Age-Dependent Impairment of Angiogenesis

Alain Rivard, MD; Jean-Etienne Fabre, MD; Marcy Silver, BS; Dongfen Chen, MD; Toyoaki Murohara, MD; Marianne Kearney, BS; Meredith Magner, BS; Takayuki Asahara, MD; Jeffrey M. Isner, MD

Background—The effect of aging on angiogenesis in ischemic vascular disease has not been studied. Accordingly, we investigated the hypothesis that angiogenesis is impaired as a function of age.

Methods and Results—Forty days after the resection of 1 femoral artery, collateral vessel development was significantly impaired in old (aged 4 to 5 years; n = 7) versus young (aged 6 to 8 months; n = 6) New Zealand White (NZW) rabbits on the basis of reduced hindlimb perfusion (ischemic: normal blood pressure ratio = 0.58 ± 0.05 versus 0.77 ± 0.06; P < 0.005), reduced number of angiographically visible vessels (angiographic score = 0.48 ± 0.05 versus 0.70 ± 0.05; P < 0.01), and lower capillary density in the ischemic limb (130.3 ± 5.8/mm² versus 171.4 ± 9.5/mm²; P < 0.001). Angiogenesis was also impaired in old (aged 2 years) versus young (aged 12 weeks) mice as shown by reduced hindlimb perfusion (measured by laser Doppler imaging) and lower capillary density (353.0 ± 14.3/mm² versus 713.3 ± 63.4/mm²; P < 0.01). Impaired angiogenesis in old animals was the result of impaired endothelial function (lower basal NO release and decreased vasodilation in response to acetylcholine) and a lower expression of vascular endothelial growth factor (VEGF) in ischemic tissues (by Northern blot, Western blot, and immunohistochemistry). When recombinant VEGF protein was administered to young and old rabbits, both groups exhibited a significant and similar increase in blood pressure ratio, angiographic score, and capillary density.

Conclusions—Angiogenesis responsible for collateral development in limb ischemia is impaired with aging; responsible mechanisms include age-related endothelial dysfunction and reduced VEGF expression. Advanced age, however, does not preclude augmentation of collateral vessel development in response to exogenous angiogenic cytokines. (Circulation. 1999;99:111-120.)

Key Words: growth substances ■ endothelium ■ aging ■ angiogenesis

Advanced age is a major risk factor for coronary and peripheral artery disease.¹ When vascular obstructions in either circulatory system are so extensive that direct revascularization techniques cannot be undertaken successfully, the severity of residual ischemia will depend in large part on the ability of the organism to spontaneously develop new collateral blood vessels. To the best of our knowledge, no study has previously evaluated the effect of aging on angiogenesis in ischemic vascular diseases. Moreover, the mechanisms by which aging could limit the formation of new blood vessels remain largely undefined.

Recent studies have demonstrated that angiogenesis, facilitated via administration of angiogenic growth factors as in recombinant protein therapy²−⁷ or gene transfer,⁸−¹⁰ may be augmented in animal models of myocardial and limb ischemia. The impact of aging in these experimental models, however, was not tested. This issue may have important implications for the utility of such therapeutic strategies in older patients who indeed represent the population subset most likely to benefit from such therapies. Accordingly, the present study was designed to investigate the hypothesis that angiogenesis is impaired as a function of age and, if confirmed, to identify potentially contributory mechanisms.

Methods

Animal Models

All protocols were approved by St. Elizabeth’s Institutional Animal Care and Use Committee. The development of angiogenesis in response to regional ischemia was investigated in 2 animal models.

Rabbit Ischemic Hindlimb Model

The first animal model involved young (aged 6 to 8 months) versus old (aged 4 to 5 years) New Zealand White (NZW) rabbits in which operative intervention was performed to establish unilateral hindlimb ischemia. The maximum age for NZW rabbits has been previously reported to be 7 years.¹¹

Surgery

Male NZW rabbits (weight, 3.6 to 4 kg) were anesthetized with a mixture of ketamine (50 mg/kg) and acepromazine (0.8 mg/kg) after...
premedication with xylazine (2 mg/kg). Young (n = 6) and old (n = 7) rabbits underwent operative resection of 1 femoral artery as previously described.2,3 In order that these groups could also serve as a basis for comparison with animals receiving exogenous cytokine therapy, all 13 rabbits received saline with 0.1% rabbit serum albumin (Sigma Chemical Co) administered via an intra-arterial route (vide infra) on postoperative day 10.

