Aging Induces Endothelial Dysfunction While Sparing Arterial Thrombosis


Objective—To assess the effects of aging on arterial thrombus formation by comparing 2-year-old with 11-week-old C57Bl6 mice.

Methods and Results—Aging is a major risk factor for cardiovascular disease. In humans, assessing the direct effects of aging on vascular homeostasis is difficult because it occurs in the presence of other risk factors. Arterial thrombosis is the critical event in cardiovascular diseases; however, it is not known whether aging per se promotes its occurrence. Mice represent an interesting system to address this issue because they age without spontaneously developing other risk factors. Organ chamber experiments confirmed the advanced level of aging of old mice. As previously shown, old mice exhibited endothelial dysfunction; however, arterial thrombosis induced by photochemical injury was unchanged. Arterial tissue factor expression and activity; expressions of tissue factor pathway inhibitor, thrombomodulin, and plasminogen activator inhibitor 1; prothrombin time; partial thromboplastin time; thrombin-antithrombin complex; and platelet activation were comparable in both groups.

Conclusion—Although these results cannot be directly extrapolated to humans, this study contributes novel important information on the direct effect of aging on arterial thrombosis and underscores the importance of controlling modifiable risk factors in aged individuals. (Arterioscler Thromb Vasc Biol. 2010;30:1960-1967.)

Key Words: aging ■ atherosclerosis ■ endothelial function ■ risk factors ■ mouse ■ thrombosis

The percentage of elderly people in our society is steadily increasing, and age is considered a major risk factor for the development of cardiovascular disease.1 Because most cardiovascular events, such as myocardial infarction and ischemic stroke, are caused by arterial thrombosis, it is tempting to speculate that advanced age promotes the development of arterial thrombosis. Yet, the direct effects of aging on arterial thrombosis have not been studied because aging in humans always occurs in parallel to other risk factors or atherosclerosis.

Unlike aged humans, old mice fed a regular diet do not spontaneously develop cardiovascular risk factors. Previous work2–5 showed that aged mice develop NO-dependent endothelial dysfunction but do not develop atherosclerotic lesions, diabetes mellitus, or hypertension. Therefore, mice represent an interesting system to study the process of aging in isolation and to investigate its direct effects on vascular homeostasis.

In light of the previous findings, we compared 2 C57Bl6 male mice groups, bred and housed in the same conditions, aged 2 years (old group) and 11 weeks (young group); and investigated age-induced changes in arterial thrombosis in the carotid artery. We used an established photochemical injury model that principally affects the intimal layer, as demonstrated by transmission electron microscopy sections (data not shown), and mimics endothelial injury, as occurring in atherothrombotic human complications.6,7 Endothelial function was assessed to document the stage of vascular aging. However, it was not our intention to establish a causal link between NO-mediated endothelial dysfunction and age-related changes in thrombosis. In fact, this hypothesis was already addressed by a previous study8 showing that mice lacking endothelial NO synthase (eNOS) exhibit no changes in arterial thrombosis. In light of the previous work linking inflammation and thrombosis,9 and inflammation and aging,10 the inflammatory state of young and old mice was assessed by immunhistochemistry and real-time PCR.

Methods

Mice

Male C57BL/6 mice from the following age groups were examined: (1) young group, aged 11 weeks; (2) intermediate group, aged 62 weeks; and (3) old group, aged 105 weeks. All mice were bred and...
housed in a specific pathogen-free animal facility with a conventional light cycle at the Institute of Laboratory Animal Science, University of Zurich, Zurich, Switzerland. All experiments were approved by the local authorities.

Organ Chamber
The thoracic aorta was dissected free, excised, and immediately placed into ice-cold Krebs-Ringer bicarbonate solution. Vessels were cut into 3-mm rings and suspended in organ chambers for isometric tension recording (System 700 MO; Danish Myo Technology A/S, Aarhus, Denmark) containing 6 mL of Krebs solution (37°C, pH 7.4) and aerated with 95% oxygen plus 5% CO2. After a 30-minute equilibration period, rings were progressively stretched to the optimal point of their length-tension curve, and the presence of a functional endothelium was tested in norepinephrine precontracted rings by adding 10⁻⁷ mol/L of acetylcholine. Endothelial function was assessed in norepinephrine precontracted rings by studying the relaxation to acetylcholine (10⁻⁷ to 10⁻⁴ mol/L; Sigma-Aldrich, Buchs, Switzerland) in the presence or absence of N²-nitro-L-arginine methyl ester (L-NAME) (3×10⁻⁴ mol/L, 30-minute incubation; Sigma-Aldrich). The NO donor, sodium nitroprusside (10⁻⁴ mol/L), was applied to test endothelium-independent relaxations. Concentration-dependent contractions were established by norepinephrine (10⁻⁹ to 10⁻⁴ mol/L; Sigma-Aldrich).

