Aging exacerbates neointimal formation, and increases proliferation and reduces susceptibility to apoptosis of vascular smooth muscle cells in mice

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Objectives: **In response to injury, aging mediates exaggerated neointimal formation, the pathologic hallmark of obliterative vascular diseases. We assessed the development of neointima in a model of mechanical vascular injury in aging mice (18 months old) and young mice (2 months old). To investigate the mechanisms by which aging affects neointimal formation, we also carried out a set of in vitro studies to characterize the biologic properties of vascular smooth muscle cells (VSMCs) derived from aging and young mice.**

Methods: **Aging and young mice were subjected to wire injury to the carotid artery. Four weeks later injured arteries were harvested, and neointimal formation was histologically assessed. The profiles of angiogenesis-related genes between aortic VSMCs derived from aging and young mice were compared with complementary DNA arrays. Expression of platelet-derived growth factor receptor-α (PDGFR-α) and proliferation in response to platelet-derived growth factor-BB (PDGF-BB) by VSMCs were assessed. Susceptibility to apoptosis in aging and young VSMCs in response to nitric oxide and serum starvation was investigated. In addition, the level of apoptosis in neointimal VSMCs (by terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling assay) was compared between aging and young animals.**

Results: **When compared with young mice, aging mice exhibited exaggerated neointimal formation (intima-media ratio, 1.17 ± 0.57 vs 0.49 ± 0.16; $P < .0001$). Aging VSMCs expressed higher levels of PDGFR-α (12.0% ± 2.7% vs 3.2 ± 0.67%; $P = .034$) and greater proliferative response (4-fold increase) to PDGF-BB, compared with young VSMCs. However, aging VSMCs were less susceptible to apoptosis when subjected to serum starvation (75% less) and exposure to nitric oxide (50% less). Furthermore, there was more apoptosis in the neointima of young arteries than in their aging counterparts (8.75% ± 3.3% vs 2.8% ± 1.9; $P = .021$).**

Conclusions: **Age-dependent increases in PDGFR-α may alter VSMC proliferation, and when coupled with resistance to apoptosis could contribute to exaggerated neointima formation in aging animals. Of significance, our findings in the mouse will enable application of abundant molecular tools afforded by this species to further dissect the mechanisms of exaggerated neointimal formation associated with aging. (J Vasc Surg 2004;40:1199-1207.)**

Clinical Relevance: **Neointimal formation is the pathologic hallmark of obliterative vascular diseases, including primary atherosclerosis, post stent restenosis, graft occlusion after vascular bypass procedures, and transplant allograft vasculopathy. Aging is an independent risk factor for development of cardiovascular diseases, and aging exaggerates neointimal formation after vascular injury. Understanding the mechanisms responsible for this phenomenon may facilitate prevention or provide new therapies for vascular occlusive diseases, which are so prevalent in the aging population. Our ability to reproduce the model in the mouse will no doubt facilitate such understanding.**

Epidemiologic studies have shown that aging is an independent risk factor for the development of cardiovascular disease.[1] Experimental data also suggest that age predisposes the vascular beds to progressive diseases. Spagnoli et al[2] demonstrated that vascular lesions were more severe in old rabbits than in young rabbits. Similar observations have also been reported in primates.[3] Furthermore, aging rats have more exaggerated neointimal formation after arterial injury than their younger counterparts do.[4] Neointimal formation is the pathologic hallmark of several obliterative vascular diseases, including primary atherosclerosis, post stent restenosis, and allograft vasculopathy. It is generally accepted that vascular injury results in migration, proliferation, and accumulation of vascular smooth muscle cells (VSMCs) in the intima, creating a neointima.[5] Possible phenotypic changes in VSMCs during neointimal formation include increased proliferation,[6-8] decreased susceptibility to apoptosis,[9-11] and exaggerated extracellular matrix deposition.[12-14] Although exaggerated neointimal formation in aging animals is well documented, the underlying mechanisms responsible for this phenomenon are not clearly understood.[4] If the mechanisms are to be studied in depth, mouse models are indispensable, because knockout and transgenic technologies are more readily available in

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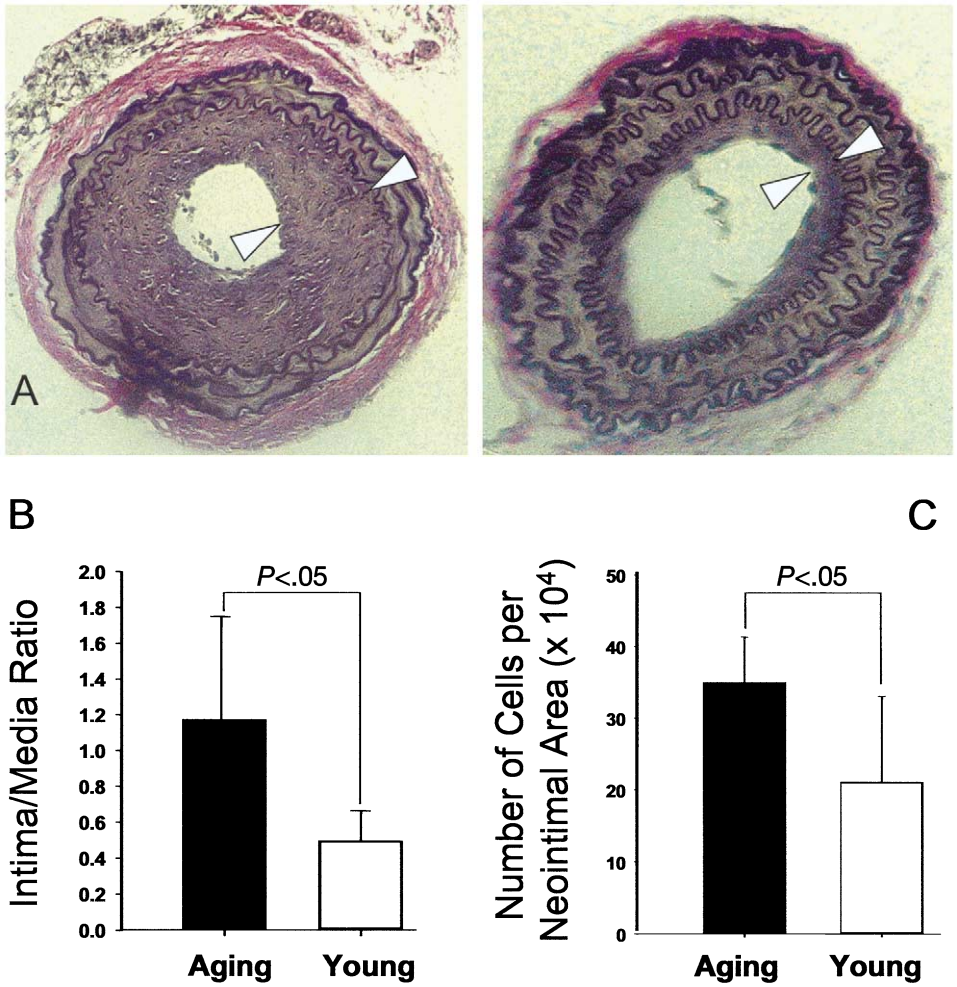