Administration of Supplemental Angiogenic Cytokines
An additional 14 NZW rabbits (7 young and 7 old) received recombinant human vascular endothelial growth factor (VEGF) (500 μg of rhVEGF(165)) in the proximal segment of the internal iliac artery of the ischemic limb as described previously2 on postoperative day 10.

Hindlimb Perfusion Pressure
Blood pressure (BP) was measured as previously described.2 The ratio of ischemic to normal hindlimb BP (BPR) was defined for each rabbit as the ratio of systolic pressure measured in the ischemic limb to systolic pressure measured in the normal limb.

Angiography
Selective angiography of the ischemic hindlimb was performed on days 10 and 40 after surgery as previously described.2,3,9 The luminal diameter of the internal iliac artery was measured with a validated automated edge-detection system (CatView, Imagemac). To quantitatively assess collateral vessel development, we used an acetate overlay with an imprinted grid composed of 2.5-mm-diameter circles arranged in rows spaced 5 mm apart to yield an angioscore as previously described.2

Capillary Density
Tissue specimens obtained as transverse sections from the adductor and semimembranous muscle groups of both limbs of each rabbit at the time of death (day 40) were embedded in OCT compound (Miles) and snap-frozen in liquid nitrogen. Tissue sections were stained for alkaline phosphatase by an indoxyl-tetrazolium method to detect capillary endothelial cells and were then counterstained with eosin.

Measurement of Nitrite Production From Rabbit Aortas Ex Vivo
Aortas were rapidly isolated after extensive washing with saline, with care being taken to preserve the endothelium intact. The retrieved aortic segments were immersed in oxygenated Krebs buffer. After 5 minutes of equilibration, Krebs buffer (10 mL) was replaced and incubated for an additional 10 minutes. Nitrite concentration was measured by Griess reaction as described previously12 and was expressed as picomoles per square millimeter of endothelial surface area.

Murine Ischemic Hindlimb Model
Surgery
Unilateral hindlimb ischemia was created in C57BL/6 female mice13,14 that were 12 weeks (young) or 2 years (old) of age. The animals were anesthetized with pentobarbital (160 mg/kg IP), after which an incision was performed in the skin overlying the middle portion of the left hindlimb. After ligation of the proximal end of the femoral artery, the distal portion of the saphenous artery was ligated, and the artery and all side branches were dissected free and excised. The skin was closed with a surgical stapler.

Monitoring of Hindlimb Blood Flow
Hindlimb perfusion was measured with a laser Doppler perfusion imager (LDPI) system (Lisca Inc.). After anesthesia, consecutive measurements were obtained after scanning of the same region of interest (leg and foot) with the LDPI. The perfusion signal was split into 6 different intervals, each displayed in a separate color. Low or no perfusion was displayed in dark blue, whereas the highest perfusion interval was displayed in red. The stored perfusion values behind the color-coded pixels were then available for analysis. To account for variables such as ambient light and temperature, the results are expressed as the ratio of perfusion in the left (ischemic) versus right (normal) limb.

Tissue Preparation
The mice were killed at predetermined arbitrary time points after surgery with an overdose of sodium pentobarbital. For immunohistochemistry, whole ischemic and nonischemic limbs were immediately fixed in methanol overnight. After bones had been carefully removed, 3-μm-thick tissue sections were cut and paraffin-embedded. For total protein and RNA extraction, isolated tissue samples were rinsed in PBS to remove excess blood, snap-frozen in liquid nitrogen, and stored at −80°C until use.

Immunohistochemistry
Histological sections (5 μm thick) prepared from paraffin-embedded tissue samples of the lower limbs were used for immunohistochemical analysis. Identification of endothelial cells was performed by immunohistochemical staining for platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) with a rat monoclonal antibody directed against mouse CD31 (Pharmaningen). Identification and localization of T lymphocytes in tissues were performed by immunohistochemical staining for CD3 (a pan T-cell surface marker) with a polyclonal anti-human CD3 antibody (Sigma). Immunohistochemical localization of VEGF was performed with a rabbit polyclonal antibody directed against human VEGF amino-terminal peptides 1 through 20 (Santa Cruz Biotechnology) that cross-reacts with murine VEGF. Immunoperoxidase staining was performed as previously described.14,15

Analysis of Capillary Density
Capillaries, identified by positive staining for CD31 and appropriate morphology, were counted by a single observer blinded to the treatment regimen under a 20× objective and a 5× lens to determine the capillary density (mean number of capillaries per square millimeter).3 A total of 20 different fields from the 2 muscles were randomly selected, and the number of capillaries was counted for each field.

