Peripheral Vascular Endothelial Dysfunction and Apoptosis in Old Monkeys

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Abstract—To determine the effects of aging on vasoactivity in a primate model (Macaca fascicularis), 13 young male monkeys (aged 7.1±0.4 years) and 9 old male monkeys (aged 19.8±0.6 years) were chronically instrumented for measurement of left ventricular and aortic pressures and cardiac output. Total cholesterol, triglyceride, and fasting blood sugar levels were not different between the 2 groups. There were no significant differences in baseline mean aortic pressure and total peripheral resistance (TPR) in the young monkeys versus the old monkeys. TPR fell less (P<0.05) with acetylcholine (1 μg/kg) in old monkeys (−25±1%) than in young monkeys (−34±2%), whereas decreases in TPR with sodium nitroprusside were similar in old and young monkeys. There was no evidence of atherosclerosis, but apoptosis of endothelial cells was enhanced (P<0.05) in the aortas and femoral arteries, but not in the media, of the old monkeys. There was a relationship (r=0.62, P=0.013) between the incidence of terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL)-positive endothelial cells and endothelial cell density in the femoral artery. The reduced endothelial cell density was also correlated (r=0.82, P<0.01) with depressed TPR responses to acetylcholine. Thus, vascular endothelial dysfunction was present in old monkeys without evidence of atherosclerosis, which may be due to endothelial apoptosis and reduced endothelial cell density. (Arterioscler Thromb Vasc Biol. 2000;20:1493-1499.)

Key Words: aging ■ endothelium-dependent vasodilation ■ apoptosis ■ vascular endothelial cell density

Cardiovascular disease is the most common cause of death among the elderly, and cardiovascular deaths increase significantly in old patients. This raises the question of whether aging acts synergistically to intensify cardiovascular disease. Before this question can be answered, the effects of aging in the absence of cardiovascular disease must be understood. Because this is difficult to study in humans, surrogates have generally been used. The effects of aging on vascular function have been evaluated in animal models, primarily in rats, but also in humans, and it is recognized that aging is associated with a variety of functional and structural changes in the vasculature.1-5 However, results are controversial, potentially because of the limitations inherent in animal models and also in human studies, which are often complicated by other cardiovascular diseases, eg, diabetes and atherosclerosis. An aging nonhuman primate model has the advantage of being phylogenetically closer to humans, and it exhibits few of the complicating effects of the cardiovascular diseases associated with aging.

The goal of the present investigation was to determine whether vascular responses in general and endothelial vasodilation in particular are diminished in aging monkeys, independent of atherosclerosis. Secondarily, we sought to determine whether there were histopathological changes that could explain the altered vasoreactivity. This topic is controversial, not only because of the difficulty of study in the absence of atherosclerosis but also in view of the studies by Celermajer et al6 and Woo et al.7 Celermajer et al found that there was deranged endothelium-dependent vasodilation in older individuals that was independent of other coronary risk factors. However, because tissue was not available, nascent atherosclerosis could not be excluded in those patients by histological examination of the vessels. Woo et al found that aging-induced endothelial dysfunction was not observed in older Chinese subjects but that it was in older white individuals, suggesting the possibility of environmental influences. The nonhuman primate model should be ideal to reconcile this controversy. To address this, peripheral vascular (total peripheral resistance [TPR]) responses to sodium nitroprusside (SNP), which is an endothelium-independent vasodilator, and acetylcholine (ACh), which is an endothelium-dependent vasodilator, were examined in conscious monkeys.

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In addition, we also examined the vascular walls histologically by using light and electron microscopy, with particular emphasis on the incidence of apoptosis on endothelial cells of the aorta and femoral artery, because that could be the mechanism for vascular endothelial dysfunction in the old monkeys.

Methods

Animals

Thirteen young (aged 7.1 ± 0.4 years) and 9 old (aged 19.8 ± 0.6 years) adult male monkeys (Macaca fascicularis) were studied. The monkeys were fed a primate diet containing 5% to 6% fat, 18% to 25% protein, and 0.2% to 0.3% sodium chloride. All of the young monkeys were bred monkeys, and their ages were determined on the basis of their actual dates of birth. Old monkeys were feral animals captured at the age of 5 to 7 years old, and the monkeys had been kept in captivity for 12 to 15 years. Age at the time of capture was estimated on the basis of eruption of dentition, general appearance, sexual development, and body weight. None of the animals had been used for any previous experimental studies. The animals used in the present study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services publication [National Institutes of Health] No. 83-23, revised 1996).