Fig 1. Exaggerated neointima formation in injured aging carotid arteries. **A,** Representative photomicrographs of cross-sections show neointimal development in aging *(left)* and young adult *(right)* mouse carotid arteries subjected to wire injury 4 weeks before harvest (elastica–van Giessen stain; original magnification ×40). **B,** Intima-media ratio assessed with morphometry in carotid arteries from aging (n = 17) and young (n = 6) mice in response to wire injury. The entire neointimal and medial areas were recorded as pixels from digitized pictures. **C,** Neointimas in arteries in aging mice (n = 6) had higher cell density than did arteries in young mice (n = 6), as determined at morphometric analysis.

mice. To our knowledge, the effects of aging on neointimal development or the in vitro cellular correlates of neointimal formation have not been studied in mice.

The purposes of this study were to investigate the effect of aging on neointimal formation in a mouse model of mechanical vascular injury and to examine possible mechanisms responsible for the effect of aging on vascular remodeling.

MATERIAL AND METHODS

In vivo experiments

Animals. Female aging (18 months old) and young adult (2 months old) C57BL/6 mice were obtained from Jackson Laboratories. Preoperatively the mice were housed in the University of Miami animal facility and were fed standard rodent chow ad libitum. After mechanical vascular injury the mice were housed in individual cages. All animals were cared for according to guidelines of the institutional Animal Care and Use Committee.

Mechanical injury model. Mice were anesthetized with isoflurane (IsoFlo; Abbott) inhalation. The external carotid artery was clamped proximally and distally, and a small arteriotomy was made between the clamps to enable passage of a 0.36-mm guide wire (Cordis). The site of arteriotomy in the external carotid artery was 4 mm from the common artery. The tip of the wire was introduced through the arteriotomy and passed proximally 5 times to create endothelial injury. The arteriotomy was then closed with 10-0 polypropylene sutures (Prolene; Ethicon). The contralateral carotid arteries were used as control arteries.

Differentially expressed angiogenesis-related genes in aging vs young vascular smooth muscle cells

Symbol	*Gene name*	*Gene bank*	*A/Y ratio**	P†
Agpt2	Angiopoietin2	NM_009621	1.407614	.014463
Cdh5	Cadherin5	NM_009868	0.465647	.056745
Csf3	G-CSF	M13926	4.756394	.007154
Erb2	Erb-2	U71126	6.814491	.077356
Fgf2	bFGF	M30644	3.554097	.02718
Fgf4	FGF4	M30642	0.252636	.004399
Fgf7	FGF7/KGF	U58503	0.281936	.028598
Fgfr3	FGFR3	M81342	0.115272	.017396
Fgfr4	FGFR4	NM_008011	10.20378	.00939
Igf1	IGF-1	NM_010512	0.13024	.017362
Nos3	NOS3	NM_008713	8.688965	.024276
Pdgfra	PDGFRa	NM_011058	11.43478	.001265
Rsn	Restin	NM_019765	10.66855	.000436

G-CSF, Granulocyte stimulating factor; *bFGF,* basic fibroblast growth factor; *FGF,* fibroblast growth factor; *FGFR,* fibroblast growth factor receptor; *IGF-1,* insulin-like growth factor; *Nos3,* endothelial nitric oxide synthase; *PDGFRa,* platelet-derived growth factor receptor-α.
*Aged vs young expression ratio.
†Calculated with *t* test with unequal variances, and n = 3.

Specimen harvest. At specimen harvesting, perfusion-fixation was performed with 4% paraformaldehyde in phosphate-buffered saline solution (PBS) at 100 mm Hg for 15 minutes, followed by en bloc excision of arteries. Specimens were fixed overnight in 4% paraformaldehyde in PBS. The following day the common carotid arteries were dissected from the specimen, and 2 segments 5 mm in length were cut from the artery and processed for paraffin embedding.

Assessment of neointimal proliferation. Histologic sections 4 to 5 micrometers thick were cut from both segments, corresponding to the midportion of each paraffin bloc, and stained with either elastica von-Giessen (EVG) or hematoxylin-eosin (American HistoLabs) stain. EVG enabled optimal delineation of the inside edge of the medial layer as well as the neointima. Images from slides stained with hematoxylin-eosin and with EVG were recorded with a digital charge-coupled device video camera on an Olympus BH-2 light microscope (Olympus America). Images were processed with PhotoShop version 5.0 software (Adobe System) color recognition, picture element dissection, and histogram determination properties. The degree of neointimal formation was calculated as total neointimal area to total medial area on full rings of EVG-stained cross-section pictures.