Quantification of T Lymphocytes in Ischemic Tissues
The number of CD3-positive cells was counted by a single observer under a 20× objective and a 5× lens. A minimum of 10 different fields from the ischemic tissues of mice were randomly selected, and the number of T lymphocytes was counted for each field. The results are expressed as the average of T lymphocytes per high-power field.

Northern Blot Analysis of VEGF mRNA Expression
Total tissue RNA was isolated from ischemic hindlimb muscles of mice by phenol/chloroform extraction.16 Twenty micrograms of RNA per lane was separated by electrophoresis on 1% agarose gel containing formaldehyde and transferred to a nylon membrane (Hybond-N, Amersham) by blotting. The membrane was hybridized with 32P-labeled probe specific for VEGF, a 675-bp EcoRI/BgII fragment of plasmid pSV1.VEGF.21 Hybridization was performed as previously described.16

Western Blot Analysis of VEGF Protein Expression
Whole-cell protein extracts were obtained after homogenization of ischemic and control muscles of both young and old animals. A total of 200 μg of protein per sample was separated on a 12% polyacrylamide gel and electroblotted on nitrocellulose membranes.18 The membrane was blocked with 10% nonfat dry milk in 0.2% Tween PBS (T-PBS) and then probed with 1:250 of rabbit polyclonal anti-human VEGF antibody (Sigma) for 3 hours at room temperature. After incubation with primary antibody, the blot was washed three times in T-PBS and was then incubated for 1 hour with 1:4000 of anti-rabbit horseradish peroxidase IgG (Santa Cruz Biotechnology). The blot was then washed in T-PBS, and antigen-antibody complexes were visualized after incubation for 1 minute with enhanced luminescence reagent (Amersham) at room temperature, followed by exposure to Kodak XAR-5 film.
T-Cell Fluorescent-Activated Cell Sorting Analysis

Fluorescent-activated cell sorting (FACS) analysis was performed on a FACScan (Becton-Dickinson); >5000 cells were analyzed per sample. Mouse blood was obtained by intracardiac puncture and placed in EDTA, and buffy coats were separated over Histopaque (Sigma). The cells were double-stained with phycoerythrin anti-mouse CD3 (Pharmingen). The cells were washed again and fixed with paraformaldehyde (1%). The absolute number of T-cells was calculated from the percentage of CD3 positive cells of each subset multiplied by the total number of leukocytes.

VEGF Promoter Activity

Vascular smooth muscle cells (VSMCs) were isolated from the aortas of young and old rabbits, seeded into 6-well plates, and maintained in DMEM supplemented with 10% FBS. The next day, cells (60% to 80% confluence) were transiently transfected with 10 μg of a reporter construct containing the luciferase gene under the transcriptional control of the VEGF promoter and 30 μg of Lipofectamine reagent (GIBCO Laboratories). To correct for differences in transfection efficiency, luciferase activity was normalized relative to the level of alkaline phosphatase activity produced from cotransfected pSVAPAP plasmid (0.5 μg), which contains the reporter gene under the control of the simian virus 40 enhancer-promoter. The cells were incubated with the transfection mixture for 3 hours and then were fed with low-serum (0.25% FBS) or high-serum (10% FBS) DMEM. After 24 hours, luciferase and alkaline phosphatase activities were measured in old and young VSMCs. Results are expressed as the ratio of luciferase to alkaline phosphatase activities.

Statistical Analysis

All results are expressed as mean ± SEM. Statistical significance was evaluated by ANOVA or 2-tailed unpaired Student’s t test for comparisons between the mean of 2 groups. A value of P<0.05 was interpreted to denote statistical significance.

Results

Postoperative Follow-Up

The consequences of hindlimb ischemia were more profound in the old animals. This was especially apparent in the old mice; severe necrosis of the distal part of the ischemic limb was noted in 5 of the 6 mice studied (Figure 1). In comparison, none of the 9 young mice studied developed limb necrosis.