Arterial Thrombosis
Mice were anesthetized with pentobarbital, 87 mg/kg IP. Rose Bengal (Fisher Scientific, Fair Lawn, NJ) was injected into the tail vein in a volume of 0.12 mL at a concentration of 50-mg/kg body weight. Mice were placed under a dissecting microscope, and the right common carotid artery was exposed after a midline cervical incision. A Doppler flow probe (model 0.5 VB; Transonic Systems, Ithaca, NY) was placed around the neck and connected to a flow meter (model T106; Transonic Systems, Germany) supplying a data acquisition system (PowerLab 4/30; AD Instruments). A 1.5-mW green light laser, 540 nm (Melles Griot, Carlsbad, Calif), was directed at the desired site of injury at a distance of 6 cm for 60 minutes or until complete occlusion. Flow was monitored for 120 minutes from the onset of injury. Occlusion was defined as flow of 0.1 mL/min or less for at least 1 minute. Data were analyzed with computer software (ChartPro Software; AD Instruments). The photochemical injury protocol leads to an endothelial-specific injury largely confined to the diameter of the laser beam, which reacts with the light-sensitive rose Bengal. As a result of the photochemical injury, endothelial cells were exposed to procoagulant factors, which, in contact with circulating coagulation factors, result in the formation of an arterial thrombus. The kinetics of and time to occlusion are monitored in real-time with a Doppler probe.

Tissue Factor Activity
The carotid arteries were homogenized in 50 μL of lysis buffer (50-mmol/L Tris·HCl, 100-mmol/L NaCl, and 0.1% Triton-X 100, pH 7.4) by manual grinding on ice. Samples were then centrifuged at 14,000 rpm for 15 minutes at 4°C, and the supernatant was transferred to a fresh tube. Tissue factor (TF) activity was assessed using a colorimetric assay (Actichrome TF; American Diagnostica, Greenwich, Conn); incubation at 37°C allowed TF to form a complex with factor VII and allowed this complex to cleave factor X to factor Xa. Absorbance was determined using naphthol AS-BI phosphate and new fuchsin as substrate. The primary antibodies were revealed by goat anti–species-specific labeled donkey antigoat antibodies. Alkaline phosphatase was visualized using naphthol AS-BI phosphate and new fuchsin as substrate. Immunohistochemistry
Aortic roots and carotid arteries were mounted in optimal cutting temperature (Tissue-Tek, Alphen aan de Rijn, The Netherlands) and first-strand cDNA primer pd(N)6. Real-time PCR was performed using a kit (SybrGreen Jump; Sigma). Primers were designed as follows: TF, forward (5'-3': AAATGAAATCTCGAATGATGTGG); and reverse (5'-3': GGAGGATGTAAGAAGATGGTGCC); tissue factor pathway inhibitor (TFPI), forward (5'-3': ACTTGTGTGTCTGT- GCCTAGCC) and reverse (5'-3': GTTCTCGTCTCCTCTCA CATCCC); plasminogen activator inhibitor (PAI) 1, forward (5'-3': ATCAATGACTGGTGATGGAGG) and reverse (5'-3': GTTGAACTTGTGTCTGAGCC); thrombomodulin (TM), (forward (5'-3': TTTTCTTCTGGTGATGTTG) and reverse (5'-3': TGCTCTG CTCCAGCAAGG); matrix metalloproteinase 9, forward (5'-3': CCTGGAACCTAACAACGCTTC) and reverse (5'-3': TGGAACCTACACGGCAGA); and eNOS, forward (5'-3': ATTAAATACGCACAAATAGGCG) and reverse (5'-3': CTCAGTGATCTCCAGGGTGCC). Data were normalized to murine S12: forward (5'-3': GAAAGTGCGAAGAGGCTTGA) and reverse (5'-3': AACTGGAACAAAAACCTTGA). The amplification program consisted of 1 cycle at 95°C for 10 minutes, followed by 40 cycles, with a denaturing phase at 95°C for 30 seconds, an annealing phase at 60°C for 1 minute, and an elongation phase at 72°C for 1 minute. For verification of the correct amplification, PCR products were analyzed on an ethidium bromide–stained 1.5% agarose gel. Quantification was performed using the 2⁻ΔΔCt method.