Implantation of Instrumentation

The animals were tranquilized with ketamine hydrochloride (2 to 3 mg/kg IM), anesthetized with thiopental sodium (5 to 10 mg/kg IV), and maintained with isoflurane (0.5 to 1.5 vol/100 mL in oxygen). After general anesthesia, an incision was made in the fourth left intercostal space with use of sterile surgical technique. Tygon catheters (Norton Elastic and Synthetic Division) were implanted in the descending aorta and left atrium, and a solid-state pressure gauge (Konigsberg Instruments) was inserted into the left ventricle (LV) through the apex. In 12 young and 9 old monkeys, an aortic flow probe (Transonic Systems) was implanted around the root of the ascending aorta to measure ascending aortic flow (cardiac output, Figure 1). The chest incision was closed in layers, and the thorax was evacuated of air. All animals were allowed to recover for 10 to 14 days before experimentation.

Experimental Measurements

Hemodynamic measurements were made with the monkeys fully awake; a tether system was used to transmit the electronic signals and catheter pressures to the recording electronics. All monkeys maintained excellent health and were active with good appetites in the tether system. All hemodynamic measurements were recorded on a digital multiple recorder (PC216Ax, Sony Precision Technology Inc) and played back on a direct-writing oscillograph (Gould-Brush). The fluid-filled catheter in the aorta was connected to a pressure transducer (Datex Ohmeda) for the measurement of aortic pressure. LV pressure and the first derivative of LV pressure (dP/dt) were measured with a miniature pressure gauge. Zero aortic flow was assumed to occur during mid and late diastole. Cardiac index (CI) was calculated as cardiac output divided by body surface area (BSA). BSA was calculated as 71.84 • (body weight)0.425•(height)0.725. TPR was calculated as the quotient of mean aortic pressure (MAP) and CI.

Experimental Protocol

Physiological responses to vasodilators were examined with the animal in the conscious state, at least 24 hours after it was placed in the tether. Bolus injections of SNP (1, 2, and 5 μg/kg; 10 young and 8 old monkeys) and ACh (0.1, 0.2, 0.5, and 1 μg/kg; 11 young and 8 old monkeys) were administered through the tether via the left atrial catheter; measurements of phasic pressure, MAP, LV pressure, LV dP/dt, and cardiac output were recorded continuously.

Blood Samples

Blood samples were obtained for determination of plasma concentrations of blood urea nitrogen, creatinine, fasting plasma glucose, cholesterol, and triglycerides with the monkey resting in the tether

Figure 1. Schematic illustration of instrumentation and representative waveforms of aortic pressure, mean arterial pressure (MAP), aortic flow (cardiac output), mean cardiac output (CO), LV pressure, and LV dP/dt in one of the young monkeys.

Figure 2. Dose-response curves for mean±SEM percent changes in total peripheral resistance (TPR) are shown for graded injections of SNP (left) and ACh (right) in young (○) and old (●) monkeys. Although dose responses of TPR to SNP are preserved in old monkeys compared with young monkeys, responses to ACh are significantly depressed (P<0.05) in old monkeys.
system in the morning before feeding. These levels were measured by standard laboratory analyses.

**Histopathology**

Histopathology was performed on perfusion-fixed paraffin-embedded sections. Sections cut at 6-μm thickness were stained with hematoxylin and eosin. Aortic tissues were embedded in epoxy resin, thin-sectioned at silver-gray interference color, stained with lead citrate and osmium, and examined on an electron microscope.

DNA fragmentation was detected in situ by using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) on paraffin sections of thoracic and abdominal aortas and femoral arteries from young and old monkeys. Briefly, the paraffin sections were deparaffinized by immersing in xylene, rehydrated through 100%, 95%, 75%, and 0% ethanol, and then incubated in PBS with 2% H₂O₂ to inactivate endogenous peroxidases. After incubation with proteinase K (20 μg/mL), the sections were washed in PBS. DNA fragments in the sections were labeled with 2 nmol/L biotin-conjugated dUTP and 0.1 U/μL TdT for 1 hour at 37°C. The incorporation of biotin-16-dUTP into DNA was determined by incubating the sections with FITC-ExtrAvidin (1:200, Sigma Chemical Co) at room temperature for 30 minutes. After the TUNEL procedure, the slides were washed in PBS, mounted in a DAPI medium (Vector Laboratories), and observed under a fluorescence microscope. The number of nuclei per linear unit in the endothelium was determined by manual counting of DAPI-stained nuclei with UV excitation. At the same magnification, whole fields in the endothelium were examined for TUNEL-positive cells. Endothelium length was measured by computer software with use of the MetaMorph system (Universal Imaging), and endothelial cell density was calculated as the number of endothelial cell nuclei per endothelium length. All morphometric measurements were performed by at least 2 independent individuals in a blinded manner.

**Statistics**

All data were reported as mean±SEM. The comparison between the groups with young and old monkeys was made by unpaired t test for grouped data. The dose-response curves were analyzed by ANOVA.