Analysis of Native Angiogenesis

As shown in Figure 2, calf BPR was similar in both old (n=7) and young (n=6) rabbits at day 10 postoperatively. By day 40, however, BPR improvement was significantly greater in young than in old rabbits (0.77±0.06 versus 0.58±0.05; P=0.02). Both young and old rabbits had a significant and similar increase in BPR when treated with rhVEGF165 protein. In young treated rabbits, BPR increased from 0.77±0.06 to 0.92±0.04 (P=0.03). In old rabbits, BPR increased from 0.58±0.05 to 0.75±0.05 (P=0.03). The ultimate level of BPR achieved by the old rabbits treated with rhVEGF165 protein was significantly lower than that of the young treated rabbits (0.75±0.05 versus 0.92±0.04; P=0.02).

Perfusion of the ischemic hindlimb in mice assessed by laser Doppler measurement (Figure 3) was also reduced in old (n=6) compared with young (n=9) mice. At day 7 after surgery, the Doppler flow ratio was significantly reduced in old mice (0.1±0.02 versus 0.26±0.04; P=0.014), and this difference was exacerbated at day 28 after surgery (0.23±0.03 versus 0.65±0.06; P=0.0005). This severe impairment of blood flow caused necrosis and autoamputation of the ischemic foot in old mice.

The number of angiographically visible collateral vessels (angiographic score) was markedly reduced in old versus young rabbits (Figure 4). At day 10, angiographic scores were similarly low for both groups (P=NS). At day 40, however, angiography disclosed significantly fewer collateral vessels in the medial thigh area of old compared with young NZW mice.
also reduced in old versus young mice (353.0±14.3/mm² versus 713.3±63.4/mm²; *P<0.01) at 28 days after surgery (Figure 5E, 5F, and 5H). Supplemental rhVEGF165 induced a significant (*P<0.001) increase in capillary density in both old and young rabbits (Figure 5C, 5D, and 5G) (191.4±7.8 and 282.7±5.0/mm², respectively, compared with 130.3±5.8/ mm² and 171.4±9.5/mm² in the untreated groups). There was no statistically significant difference (*P=NS) between old and young rabbits in the magnitude of improvement observed for these end points after rhVEGF165 treatment. However, the ultimate level of capillary density achieved in the ischemic hindlimbs of VEGF treated animals was still lower in the old rabbits than in the young rabbits (*P<0.001).

**Vasomotor Reactivity**
Because endothelial cells constitute the principal cellular element responsible for neovascularization,10 we considered that dysfunctional endothelial cells could represent a putative basis for age-dependent angiogenesis. To assess the integrity of endothelial function in vivo, the magnitude of vasorelaxation induced by the endothelium-dependent agonist acetylcholine was determined by angiography in untreated rabbits. As shown in Figure 6A, vasorelaxation induced by acetylcholine was significantly (*P<0.05) reduced in old versus young NZW rabbits (3.9±0.13% versus 8.8±1.8%). That this was not due simply to a generic reduction in vasomotor responsivity was demonstrated by equivalent vasorelaxation in response to nitroprusside for both groups of rabbits (4.1±3.8% versus 3.1±2.1%; *P=NS).

**Measurement of NO**
To further characterize the extent of endothelial dysfunction in old versus young rabbits, we measured NO production from freshly isolated aortic rings. In aortic rings from old rabbits, NO production was significantly reduced compared with that in young rabbits (Figure 6B), with nitrite values of 31.6±3.6 versus 158.4±54.8 pmol/mm², respectively (*P<0.03).

**VEGF mRNA Expression**
Expression of VEGF mRNA in ischemic tissues was markedly reduced in old versus young mice. The difference in mRNA levels between the 2 groups was especially apparent at day 7 and day 14 after development of limb ischemia, as shown on the Northern blot in Figure 7.