Culture of VSMCs. Cells were harvested and cultured from the thoracic aortas of young and aging adult mice as described.[15] Animals were anesthetized and exsanguinated, and a 1-cm length of thoracic aorta was digested with culture medium (Dulbecco minimum essential medium [DMEM] supplemented with F12 and fetal bovine serum [FBS], 60:30:10) containing 2 mg/mL of collagenase for 2 hours. The tissue was then removed and cut into 1 × 1-mm pieces, and cultured in 6-well plates. When confluent areas of cell growth could be seen at inverted light microscopy, cells were trypsinized, expanded, and frozen in liquid nitrogen for further experiments. SMCs were identified at immunostaining with anti–smooth muscle actin monoclonal antibody clone 1A4 (Sigma). Confirmatory Western blot analysis was also performed for anti–smooth muscle alpha actin and heavy chain myosin production by the cultured cells.

Microarray experiments

Using mouse angiogenesis GE array Q series (MM-009; SuperArray), we compared aging versus young VSMCs with respect to expression of 96 genes involved in modulating biologic processes of angiogenesis. For these experiments total RNA was extracted from cells with TriReagent (MRC). Integrity of RNA was assessed with visualization of ethidium bromide–stained gels. The microarrays were used according to the manufacturer's instructions. In brief, complementary DNA was prepared from total RNA with reverse transcription with MMLV reverse transcriptase, radiolabeled with phosphorus 32–deoxycytidine triphosphate; 3000 Ci/mmol/L; Amersham Pharmacia Biotech), then hybridized to a positively charged nylon membrane containing the arrayed DNA. After washing, the arrays were visualized at autoradiography. Gene expression was quantitated with scanning densitometry. Loading was adjusted on the basis of intensity of hybridization signals to the housekeeping genes. Each experiment was performed at least twice, with 2 independent cell lines per group, to ensure reproducibility of results.

Semiquantitative reverse transcriptase polymerase chain reaction. Reverse transcription was performed on 2 μg of total RNA with oligo$(dT)_{15}$ primer and MMLV reverse transcriptase (Roche). Primers 5′GCTATCAGGAGCATCCG3′ (forward) and 5′CCCAAAGCGACATCAG3′ (reverse) were used to amplify a 407 base pair of PDGFR-α messenger RNA (mRNA). α-Actin was used as a housekeeping gene, because its expression remained stable in the young and aging mouse VSMCs. The amplified samples were separated on 3% agarose gel, and were analyzed with densitometry (NIH Image software). Data were expressed as relative mRNA concentrations determined by