Blood Glucose, Total Cholesterol, and Plasma Triglycerides
All measurements were performed in the morning after a 14-hour fasting period. Glucose was measured in whole blood with a commercially available system (ACCU-CHEK aviva system; Roche Diagnostics). Cholesterol and triglyceride levels were determined in heparinized plasma (15-minute centrifugation at 2500g and 4°C) by the enzymatic method (CHOD–peroxidase–antiperoxidase and glycine phosphate oxidase (GPO)–peroxidase–antiperoxidase methods (Roche Diagnostics), respectively).

Immunohistochemistry
Aortic roots and carotid arteries were mounted in optimal cutting temperature (Tissue-Tek, Alphen aan de Rijn, The Netherlands). Specimens were cut into 8-μm sections and fixed with 4% paraformaldehyde. Sections were stained with oil red O for 1 hour at room temperature. For the assessment of myeloperoxidase (Labvision, Cheshire, UK), CD68 (Serotec, Dusseldorf, Germany), and tumor necrosis factor α (Abcam, Cambridge, UK), sections were fixed in aceton, blocked with 1% FCS, and incubated with specific primary antibodies. The primary antibodies were revealed by goat anti–species-specific immunoglobulin antibodies, followed by alkaline phosphatase–labeled donkey anti–goat antibodies. Alkaline phosphatase was visualized using naphthol AS-BI phosphate and new fuchsin as substrate. Sections were counterstained with hemalum and coverslipped.

Results

Vascular Tone
Endothelium-dependent relaxation to acetylcholine was impaired in old compared with young mice (maximal relaxation, Shear Stress–Dependent Platelet Function
Shear stress–dependent platelet function was assessed with a cone and a platelet analyzer. Citrated whole blood, 200 μL, was circulated in polystyrene wells at a shear rate of 1875sec for 2 minutes, with a rotating polytetrafluoroethylene cone as described. Wells were washed, stained with May-Grünwald dye, and analyzed with a microscope connected to an image analysis system (ImageJ). The results are expressed as the percentage of surface covered by platelets.

Real-Time PCR
Aortic arches were snap frozen in liquid nitrogen and stored at −80°C until use. Total RNA was isolated using a reagent (TRIzol; Invitrogen, Basel, Switzerland). cDNA was generated using Ready-To-Go You-Prime First-Strand Beads (Amersham Bioscience, Glattbrugg, Switzerland) and first-strand cDNA primer pd(N)6. Real-time PCR was performed using a kit (SybrGreen Jump; Sigma). Primers were designed as follows: TF, forward (5'-3': CAATGAATTCTCTGATGTGG); and reverse (5'-3': GGAGGATGTAAGAAGATGGTGCC); tissue factor pathway inhibitor (TFPI), forward (5'-3': ACTTGTGTGTCTGTGCTAGCC) and reverse (5'-3': GTTCTCGTCTCCTCTCATCCC); plasminogen activator inhibitor (PAI) 1, forward (5'-3': ATCAATGACTGGTGATGGAGG) and reverse (5'-3': GTTGAACTTGTGTCTGAGCC); thrombomodulin (TM), (forward (5'-3': TTTTCTTCTGGTGATGTTG) and reverse (5'-3': TGCTCTGCTCCAGCAAGG); matrix metalloproteinase 9, forward (5'-3': CCTGGAACCTAACAACGCTTC) and reverse (5'-3': TGGAACCTACACGGCAGA); and eNOS, forward (5'-3': ATTAAATACGCACAAATAGGCG) and reverse (5'-3': CTCAGTGATCTCCAGGGTGCC). Data were normalized to murine S12: forward (5'-3': GAAAGTGCGAAGAGGCTTGA) and reverse (5'-3': AACTGGAACAAAAACCTTGA). The amplification program consisted of 1 cycle at 95°C for 10 minutes, followed by 40 cycles, with a denaturing phase at 95°C for 30 seconds, an annealing phase at 60°C for 1 minute, and an elongation phase at 72°C for 1 minute. For verification of the correct amplification, PCR products were analyzed on an ethidium bromide–stained 1.5% agarose gel. Quantification was performed using the 2⁻ΔΔCt method.