**VEGF Protein Expression in Tissues From Ischemic and Control Limbs**
Endogenous expression of VEGF protein was determined for old and young animals by Western blot analysis of protein extracts obtained from muscles of both the ischemic and normal limbs harvested at different time points after surgery. Figure 8A shows the expression of VEGF protein in young and old rabbits in ischemic muscles harvested 7 days after hindlimb surgery. The level of VEGF protein was significantly reduced in old versus young rabbits. Similar results were obtained in old and young mice: the upper panel of Figure 8B shows that basal expression of VEGF is low in the nonischemic limbs of unoperated mice. There were no rabbits (angioscore of 0.48±0.05 versus 0.70±0.05; *P=0.008). Treatment with rhVEGF165 protein resulted in a significant and similar increase in the number of angiographically visible collaterals in both young and old rabbits (Figure 4F). The angioscore for young animals treated with rh-VEGF165 (0.91±0.08) was significantly higher than the corresponding value (0.70±0.05) obtained in untreated animals (*P=0.03). In old treated rabbits, the angioscore (0.69±0.04) was also significantly higher than that recorded for the untreated group (0.48±0.05; *P=0.005). The ultimate magnitude of angiographically visible collaterals observed in the old treated rabbits, however, remained inferior to that of the young treated rabbits (0.69±0.04 versus 0.91±0.08; *P=0.014).

Tissue sections from the medial thigh muscles of rabbits were examined histologically at day 40 as described above. As shown in Figure 5A and 5B, capillary density was significantly lower in old NZW rabbits (130.3±5.8/mm²) than in young rabbits (171.4±9.5/mm²; *P<0.001). Likewise, capillary density as assessed by CD31 immunostaining was
significant differences in basal expression of VEGF in young versus old mice. The lower panel of Figure 8B shows the time course of VEGF expression in young and old mice after operative induction of hindlimb ischemia. VEGF was upregulated as early as day 3 after surgery, reached a maximum by day 7, and decreased thereafter. The level of VEGF protein was significantly reduced in old versus young mice for all time points studied. Immunostaining confirmed the results of the Western blot by showing a lower level of VEGF expression in the tissues retrieved from old versus young mice at day 7 after surgery (Figure 8C). Tissue immunohistochemistry further established that the cell types responsible for VEGF expression included skeletal myocytes and T lymphocytes infiltrating the ischemic tissues (Figure 9).

**T Cells in Ischemic Tissues**

Immunostaining for CD3 revealed a lower number of infiltrating T cells (3.3±0.2 versus 11.7±1.8 per high-power field, \( P<0.05 \)) in old mice than in young mice (Figure 9). This difference in T-cell infiltrate could not be attributed simply to a reduction in circulating peripheral blood T cells. Although the total number of white blood cells was lower in old animals, FACS analysis performed in 10 unoperated mice (5 young and 5 old) established that the absolute number (as opposed to the percentage) of peripheral blood T cells was similar in both groups (Figure 9D).

**VEGF Promoter Activity**

Using VSMCs isolated from aortas of young or old rabbits, we studied the expression of the VEGF promoter in low- or high-serum conditions. The cells were transfected with a plasmid that contains the firefly luciferase gene under the control of the VEGF promoter. As seen in Figure 10, although the VEGF promoter was induced by serum in both young and old VSMCs, the level of promoter activity was dramatically reduced in old cells, especially in high-serum conditions. This result implies that aging impairs VEGF expression at the transcriptional level.

**Discussion**

The results of the present experiments establish that angiogenesis is impaired as a function of age. The reduced capability for collateral vessel development in response to ischemia was confirmed in 2 different animal models. The ultimate hindlimb BPR achieved at 40 days after surgery was significantly less in old than in young NZW rabbits. In old mice, perfusion of the ischemic hindlimb, reflected by the Doppler flow ratio, was significantly reduced compared with young mice; this difference was apparent as soon as 7 days after surgery and persisted throughout the duration (28 days) of the study. Likewise, the number of blood vessels that were angiographically visible in rabbits and the number of capillaries per unit area identified histologically in mice and...
rabbits were both significantly reduced in old versus young animals. The latter finding is consistent with the observation that myocardial angiogenesis related to left ventricular hypertrophy is attenuated in an age-dependent manner.20

The mechanisms by which aging can affect angiogenesis are potentially diverse. Angiogenesis is a complex process that includes activation, migration, and proliferation of endothelial cells.19 Recent studies21–24 have indicated that the integrity of endothelial cell function may be compromised as a function of advanced age. We confirmed that endothelial function was abnormal in old versus young rabbits by documenting impaired vasodilation in response to the endothelium-dependent vasodilator acetylcholine in vivo and reduced release of NO from isolated blood vessels studied ex