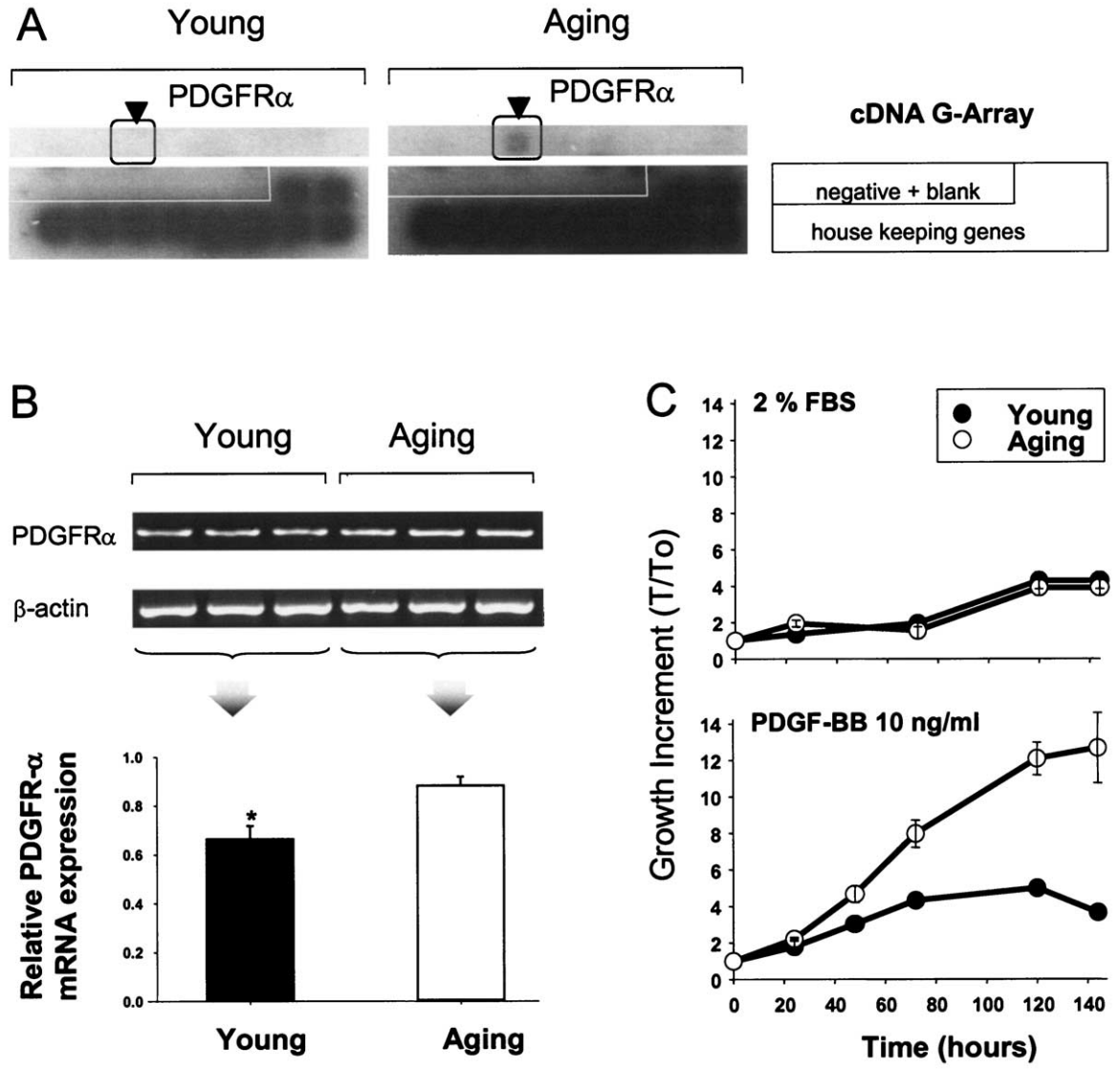

Fig 2. Upregulation of PDGFR-α gene expression in vascular smooth muscle cells (VSMCs) in aging mice. **A,** Aging increases PDGFR-α in VSMCs as shown on representative complementary DNA arrays. **B,** Reverse transcriptase polymerase chain reaction confirmation of messenger RNA levels for PDFGR-α in 3 aging and young individual VSMC lines. $*P < .05$. **C,** Aging VSMCs respond more readily to PDGF-BB (10 ng/mL) than their younger counterparts do *(lower panel)*. In contrast, aging and young VSMCs proliferate at the same rate in control media (ie, supplemented with 2% fetal calf serum only; *upper panel*). Cell counts represent the average of at least 3 repeated experiments.

the ratio of the target transcript with respect to α-actin mRNA.

Immunohistochemistry

Sections from mouse arteries were immunohistochemically stained for α-actin (anti–smooth muscle α-actin, 1:100; 1A4/M851; Dako) and PDGFR-α (anti-PDGFR-α sc-338, 1:200; Santa Cruz Biotechnology). Arterial sections were deparaffinized, rinsed in xylene, and rehydrated. Subsequently they were quenched with 3% hydrogen peroxide, washed in water, treated with 2% ovine albumin in PBS, and incubated with the primary antibodies at 37°C for 2 hours. After being washed in PBS the primary antibody was detected with biotinylated anti-mouse or anti-rabbit immunoglobulin G for 60 minutes at room temperature. Sections were washed in PBS, reacted with horseradish peroxidase–conjugated streptavidin (1:5000; Dako), and developed with 3,3′-diaminobenzidine. Negative controls were prepared by substitution of the primary antibody with an irrelevant antibody.

VSMC growth curves. VSMCs cultured from aortas of aging and young mice were plated into separate 24-well

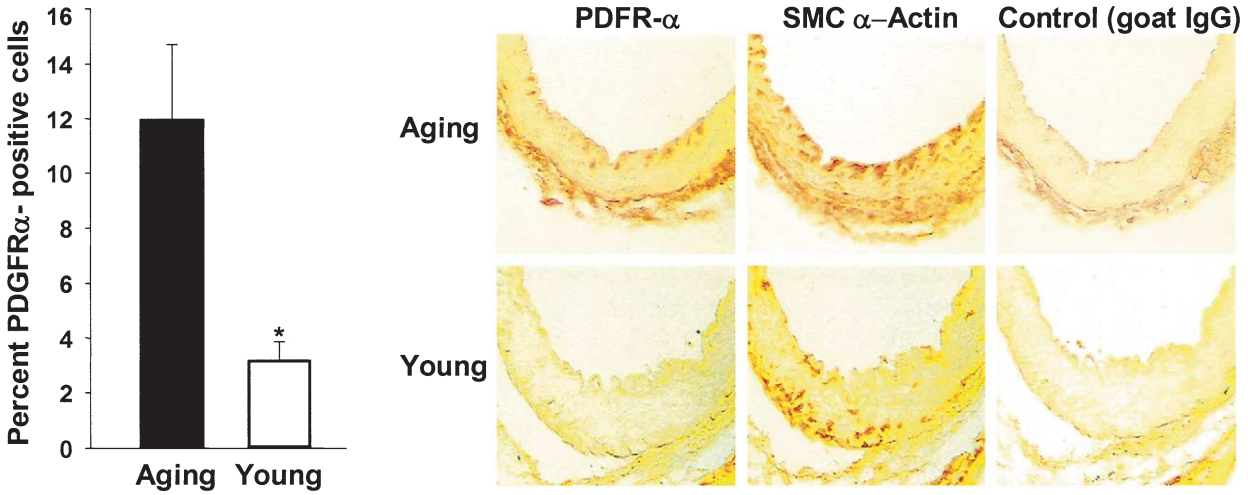

Fig 3. Expression of PDGFR-α in mouse aorta. Abundance of PDGFR-α is present in uninjured aging aortas, as assessed with immunohistochemical staining, in contrast with the barely stained young vessels *(right panel)*. Percent positively stained cells was counted against unstained vascular smooth muscle cells (VSMCs; left panel). $*P < .05$. VSMC phenotype was ascertained with VSMC α-actin monoclonal antibody. In the negative controls, the primary antibody was substituted with the immunoglobulin G fraction of normal goat serum.