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Arterial Thrombosis

Age-related changes in arterial thrombosis were examined by a carotid artery photochemical injury model and assessed by real-time blood flow measurement. Both time to thrombotic occlusion and the kinetics of thrombus formation were unaltered in old (45.5±3.7 minutes; n=5) compared with middle-aged (53.0±5.8 minutes; P=0.6126; n=6) or young (45.6±5.9 minutes; P=0.9889; n=5) (Figure 2A and B) mice. Carotid artery blood flow before initiation of photochemical injury was similar in old and young mice (0.81±0.07 versus 0.77±0.07 mL/min; P=0.6978; n=5).

Figure 1. Isometric tension studies in aortic rings of old and young mice. A, Endothelium-dependent relaxations to acetylcholine are impaired in old vs young mice (\(P<0.01\) and \(**P<0.001; n=5\)). Pretreatment with L-NAME abrogates relaxations in both groups (\(P=NS\) for old vs young mice and \(P<0.001\) for control vs L-NAME; \(n=5\)). B, Endothelium-independent relaxations to sodium nitroprusside do not differ between old and young mice (\(P=NS; n=5\)). C, Contractions to potassium chloride (KCl), 80 mmol/L, are similar in old and young mice (\(P=NS; n=5\)). D, Contractions to L-NAME (3×10^{-4} mol/L) are similar in old and young mice (\(P=NS; n=5\)). E, Contractions to norepinephrine are similar in old and young mice (\(P=NS; n=5\)). w indicates weeks.

Figure 2. Aging does not affect arterial thrombus formation. A, Old and young mice exhibit similar time to thrombotic occlusion (\(P=NS; n=5\)). B, The real-time blood flow plot analysis reveals no difference in the flow pattern over time (representative records).
To improve our understanding of the laser-induced damage to the arterial wall, we examined sections of noninjured and injured carotid arteries by electron microscopy. Both the internal elastic membrane and the vascular media remained morphologically intact after the laser injury under our experimental conditions, indicating that the injury is largely confined to the endothelial layer (data not shown).

Analysis of Coagulatory and Fibrinolytic Factors and Platelets

TF activity in the carotid artery did not differ between old and young mice (OD405, 0.68±0.01 versus 0.50±0.05; P=0.1338; n=5) (Figure 3A). In line with this finding, TF mRNA levels (ΔΔCt, 3.7±1.1 versus 4.8±1.5; P=0.5755; n=5) (Figure 3A) and levels of its antagonist TFPI (ΔΔCt, 0.0134±0.0028 versus 0.0120±0.0017; P=0.6751; n=5) (Figure 3B) were comparable in old and young mice. The expressions of PAI-1 (ΔΔCt, 0.210±0.049 versus 0.408±0.088; P=0.0862; n=5) (Figure 3B) and TM (ΔΔCt, 0.342±0.087 versus 0.434±0.153; P=0.6429; n=5) (Figure 3B) were not different between the 2 groups. Extrinsic and intrinsic coagulation pathways were examined by measuring PT and aPTT, respectively. No changes were observed between old and young mice, neither for PT (10.70±0.23 versus 10.90±0.25 seconds; P=0.613; n=5) (Figure 3C) nor aPTT (26.7±2.8 versus 26.2±1.5 seconds; P=0.8873; n=5) (Figure 3C). The thrombin-antithrombin complex was assayed in plasma, and no difference was found between young and old mice (4.79±0.16 versus 4.47±0.093 ng/mL; P=0.12; n=5; data not shown). Platelet function was assessed by determining shear stress-dependent platelet adhesion. Old and young mice exhibited similar values (5.7±2.0% versus 4.1±1.5%; P=0.5495; n=5) (Figure 3D).