**Figure 3.** a and b, Photomicrographs of the thoracic aorta from young and old monkeys. Hematoxylin- and eosin-stained cross sections from young and old aortas showed no atherosclerotic lesions in the aortic intima. The old animals show a mild increase in intimal thickness indicated by the arrows. Bar=50 μm. c and d, Transmission electron photomicrographs show sections taken from the thoracic aorta of a young monkey (left) and an old monkey (right). The old animal has intimal thickening characterized by smooth muscle cells (smc) and increased amounts of matrix. The endothelium (en) is intact, and the internal elastic lamina (iel) is present between the intima and the underlying media. Bar=5 μm.
for repeated measurements. The linear regressions for endothelial cell density, percent apoptosis of endothelial cells, and TPR response to ACh (1 \( \mu \)g/kg) relationships were fitted by using commercially available software (StatView, SAS Institute Inc). A value of \( P<0.05 \) was taken as the minimal level of significance.

**Results**

**Analyses of Body Weight, Height, and BSA and Blood Examinations**

There were no significant differences in body weight, height, and BSA between the young and old monkeys. Measurements of cholesterol, triglycerides, and fasting plasma glucose in the blood were also not different between the young and old monkeys.

**Baseline Hemodynamics**

In the old monkeys, the values for heart rate (154 ± 8 bpm), MAP (81 ± 3 mm Hg), LV systolic pressure (103 ± 3 mm Hg), LV dp/dt (2989 ± 203 mm Hg \( \cdot \) s\(^{-1} \)), CI (1.59 ± 0.08 L \( \cdot \) min\(^{-1} \), m\(^{-2} \)), or TPR (52 ± 3 mm Hg \( \cdot \) L\(^{-1} \) \cdot \) min\(^{-1} \) \cdot \) m\(^{2} \)) were not different from the values in young monkeys.

**Peripheral Vasoactivity**

**Response to SNP**

Peripheral vascular responses to an endothelium-independent vasodilator were not impaired in old monkeys compared with young monkeys. Dose responses of TPR to SNP were similar in old and young monkeys (Figure 2). The percentage decreases from baseline for MAP (−21±2% versus −24±2%) and CI (20±2% versus 18±4%) were also not significantly different between young and old monkeys; however, heart rate increased less in old monkeys (20±5%) than in young monkeys (38±6%).

**Response to ACh**

Endothelial function was impaired in old monkeys compared with young monkeys. In contrast to responses to SNP, dose responses of TPR to ACh were significantly decreased in old monkeys compared with young monkeys (Figure 2). ACh decreased MAP in young monkeys (−22±2%) and old monkeys (−20±2%) similarly, but ACh increased CI significantly less (\( P<0.05 \)) in old monkeys (8±3%) compared with young monkeys (20±4%). Responses of heart rate to ACh tended to be less in old monkeys (14±5% [young monkeys] versus 6±2% [old monkeys]).

**Histopathology**

Hematoxylin- and eosin-stained cross sections of young and old aortas showed no atherosclerotic lesions in the arterial intima (Figure 3a and 3b). Animals in the old group often had mild intimal thickening, which was not characterized by lipid. Electron microscopy of these regions of intimal thickening demonstrated only modified smooth muscle cells and matrix connective tissue deposition in the intima beneath an intact endothelium (Figure 3c and 3d).

TUNEL-positive cells were observed in the endothelium of the aorta (Figure 4) and femoral artery but not in the media of the aorta or femoral artery from either young or old monkeys. There was a higher incidence of TUNEL-positive cells in the endothelium of old monkeys compared with young monkeys (Table). Furthermore, the higher incidence of TUNEL-positive endothelial cells was accompanied by reduced endothelial cell density in old monkeys (Figure 4). There was a regression relationship (\( r=0.62, \) \( P<0.02 \)) between the incidence of TUNEL-positive endothelial cells and endothelial cell density in the femoral artery (Figure 5A). The higher incidence of TUNEL-positive endothelial cells was correlated (\( r=0.65, \) \( P<0.01 \)) with diminished TPR responses to ACh (Figure 5B). There was also a significant regression relationship (\( r=0.82, \) \( P<0.01 \)) between the endothelial cell density and TPR response to ACh. The lower endothelial cell density was correlated with reduced TPR responses to ACh (Figure 5C).