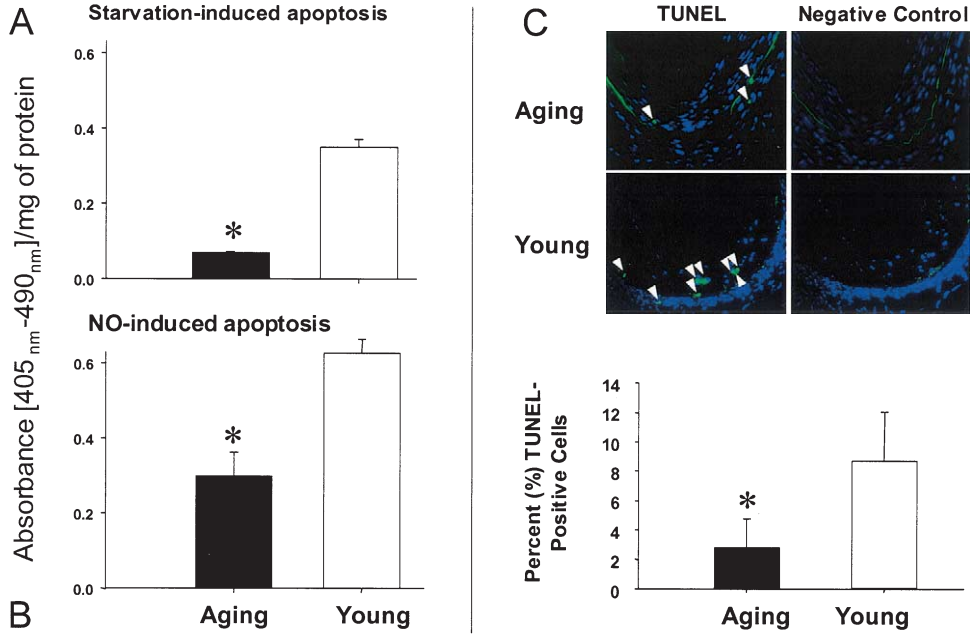

Fig 4. Vascular smooth muscle cells (VSMCs) from aging mice are less susceptible to apoptosis in vitro and in vivo. Aging VSMCs are less susceptible to apoptosis under serum starvation **(A)** than to exposure to nitric oxide (*NO*; at 200 μmol/L; **(B)**, as assessed with a Cell Death ELISA kit. Data were normalized for total amount of protein present in each sample. $*P < .05$). Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeled staining *(TUNEL)* shows more apoptotic cells in the neointima of young compared with aging aortas **(C)**. $*P < .01$.

plates at a density of 5×10^3 cells per well. Cells were allowed to attach to the plates in culture medium overnight, and the medium was then replaced with low serum medium (DMEM supplemented with 0.1% FBS). Cells were counted at time zero and every 24 hours thereafter for 6 days. Growth curves were additionally established for VSMCs cultured in DMEM supplemented with 2% FBS and 10 ng/mL of human PDGF-BB (Invitrogen), a well-known VSMC mitogen. Four wells were counted for each time point, with a cell counter (Coulter Electronics). Each

growth curve was repeated at least 3 times with 2 different cell lines.

Apoptosis assays. Cells were plated in 24-well plates at a density of 5×10^3 cells per well and allowed to attach to the plates in culture medium overnight. The following morning, apoptosis was induced with either serum starvation condition (DMEM–0.1% FBS) or exposure to nitric oxide (NO). In the serum starvation experiment the culture media were replaced with low serum medium, and cells were incubated for 48 hours before being assayed for apoptosis. In the NO induced-apoptosis experiment cells were kept in DMEM–0.1% FBS supplemented with 200 μmol/L of the NO donor *S*-nitroso-*N*-acetylpenicillamine (Sigma) for 2 hours.

Apoptosis was quantified by measuring the level of histone-associated DNA fragments, with the Cell Death ELISA [enzyme-linked immunosorbent assay] Kit Plus (Roche Molecular Biochemicals). After the specified treatment, medium was removed and cells were incubated in 200 μL of lysis buffer for 30 minutes. Cell extracts were spun for 15 minutes at 10,000 rpm, and 20 μL of the supernatant was transferred to a 96-well microtiter plate. Subsequently, 80 μL of the antibody mix (1 anti-histone:1 anti-DNA-horse radish peroxidase [HRP]) was added, and the plate was incubated at room temperature for 2 hours. Excess antibody was washed, and 100 μL of substrate solution was added. The absorbance values were determined at 405 nm with a microtiter plate reader (Bio-Rad Labs) and normalized according to the amount of soluble proteins determined with the Bradford method.[16] Increased absorbance corresponds to increased apoptosis with this technique.

In situ detection of apoptotic VSMCs in mouse arteries. Paraffin sections of injured arteries were pretreated (30 minutes at 37°C) with proteinase K in 50 mmol/L of Tris hydrochloride, pH 8.0, and rinsed twice in PBS. Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeled (TUNEL)–positive cells were detected with a TUNEL kit (Upstate). Sections were incubated at 37°C for 1 hour with biotin–deoxyuridine triphosphate in the presence of terminal deoxynucleotidyl transferase. Control sections were incubated with biotin–deoxyuridine triphosphate in the absence of deoxynucleotidyl transferase. The sections were then incubated with Avidin–fluorescein isothiocyanate for 30 minutes at 37°C and mounted with Vectashield mounting medium with 4,6-diamino-2-phenylindole (Vector Laboratories). TUNEL-positive cells were detected with fluorescent microscopy.

Matrix metalloproteinase (MMP)–2 and MMP-9 activity. Cell supernatants were collected 72 hours after cell starvation. MMP-2 and MMP-9 activity was assessed with 10% zymogram gels as described.[17] Medium was diluted to normalize for cell number before the addition of 5X Laemmli buffer under nonreducing conditions. After electrophoresis, gels were washed for 1 hour in 2.5% Triton X-100, and incubated for 40 hours in 50 mmol/L of Tris buffer. The gels were stained with Coomassie blue, and air-dried. Densitometry, with NIH Image 1.6 software, was used to assess relative enzyme levels.

Statistical analysis

Unless otherwise noted, quantitative data are expressed as mean ± SD. Comparisons were made wiht *t* tests for independent samples with unequal variance.