Arterial Morphology

Aortic roots, descending aortae, and carotid arteries were stained by hematoxylin-eosin and oil red O. Histological features of the arteries were comparable in old and young mice (Figure 4A). No lipid deposits or fatty streaks were detected in either group. In the aortic root, sporadic single subendothelial cells stained positive for oil red O in old, but not in young, mice (Figure 4B). The descending aorta (Figure 4C) and the carotid arteries (Figure 4D) exhibited similar histological features in old and young mice.

Blood Glucose, Plasma Cholesterol, and Plasma Triglycerides

Fasting blood glucose (81.1±8.3 versus 60.8±1.3 mg/dL; P=0.0922) and total plasma cholesterol (81.2±2.2 versus 59.3±20.3 mg/dL; P=0.2575) did not differ in old and young mice. Plasma triglyceride levels after 14 hours of fasting were significantly lower in old mice compared with young controls (35.6±7.9 versus 99.8±4.2 mg/dL; P<0.001).
Matrix Metalloproteinase 9 Expression and Immunohistochemical Analysis of Inflammation

Levels of matrix metalloproteinase 9 mRNA expression were comparable in both groups (ΔΔCt, 0.00023±0.00004 versus 0.00026±0.00006; P=0.7028; n=5) (Figure 5). Immunohistochemical analysis of carotid arteries revealed comparable expression levels of the inflammatory markers myeloperoxidase, CD68, and tumor necrosis factor in young and old mice, indicating a similar and low level of inflammation in both groups (Figure 5).

Discussion

Increasing age is considered a major risk factor for the development of cardiovascular disease. Thus, studying its molecular mechanisms is an important scientific goal, especially in view of the growing percentage of elderly people in our society. This study demonstrates that aging per se does not promote arterial thrombosis. Endothelial function was assessed to document the advanced stage of vascular aging of old mice but not to establish a direct link between NO-mediated endothelial function and thrombosis. This link was investigated in a previous study in which deletion of the eNOS gene was shown not to affect arterial thrombus formation. Old mice exhibited impaired endothelium-dependent relaxation while maintaining an unaltered thrombotic potential. Moreover, the kinetics of thrombus formation, arterial TF activity, plasma coagulation times, and platelet adhesion and modulators of coagulation (TFPI, PAI-1, and TM) remained unchanged when compared with young controls. Although levels of PAI-1 and eNOS were not statistically different, a trend was visible and we cannot fully exclude that many animals might have yielded a different result. Mice have a life expectancy of roughly 2 years, and the aged mice used in this study were older than 104 weeks; thus, their age corresponded roughly to 70 or 80 years for a human.

Mice fed a regular diet do not exhibit an elevated blood pressure, glucose level, or cholesterol level with age. Indeed, fasting blood glucose and plasma cholesterol levels...
remained unaltered in old mice, and fasting triglyceride levels were even decreased in these animals; therefore, in line with previous studies, old mice fed their entire lifespan a normocaloric diet did not exhibit glucose, cholesterol, or triglyceride levels known to be associated with vascular disease. Consistent with these observations, histological analysis did not reveal atherosclerotic lesions, such as fatty streaks in aortic roots, carotid arteries, or descending aortas of old mice. Sporadic single cells stained positive for oil red O in the aortic roots, carotid arteries, or descending aortas of old mice. Notably, old mice fed a high-fat diet did not exhibit glucose, cholesterol, or triglyceride levels known to be associated with vascular disease. Consistent with these observations, histological analysis did not reveal atherosclerotic lesions, such as fatty streaks in aortic roots, carotid arteries, or descending aortas of old mice. Sporadic single cells stained positive for oil red O in the aortic roots, carotid arteries, or descending aortas of old mice.

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The rupture of atherosclerotic plaques and consequent thrombus formation are the main cause of acute cardiovascular events in humans. An important question in this context is whether the increasing incidence of thrombosis with aging solely depends on more severe atherosclerosis or whether aging promotes thrombosis by itself. In light of these considerations, the absence of atherosclerosis in our mouse model is a prerequisite for this study. Thus, we were especially interested in studying aging-related changes in the absence of atherosclerosis to dissect these 2 parameters. In light of the previous findings and despite the obvious differences to humans, mice represent an interesting model for the independent assessment of aging on vascular function or, as in the present study, for investigating the direct effect of aging on arterial thrombus formation.

