was low in both age groups and the differences were not significant. The difference between the groups was twofold at 14 days, was decreased at 17 days, and was not significant at 21 days.

aged mice and, like the expression of TGF- $\beta 1$, was correlated with the delay of angiogenesis into the PVA sponge. It is likely that a lack of synthesis of type I collagen by fibroblasts during the proliferation and branching of endothelial cells contributes to delayed angiogenesis and to impaired wound repair. TGF- $\beta 1$ increases the amount of type I collagen produced by fibroblasts from aged humans and rodents^{9,33} and thereby also enhances neovascularization indirectly.

In this paper we examined angiogenesis and the expression of TGF- $\beta 1$ and type I collagen in aged mice relative to young mice during fibrovascular invasion into implanted PVA sponges. We demonstrate that, although the morphology was similar, maximal levels of angiogenesis and the coincident expression of TGF- $\beta 1$ and type I collagen were delayed significantly in the aged mice. Moreover, implants from the later time points showed a comparable degree of fibrovascular invasion and expression of both proteins between the aged and young mice. We also found that the levels of circulating TGF- β were decreased significantly in normal aged mice relative to

young mice. We propose that decreases in the synthesis of growth factors and matrix proteins contribute to the delay in angiogenesis associated with aging. Additional studies will determine whether replacement of a growth factor (eg, TGF- $\beta 1$) or modulation of a matrix or matricellular protein (eg, type I collagen) will enhance angiogenesis in aged cells and animals. In particular, we plan to examine the response of isolated microvessels from aged rodents to specific growth factors. The modulation of growth factors and extracellular matrix proteins that regulate the formation of blood vessels has important implications for the treatment of delayed wound repair with aging.

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