RESULTS

Aging mice exhibit exaggerated neointimal development after mechanical vascular injury. Four weeks after injury significantly more neointima developed in the aging (n = 17) mice than in their young (n = 6) counterparts (intima-media ratio, 1.17 ± 0.58 vs 0.50 ±0.16; $P < .001$; Fig 1). Neointimal cellular density was significantly higher in aging animals than in young animals (34.63 ± 5.7 vs 22.34 ± 11.13; $P = .044$). The contralateral uninjured common carotid arteries showed no neointimal formation (data not shown). Potential cellular mechanisms for these findings were explored in the following experiments.

Angiogenesis-related genes are differentially expressed in aging and young VSMCs. The Table summarizes angiogenesis-related genes differentially expressed by aging VSMCs. Compared with young VSMCs, expression of PDGFR-α (11.43-fold; $P = .0012$), restin (10.6-fold; $P = .000436$), Nos3 (8.6-fold; $P = .024$), Fgfr4 (10.20-fold; $P = .009$), and Erb2 (6.8-fold; $P = .077$) were notably increased in aging VSMCs. In contrast, expression of Fgf4 (0.25-fold; $P = .0043$), Fgfr3 (0.11-fold; P = .017), and cadherin 5 (0.46-fold; $P = .056$) were lower in aging cells.

VSMCs derived from aging mice proliferate faster in the presence of PDGF-BB. PDGF is a well-known VSMC mitogen that has an important role in neointimal development after vascular injury.[18,19] Because our array data (Table) demonstrated that expression of PDGFR-α was significantly higher in aging VSMCs, we decided to investigate the role of the PDGF/PDGFR pathway on the proliferative responses of VSMCs from aging and young mice. Our reverse transcriptase polymerase chain reaction data confirmed that expression of PDGFR-α was significantly higher in the aging cells (Fig 2, *A* and *B*). Increased expression of PDGFR-α in aging VSMCs compared with young cells was consistently seen in 3 independent cell lines. Moreover, aging cells exhibited significantly increased overall growth throughout the growth curve in the presence of the commonly used in vitro concentration (10 ng/mL) of PDGF-BB in culture medium containing 2% FBS (Fig 2, *C*). PDGF-BB activates both PDGFR-α and PDGRR-β.[20] Therefore aging cells exhibit increased proliferation in a PDGF-enhanced environment, which is designed to mimic the vascular injury microenvironment.[21]

Medial VSMCs in aging mouse aortas express more PDGFR-α. Guided by our in vitro data on the role of the PDGF/PDGF pathway on aging and young VSMCs, we next investigated the presence of PDGF-R in whole aortas. With immunohistochemical analyses we labeled the medial VSMCs of the injured mouse aortas with either anti-PDGFR-α or VSMC actin. Our data demonstrated that

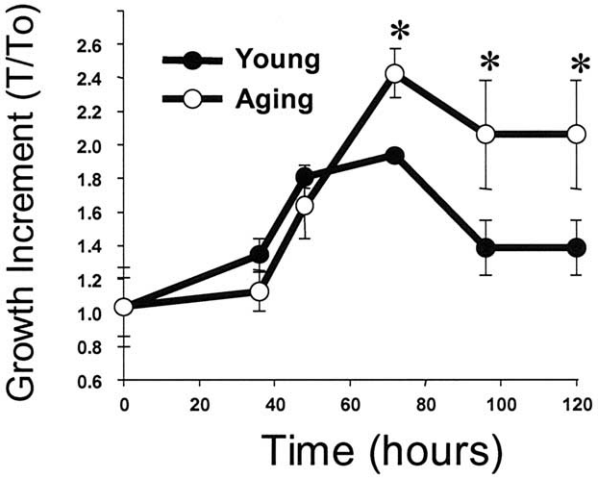

Fig 5. Aortic vascular smooth muscle cells from aging mice exhibit greater growth potential in serum-poor (0.1% fetal bovine serum) medium (*$P < .05$) than those from young mice. Growth increment was expressed as a ratio of the number of cells at a given time *(T)* and at time zero *(To)*; see Material and methods.

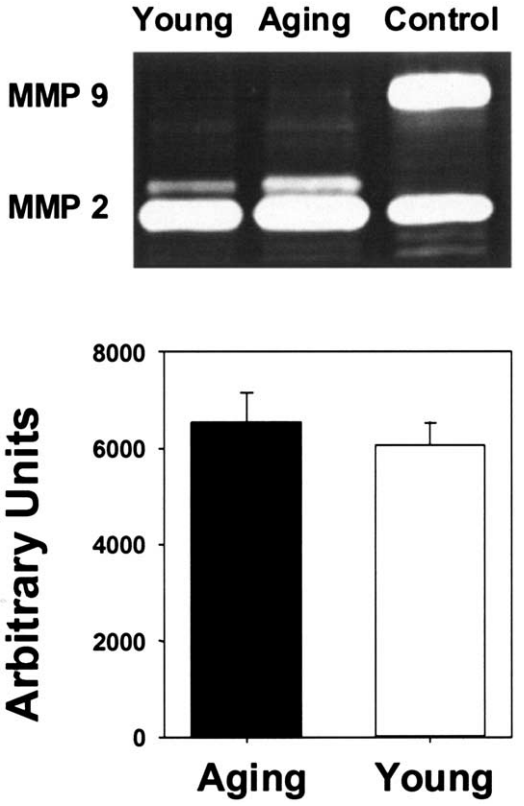

Fig 6. No significant differences were noted between aging and young vascular smooth muscle cells with regard to matrix metalloproteinase (*MMP-2* and *MMP-9*) activity at gelatin zymography.

medial VSMCs of aging aortas expressed more PDGFR-α than their young counterparts did (Fig 3).