Figure 4. Representative examples of collateral vessel formation in old rabbits (A), old VEGF-treated rabbits (B), young rabbits (C), and young VEGF-treated rabbits (D). The number of angiographically visible collateral vessels (angiographic score) was markedly reduced in old vs young rabbits. E and F, Angiographic scores at 10 and 40 days after surgery. At day 10, angiographic scores were similarly low for all groups. At day 40, however, angiography disclosed significantly fewer collateral vessels in the medial thigh area of old compared with young NZW rabbits. Treatment with rhVEGF165 protein significantly increased the number of collateral vessels in both groups, although the level achieved in young animals was higher than that in old animals.
vivo. Previous in vitro studies 25, 26 have also suggested that aged endothelial cells show impaired proliferation and migration in response to cytokines such as platelet-derived growth factor and fibroblast growth factor. Taken together, these observations support the notion that age-dependent endothelial dysfunction contributes to impaired angiogenesis in the setting of tissue ischemia.

Growth factors, particularly endothelial cell mitogens, represent a second essential element in the promotion and regulation of angiogenesis. Numerous reports suggest that VEGF, an endothelial cell–specific mitogen, is a critical growth factor in therapeutic 2, 3, 6, 7, 9, 10 and pathological 27–29 angiogenesis. In the present study, we demonstrated that the magnitude of VEGF expression in tissues harvested from ischemic limbs of old mice and rabbits was reduced compared with that observed in young animals.

The observed reduction in VEGF expression appears to be at least bifactorial. First, immunostaining of tissue specimens harvested from the murine hindlimbs disclosed less VEGF protein expression in skeletal myocytes from old versus young mice at time they were euthanized (E and F) showed a significant reduction in capillary density in old animals (H).

**Figure 5.** Alkaline phosphatase staining of ischemic muscles from old control and old VEGF-treated rabbits (A and B) and from young untreated and young VEGF-treated rabbits (C and D) 40 days after surgery. Capillary density was significantly lower in old NZW rabbits than in young rabbits. Treatment with rhVEGF 165 induced a similar increase in capillary density in both groups, although the ultimate level achieved in old treated rabbits was lower than that of young treated rabbits (G). CD31 staining of ischemic muscles from old and young mice at time they were euthanized (E and F) showed a significant reduction in capillary density in old animals (H).

**Figure 6.** A, Vasomotor response to acetylcholine and nitroprusside in young and old rabbits. Vasorelaxation induced by acetylcholine was significantly (*P*<0.05) reduced in old vs young NZW rabbits, whereas vasorelaxation induced by nitroprusside was similar in both groups. B, Ex vivo production of nitrite from aortic rings isolated from young and old rabbits. In aortic rings from old animals, NO production was significantly reduced compared with young rabbits.

**Figure 7.** VEGF mRNA expression in old and young mice. Northern blot analysis shows marked reduction in expression of VEGF mRNA in ischemic tissues from old vs young animals. The difference in mRNA level between the 2 groups was especially apparent at day 7 (Y7 vs O7) and at day 14 (Y14 vs O14) after surgery.
young mice. Moreover, the finding that VEGF promoter activity was reduced in old versus young VSMCs suggests that the reduction in VEGF expression observed in old animals is due at least in part to a defect in transcriptional regulation. Second, T lymphocytes, shown immunohistochemically to constitute a source of VEGF protein in young mice, were markedly reduced in tissue sections retrieved from old mice and stained with antibodies to CD3. T cells have previously been shown to constitute a potentially important source of VEGF that contributes to the growth of malignant neoplasms. More recently, studies performed in our own laboratory have shown that the development of hindlimb ischemia in nude mice is quickly followed by limb necrosis and autoamputation (T. Couffinhal, MD, unpublished data, 1996). These findings thus reinforce the potential contribution of T cells to VEGF expression in the setting of tissue ischemia and are consistent with the interpretation that the lower level of T cells detected in the ischemic hindlimbs of old mice may be responsible, at least in part, for the local reduction in VEGF expression. Although conflicting results