**Discussion**

Although most investigations of aged animals\(^ {12–17} \) and humans\(^ {18,19} \) have shown that endothelium-independent relaxation is not affected, it is still controversial whether endothelial function is impaired by aging. Endothelial dysfunction has been demonstrated in aged animals in vitro\(^ {16,17,20,21} \) and in vivo\(^ {12,13} \) and in human studies.\(^ {18,19,22} \) On the other hand, some studies have demonstrated that endothelium-dependent relaxation is unchanged in old rats\(^ {14,21,23} \) and dogs\(^ {15,24} \) and in human coronary arteries.\(^ {25} \) Although Celermajer et al\(^ {6} \) demonstrated in patients that a reduction in flow-mediated vasodilation was associated with age, independent of other coronary risk factors, it was not determined whether nascent atherosclerosis was present, which could have affected the endothelium in those patients and consequently could explain the results. For obvious reasons, histological examination of the vessels was not possible in that study or in other clinical studies. On the other hand, Woo et al\(^ {7} \) found that older
Chinese individuals did not demonstrate endothelial dysfunction but that older white individuals did. Again, histology was not possible in that study. In fact, coronary artery plaques have been shown to be present at an early age, and even angiographically normal vessels may have early atheromatous lesions in young adults.

The present investigation used a novel monkey model of aging, phylogenetically closer to humans but devoid of complications secondary to associated cardiovascular diseases. The old monkeys (aged 20 years) in the present study are thought to be age equivalent to humans aged 60 to 70 years. In addition, environmental factors, which might be related to endothelial dysfunction in humans, were similar among the monkeys. The present results demonstrate that peripheral vasodilatation to ACh, but not to SNP, is impaired with aging in conscious old monkeys, suggesting diminished endothelial control. Importantly, baseline hemodynamics in the old monkeys were not different from those in young monkeys, and cardiovascular diseases associated with aging, eg, atherosclerosis, were not present in the old monkeys. In view of these considerations, it is likely that the reduced vasodilator response to ACh with aging is related to decreased endothelial and nitric oxide (NO) control. The responses of MAP to SNP in young and old monkeys. However, in response to SNP, heart rate was increased more in young monkeys than in old monkeys, which is likely due to an impaired baroreflex function in the old monkeys.

In the present study, a potential mechanism for the endothelial dysfunction, which has not been described previously, was the increased density of apoptotic cells observed in the endothelium of the aorta and femoral artery in old monkeys compared with young monkeys. This was associated with reduced endothelial cell density, which could account for the endothelial dysfunction in vivo. Apoptosis has been observed previously in human atherosclerotic plaques, smooth muscle, and endothelial cells in culture and in other disease states, but it has not previously been shown that apoptosis occurs in nondiseased arteries, in general, and in endothelial cells, in particular, with advancing age. Although the initial cause of increased apoptosis in aging monkey endothelium is not clear, it may be that endothelial cell loss through apoptosis results in further decreased NO production. This process could then accelerate the attenuation of endothelium-dependent smooth muscle cell relaxation. Intimal thickening, although it was only moderate (and not due to atherosclerosis; ie, no lipid deposition was observed), theoretically could also play a role in the decreased vasodilator response to ACh.

Figure 4. Thoracic aortas from a young monkey (a and c) and an old monkey (b and d) stained with DAPI (a and b) to show all nuclei. The same areas were subjected to the TUNEL procedure (c and d), with positive TUNEL-stained endothelial nuclei apparent in the old monkeys (arrows). Bar=25 μm (all panels).
because of the simple physical separation of the endothelium from the media and the resulting impaired endothelial–smooth muscle cell contact.

Additional possible mechanisms of the impaired endothelium-dependent vasodilation are (1) release of endothelium-derived contracting factors (EDCFs), (2) inactivation of NO, and (3) attenuated smooth muscle response to NO. For the first possible mechanism, it has been demonstrated that the production of EDCF induced by ACh is augmented with age in rat aorta28,30, therefore, a concomitant release of EDCF may be related to the impaired vasodilator response to ACh. The second possible mechanism of endothelial dysfunction is inactivation of NO by reactive oxygen species.31 Oxidative inactivation of NO may be important in the pathogenesis of endothelial dysfunction in hypercholesterolemia32 and in hypertension.33 The third possible mechanism, i.e., attenuated smooth muscle response to NO, is not likely, because endothelium-independent relaxation (SNP) was not impaired in the old monkeys.

**Limitation to Experimental Design**

There is one potential limitation to the conclusion that the endothelial apoptosis in aged monkeys was responsible for the impaired endothelial vasodilator responses. The data demonstrating endothelial apoptosis were collected in the aorta and femoral artery, which are not resistance vessels. It would be important to determine whether endothelial apoptosis also occurred in resistance vessels. Nonetheless, this first demonstration of endothelial cell apoptosis and reduced endothelial density associated with deranged endothelial vascular regulation in aged monkeys will stimulate further studies to elucidate the extent to which these findings are causal.

In summary, aging monkeys demonstrate depressed endothelial function independent of atherosclerosis and other age-related diseases. The endothelial dysfunction could be due to the increase in vascular endothelial apoptosis and reduced endothelial cell density in old monkeys.

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