VSMCs from aging mice are less susceptible to apoptosis. Resistance to apoptosis may also contribute to the increased neointima seen in aging animals in response to injury. To test this hypothesis we carried out a series of in vitro and in vivo experiments. We first challenged both aging and young mouse VSMCs to serum starvation, a condition that induces apoptosis. After 48 hours of serum starvation, aging cells demonstrated significantly less susceptibility to apoptosis (75% less) than did young mouse VSMCs (Fig 4, *A*). We observed that NO, whose production is enhanced at sites of vascular injury[22] but not in atherosclerotic lesions, caused dose-dependent increases in apoptosis in mouse VSMCs (data not shown). We next exposed aging and young VSMCs to a NO donor, *S*-nitroso-*N*-acetyl-penicillamine. In response to NO exposure, aging cells underwent less apoptosis (50% less) than their young counterparts did (Fig 4, *B*). Aortic VSMCs from aging mice exhibit greater growth capacity in serum poor (0.1% FBS) medium than do those from young mice (Fig 5). It is possible that, because of their reduced susceptibility to apoptosis, aging VSMCs survive better in serum-starved conditions, resulting in higher cell counts.

Finally, in agreement with our in vitro results, the incidence of apoptosis in neointimal VSMCs, as determined with the TUNEL assay, was higher in the injured arteries of young mice compared with those of aging mice (8.75% ± 3.3 vs 2.8% ± 1.9; $P = .021$). Mean numbers of apoptotic cells in arterial rings in each group are shown in Fig 4, *C*.

Production and activation of ECM degrading enzymes did not differ between aging and young mice. VSMCs from aging and young mice expressed similar levels of MMP-2. Neither set of cells expressed substantial MMP-9 in response to serum starvation (Fig 6).

DISCUSSION

Neointimal proliferation is well established as a major contributor to cardiovascular and peripheral vascular diseases that involve luminal obstruction. These include primary atherosclerosis, recurrent stenosis after coronary artery stenting, and allograft vasculopathy. The common characteristic of these diseases is that initial arterial injury ultimately results in expansion of a pathologic neointimal layer and progressive occlusion of the vessel. The current study examined the extent of neointimal formation in aging and young mice with mechanical injury. Our data indicate that aging exaggerates neointimal formation in response to vascular injury in mice. Furthermore, we have demonstrated that the cellular events that lead to neointimal formation, specifically, VSMC proliferation and resistance to apoptosis, increase in aging mice.

Aging increases neointimal formation in rats.[4] In addition, injured vessels or cultured VSMCs from aging rats exhibit increased proliferation[6,23-25] and less apoptosis[26] when compared with young counterparts. To our knowledge, no other comprehensive reports demonstrate all of these features of neointimal formation in a mouse model.

The importance of the parallels between the mouse model of age-related exacerbation of vascular response to injury and previous findings in rats[4] and larger animals[2,3] lies in the relevance of the mouse model for understanding the genetic and molecular mechanisms of this pathologic phenomenon. Molecular biology tools are more readily available for the mouse than for any other animal model. More important, genetically manipulated mice with transgenic and knockout technology are readily available. This enables specific genes to be studied in greater detail than can be attained in experiments involving larger animals.

PDGF/PDGFR signaling has an important role in neointimal development after arterial injury.[27] Daily administration of anti-PDGFR-α antibody significantly reduced neointimal formation in the rat cardiac allograft model[20] and in cholesterol-fed rabbits.[28] Moreover, PDGF binding sites increase in senescent VSMCs.[29] Our results reported herein further support the important role of the PDGF/PDGFR pathway in exaggerated neointimal development associated with aging. We demonstrate both in vitro and in vivo that aging VSMCs express more PDGFR-α than young cells do (Fig 2, *A* and *B*, and Fig 3). We also show that VSMCs from aging mice have an increased proliferative response to PDGF-BB (Fig 2, *C*), which is able to activate both PDGF receptors.[27] Collectively, our findings, along with those of others, suggest an important role for the PDGF/PDGFR pathway in the exaggerated neointimal formation associated with aging.

Of note, our data do not demonstrate age-related differences in either MMP-2 or MMP-9 in mouse VSMC cultures. Increased MMP-2 activity has been demonstrated in the neointima of rats and rabbits after vascular injury.[30-32] However, published data on age-related differences in MMP-2 activity are contradictory, showing both increased and decreased levels in aging neointimal tissue.[31,33] We believe that such discrepancies are due more to conditions of culture than to the model itself. Previous in vitro studies have not addressed the production of MMP-2 and the other extracellular matrix proteins in cultured VSMCs.

In conclusion, our results indicate that aging exaggerates vascular response to injury in mice. We show that aging increases the ability of VSMCs to proliferate and decreases their susceptibility to undergo apoptosis. We also demonstrate the important role of the PDGF/PDGFR pathway in the exaggerated neointimal formation associated with aging. Understanding the mechanisms responsible for this phenomenon may facilitate prevention or provide new therapies for vascular occlusive diseases. Our ability to reproduce the model in the mouse will no doubt facilitate such understanding.