**Figure 8.** Expression of VEGF protein. A, Western blot analysis of VEGF protein expression in ischemic muscles harvested 7 days after hindlimb surgery in young (Y) and old (O) rabbits. The level of VEGF protein was significantly reduced in old vs young rabbits. B, Top, Western blot analysis in 2 unoperated (ie, nonischemic) young (Ya and Yb) and old (Oa and Ob) mice. + indicates positive control consisting of 50 ng rhVEGF165. Low basal expression of VEGF was detected in nonischemic muscles. B, Bottom, Western blot time course of VEGF protein expression in ischemic tissues harvested at days 3, 7, and 21 after surgery in old (O3, O7, O21) and young (Y3, Y7, Y21) mice. VEGF was upregulated as early as day 3 after surgery, reached its maximal value at day 7, and decreased thereafter. Level of VEGF protein was significantly reduced in old vs young animals for all time points studied. C, Immunostaining for VEGF in ischemic tissues of young and old mice 7 days after surgery. Immunostaining confirmed results of Western blot analysis, showing a lower level of VEGF expression in ischemic muscles of old vs young animals.

**Figure 9.** Immunostaining for T lymphocytes (CD3) in ischemic tissues of young (A) and old (B) mice. The number of T cells (arrows) infiltrating tissues was reduced in old animals. Lower right inserts in A and B show CD3-positive cells in higher magnification of region indicated by asterisk. Lower left insert in A shows infiltrating T lymphocytes positively stained for VEGF. C, Number of infiltrating T lymphocytes in tissue sections of ischemic limb was markedly reduced in old vs young mice. D, Left, Absolute number of peripheral white blood cells (WBC) was lower in old animals; right, FACS analysis performed in 10 mice (5 young and 5 old) revealed a similar level of peripheral blood T cells in both groups.
have been reported in human studies regarding the effects of age on the level of CD4+ cells, the notion that T-cell immunity is compromised as a function of age is well documented. T-cell proliferation in vitro and in vivo declines with age in both mice and humans, and there is a shift away from naive CD4+ cells toward a relative increase in memory subsets.

The fact that we observed fewer T cells in the ischemic tissues of older mice despite similar peripheral T-cell counts in old and young mice is consistent with the notion of an age-dependent defect in transendothelial migration of T lymphocytes into the target ischemic tissues. Although the precise mechanisms responsible for T-cell migration remain enigmatic, recent reports suggest that a combination of signals is required to trigger the migratory T-cell phenotype and that CD4+ activated T cells are more likely to transmigrate than CD4− cells.

These observations suggest that age-related reduction in activated T cells migrating into the tissues of the ischemic limbs may obviate a source of VEGF that is potentially important to upregulate expression of this angiogenic growth factor in the setting of limb ischemia. This finding may in fact represent a conceptual link to studies performed in mice injected with tumor cells in which old animals were shown to have a slower rate of tumor growth than younger animals; such altered tumor growth was associated with a reduced capacity to vascularize the tumors.

The favorable response to VEGF replacement therapy described above strongly implicates VEGF as the pivotal cytokine deficiency responsible for impaired angiogenesis. This interpretation is consistent with the fact that absence of a single VEGF allele in the developing embryo is sufficient to prohibit vascular development and with the finding that VEGF appears to lie downstream of several, if not all, other angiogenic cytokines.

Involvement of other angiogenic growth factors cannot be excluded on the basis of the data given in the present study. Similarly, the present findings do not exclude the possibility that age-impaired angiogenesis is due in part to upregulated expression of a natural endogenous inhibitor of angiogenesis, although experiments recently performed in our own laboratory have failed to provide evidence that impaired angiogenesis is associated with the candidate inhibitor thrombospondin (C. Kalka, MD, unpublished data, 1997).

The magnitude of improvement in end points used to assess limb perfusion after rhVEGF165 replacement therapy for ischemia was similar for old and young rabbits. This finding suggests that the expression and function of VEGF receptors are preserved in old animals. Indeed, we have found that expression of the principal VEGF receptor KDR, as assessed by Western blotting, was not reduced in normal hindlimbs of old mice compared with young mice (data not shown).

However, the ultimate level of recovery achieved in old animals after rhVEGF165 therapy was still inferior to that observed in young rabbits after identical treatment. This finding suggests that persistent endothelial dysfunction may represent the rate-limiting factor that affects angiogenesis. Additional studies are therefore required to characterize the full complement of responsible mechanisms that might allow optimization of strategies designed to address critical ischemia in the expanding population of elderly individuals.

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