After the photochemical injury protocol, old mice displayed an unaltered time to thrombotic occlusion compared with young mice. Furthermore, the kinetics of arterial thrombus formation, as assessed by measuring real-time blood flow after photochemical injury, were comparable. Both the internal elastic membrane and the vascular media remained morphologically intact after the laser injury under our experimental conditions, as confirmed by transmission electron microscopy (data not shown). Altogether, the model has accepted parameters for assessing changes in coagulation; for assessing the activity of coagulation pathways. Age-related changes of these parameters have not been described, and clinically used reference ranges are not age dependent. In line with these observations and with the reported similar kinetics of arterial thrombosis in vivo, the PT, aPTT, and thrombin-antithrombin complexes remained unchanged in old mice. Therefore, no functionally relevant alteration of the coagulation system was apparent. In addition, given the unchanged thrombotic potential and unchanged PAI-1 and TM were not altered in these animals. The PT, aPTT, and thrombin-antithrombin complexes are globally accepted parameters for assessing changes in coagulation; the integral character of these tests renders them well suited for assessing the activity of coagulation pathways. Age-related changes of these parameters have not been described, and clinically used reference ranges are not age dependent.

In both humans and mice, age-related changes of coagulation factors have been reported. Factor VIII, factor VII, and fibrinogen plasma levels increase with age in humans and mice. In the former, some of these alterations are associated with coronary artery disease or overall mortality, although no causal relationship has been established. Such prothrombotic alterations may well be counterbalanced in humans by increased plasma levels of anticoagulant proteins, such as TFPI or protein C, which have also been reported. Moreover, such changes may occur secondary to activation of coagulation by endothelial damage or endothelial erosion rather than playing a direct role in thrombosis. In addition, many of these parameters are acute-phase reactants and may simply represent an enhanced inflammatory burden. Thus, the clinical relevance of such changes in plasma coagulation factors with aging is questionable. Consistent with this interpretation, studies in centenarians revealed that these individuals exhibit higher levels of procoagulant factors compared with old controls, suggesting that such changes correlate with health and longevity and do not necessarily lead to a higher risk for arterial thrombosis. In addition, mRNA expression and protein activity of TF, the main trigger of coagulation, and expression of its antagonist TFPI and PAI-1 and TM were not altered in these animals. The PT, aPTT, and thrombin-antithrombin complexes are globally accepted parameters for assessing changes in coagulation; the integral character of these tests renders them well suited for assessing the activity of coagulation pathways. Age-related changes of these parameters have not been described, and clinically used reference ranges are not age dependent. In line with these observations and with the reported similar kinetics of arterial thrombosis in vivo, the PT, aPTT, and thrombin-antithrombin complexes remained unchanged in old mice. Therefore, no functionally relevant alteration of the coagulation system was apparent. In addition, given the unchanged thrombotic potential and unchanged PAI-1 levels observed in young and old mice, it is conceivable that activation of the fibrinolytic system did not vary with age.

Platelets are crucially involved in arterial thrombus formation. In aged humans, when classic risk factors are not controlled for, elevated levels of β-thromboglobulin and platelet factor 42 and a lower aggregation threshold to adenosine diphosphate and collagen have been reported. In addition, von Willebrand factor expression is known to increase with age and correlate with age-related risk factors;
in these subjects, it may enhance platelet activation in vivo. Furthermore, the phospholipid composition of platelets differs with age in healthy subjects, suggesting an age-related alteration in transmembrane signaling. In the present study, platelet function was assessed by shear stress–induced adherence. No age-dependent alteration in platelet function was recorded in these experiments, suggesting comparable platelet function among young and old mice, which implies that the age-related changes described may not be directly influenced by aging but rather by other risk factors.

In summary, this study shows that aging per se does not affect arterial thrombosis in the mouse and that age-dependent vascular dysfunction in the absence of additional risk factors does not alter arterial thrombus formation. Although limited by the use of mice, this study contributes novel important information concerning the direct effect of aging on arterial thrombosis and underscores the importance of controlling modifiable risk factors in aged individuals. Studying the effect of additional risk factors and/or atherosclerosis in concert with aging on arterial thrombosis could certainly represent an interesting future development of this study.

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Disclosures
None.

References