REFERENCES

1. Kannel WB, Hjortland MC, McNamara PM, Gordon T. Menopause and risk of cardiovascular disease: the Framingham study. Ann Intern Med 1976;85:447-52.
2. Spagnoli LG, Sambuy Y, Palmieri G, Mauriello A. Age-related modulation of vascular smooth muscle cells proliferation following arterial wall damage. Artery 1985;13:187-98.
3. Weingand KW, Clarkson TB, Adams MR, Bostrom AD. Effects of age and/or puberty on coronary artery atherosclerosis in cynomolgus monkeys. Atherosclerosis 1986;62:137-44.
4. Hariri RJ, Alonso DR, Hajjar DP, Coletti D, Weksler ME. Aging and arteriosclerosis. I: Development of myointimal hyperplasia after endothelial injury. J Exp Med 1986;164:1171-8.
5. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993;362:801-9.
6. McCaffrey TA, Nicholson AC, Szabo PE, Weksler ME, Weksler BB. Aging and arteriosclerosis: the increased proliferation of arterial smooth muscle cells isolated from old rats is associated with increased platelet-derived growth factor-like activity. J Exp Med 1988;167:163-74.
7. Hultgardh-Nilsson A, Krondahl U, Querol-Ferrer V, Ringertz NR. Differences in growth factor response in smooth muscle cells isolated from adult and neonatal rat arteries. Differentiation 1991;47:99-105.
8. Sarzani R, Arnaldi G, Takasaki I, Brecher P, Chobanian AV. Effects of hypertension and aging on platelet-derived growth factor and platelet-derived growth factor receptor expression in rat aorta and heart. Hypertension 1991;18:III-93-9.
9. Durand E, Mallat Z, Addad F, Vilde F, Desnos M, Guerot C, et al. Time courses of apoptosis and cell proliferation and their relationship to arterial remodeling and restenosis after angioplasty in an atherosclerotic rabbit model. J Am Coll Cardiol 2002;39:1680-5.
10. George SJ, Angelini GD, Capogrossi MC, Baker AH. Wild-type p53 gene transfer inhibits neointima formation in human saphenous vein by modulation of smooth muscle cell migration and induction of apoptosis. Gene Ther 2001;8:668-76.
11. Mayr U, Mayr M, Li C, Wernig F, Dietrich H, Hu Y, et al. Loss of p53 accelerates neointimal lesions of vein bypass grafts in mice. Circ Res 2002;90:197-204.
12. Tsukioka K, Suzuki J, Fujimori M, Wada Y, Yamaura K, Ito K, et al. Expression of matrix metalloproteinases in cardiac allograft vasculopathy and its attenuation by anti MMP-2 ribozyme gene transfection. Cardiovasc Res 2002;56:472-8.
13. Nili N, Zhang M, Strauss BH, Bendeck MP. Biochemical analysis of collagen and elastin synthesis in the balloon injured rat carotid artery. Cardiovasc Pathol 2002;11:272-6.
14. Gurjar MV, DeLeon J, Sharma RV, Bhalla RC. Mechanism of inhibition of matrix metalloproteinase-9 induction by NO in vascular smooth muscle cells. J Appl Physiol 2001;91:1380-6.
15. Pauly RR, Bilato C, Cheng L, Monticone R, Crow MT. Vascular smooth muscle cell cultures. Methods Cell Biol 1997;52:133-54.
16. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochem 1976;72:248-54.
17. Jacot TA, Striker GE, Stetler-Stevenson M, Striker LJ. Mesangial cells from transgenic mice with progressive glomerulosclerosis exhibit stable, phenotypic changes including undetectable MMP-9 and increased type IV collagen. Lab Invest 1996;75:791-9.
18. Ferns GA, Raines EW, Sprugel KH, Motani AS, Reidy MA, Ross R. Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. Science 1991;253:1129-32.
19. Jawien A, Bowen-Pope DF, Lindner V, Schwartz SM, Clowes AW. Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. J Clin Invest 1992;89:507-11.
20. Mancini MC, Evans JT. Role of platelet-derived growth factor in allograft vasculopathy. Ann Surg 2000;231:682-8.
21. Zhang Y, Stewart KG, Davidge ST. Estrogen replacement reduces age-associated remodeling in rat mesenteric arteries. Hypertension 2000;36:970-4.
22. Joly GA, Schini VB, Vanhoutte PM. Balloon injury induces nitric oxide synthase activity in rat carotid arteries. J Cardiovasc Pharm 1992; 20(suppl):S151-4.
23. Hariri RJ, Hajjar DP, Coletti D, Alonso DR, Weksler ME, Rabellino E. Aging and arteriosclerosis: cell cycle kinetics of young and old arterial smooth muscle cells. Am J Pathol 1988;131:32-6.

24. Porreca E, Ciccarelli R, Di Febbo C, Cuccurullo F. Protein kinase C pathway and proliferative responses of aged and young rat vascular smooth muscle cells. Atherosclerosis 1993;104:137-45.
25. Stemerman MB, Weinstein R, Rowe JW, Maciag T, Fuhro R, Gardner R. Vascular smooth muscle cell growth kinetics in vivo in aged rats. Proc Natl Acad Sci U S A 1982;79:3863-6.
26. Fukagawa NK, Li M, Timblin CR, Mossman BT. Modulation of cell injury and survival by high glucose and advancing age. Free Radical Biol Med 2001;31:1560-9.
27. Raines EW. PDGF and cardiovascular disease. Cytokine Growth Factor Rev 2004;15:237-54.
28. Sihvola RK, Tikkanen JM, Krebs R, Aaltola EM, Buchdunger E, Laitinen O, et al. Platelet-derived growth factor receptor inhibition reduces allograft arteriosclerosis of heart and aorta in cholesterol-fed rabbits. Transplantation 2003;75:334-9.
29. Aoyagi M, Fukai N, Ogami K, Yamamoto M, Yamamoto K. Kinetics of 125I-PDGF binding and down-regulation of PDGF receptor in human arterial smooth muscle cell strains during cellular senescence in vitro. J Cell Physiol 1995;164:376-84.
30. Li Z, Froehlich J, Galis ZS, Lakatta EG. Increased expression of matrix metalloproteinase-2 in the thickened intima of aged rats. Hypertension 1999;33:116-23.
31. Wang M, Lakatta EG. Altered regulation of matrix metalloproteinase-2 in aortic remodeling during aging. Hypertension 2002;39:865-73.
32. Wang H, Keiser JA. Expression of membrane-type matrix metalloproteinase in rabbit neointimal tissue and its correlation with matrix-metalloproteinase-2 activation. J Vasc Res 1998;35:45-54.
33. Zhang Y, Stewart KG, Davidge ST. Estrogen replacement reduces age-associated remodeling in rat mesenteric arteries. Hypertension 2000;36:970-4.

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