

Aging Is Associated with a Proapoptotic Endothelial Progenitor Cell Phenotype

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Key Words

Age · Apoptosis · Progenitor cells

Abstract

The aim of this study was to determine if aging is associated with enhanced endothelial progenitor cell (EPC) sensitivity to apoptosis. Cells with phenotypic EPC characteristics were isolated from healthy, nonobese young (age 25 ± 1 years) and older (61 ± 1 years) men. Intracellular active caspase-3 concentrations in response to staurosporine stimulation were approximately 35% higher ($p < 0.05$) in EPCs from older (3.15 ± 0.29 pg/ml) compared with young (2.33 ± 0.24 pg/ml) men. Protein expression of Akt, p70 S6-kinase and Bcl-2 was markedly lower (approx. 35, 75 and 60%, respectively, all $p < 0.05$) in EPCs from older compared with young men, whereas there were no age-related differences in either 14-3-3 ϵ or Bax expression. Additionally, EPC telomerase activity was 57% lower ($p < 0.05$) in older (0.18 ± 0.11 AU) versus young (0.43 ± 0.11 AU) men. These results indicate that aging is associated with a proapoptotic EPC phenotype characterized by decreased expression of key antiapoptotic proteins associated with the PI-3-kinase signaling pathway and reduced telomerase activity. These age-related changes like-

ly contribute, in part, to the diminished ability of EPCs to resist an apoptotic stimulus in older men. Increased susceptibility to apoptosis may contribute to the numerical and functional impairments observed in EPCs with aging.

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Introduction

Aging is associated with deleterious modifications in the structure and function of the vasculature, such as increased carotid intima-media thickness and decreased endothelial vasomotor as well as fibrinolytic function [1–3]. Age-related alterations in the vasculature precede and predispose individuals to cardiovascular disease and its clinical sequelae (for example, arterial spasm, myocardial infarction and stroke) [1]. In addition, diminished bioavailability or functional impairments in endogenous vascular repair mechanisms, such as endothelial progenitor cells (EPCs), are thought to contribute etiologically to the manifestation of cardiovascular disease [4]. EPCs are bone marrow-derived circulating cells that are now recognized to play an important role in vascular repair and neovascularization [5]. Indeed, EPCs home to sites

of vascular injury and elicit proangiogenic, prosurvival and anti-inflammatory effects that contribute to the reparative process [6, 7]. Moreover, EPCs can integrate into newly forming blood vessels and are able to transdifferentiate into a variety of cell phenotypes, such as mature endothelial cells and smooth muscle cells [8]. Aging is associated with reduced number and function of EPCs [9, 10], and this may contribute to the greater cardiovascular risk and reduced angiogenic capacity in middle-aged and older adults [11, 12].

Apoptosis, or programmed cell death, is a fundamental cellular program that is highly regulated to protect against aberrant cell death and/or proliferation [13, 14]. Initiation of apoptosis, either by extracellular or internal events, triggers a hierarchy of caspases, a family of cysteine-aspartic acid proteases, leading to cell destabilization and destruction [15]. Induction of apoptosis is highly conserved in biological systems [16], and dysregulation of apoptotic programs can lead to blunted cell viability or unrestricted cell/tissue growth [14]. The phosphoinositide 3-kinase (PI-3-kinase)/Akt signaling pathway plays a key role in regulating caspase-mediated apoptosis. Activation of Akt phosphorylates the downstream protein Bad, leading to its sequestration of Bad via a 14-3-3 ϵ complex. Nonphosphorylated Bad translocates to the mitochondria and inhibits the expression of the antiapoptotic protein Bcl-2. Inhibition of Bcl-2 promotes the release and activation of downstream mitochondrial-associated proapoptotic proteins, such as Bax and cytochrome-c, resulting in caspase-mediated apoptosis [15]. In addition, telomerase activity has been linked to a regulatory role in caspase signaling and apoptosis [17]. The influence of aging on EPC apoptosis is currently unknown. Increased susceptibility to apoptosis may contribute to the numerical and functional impairments observed in EPCs with age [18, 19] and, in turn, age-related CVD risk.

The aim of the current study was to determine if aging is associated with enhanced EPC sensitivity to apoptosis. To address this aim, we assessed active intracellular caspase-3 concentrations and determined the expression of key PI-3-kinase/Akt pathway proteins to gain insight into signaling mechanisms that may contribute to altered apoptotic sensitivity with age.

Methods

Subjects

Peripheral blood samples were obtained from 16 young (age 21–34 years) and 23 older (age 56–67 years) healthy, sedentary men. All subjects were nonobese (BMI \leq 30.0), nonsmokers,

normotensive (arterial blood pressure \leq 140/90 mm Hg), non-medicated and free of overt cardiovascular as well as metabolic disease as assessed by medical history, physical examination and fasting blood chemistries. The older men were further evaluated for clinical evidence of coronary artery disease with electrocardiogram and blood pressure at rest and during incremental exercise performed to exhaustion. Before participation, all subjects provided written informed consent according to the guidelines of the Institutional Review Board of the University of Colorado at Boulder.

EPC Isolation

Putative EPCs were cultured as previously described by our laboratory [10]. Briefly, EPCs were isolated via Ficoll-density gradient centrifugation (Histopaque 1077; Sigma, St. Louis, Mo., USA), washed, resuspended in growth medium (Medium 199; Gibco, Grand Island, N.Y., USA), supplemented with 20% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 mg/ml), and plated on human fibronectin (BD Biosciences, San Jose, Calif., USA) coated 6-well plates for 48 h at 37°C. Thereafter, nonadherent cells were collected for analysis. Endothelial phenotype of these putative EPCs was confirmed by immunofluorescent staining for the uptake of DiI-ac-LDL (Biomedical Technologies, Stoughton, Mass., USA) and expression of von Willebrand factor (Dako, Glostrup, Denmark), VE-cadherin, CD31 and VEGFR-2 (Invitrogen, Carlsbad, Calif., USA). In addition, fluorescence-activated cell-sorting analysis utilizing antibodies recognizing cell surface expression of VEGFR-2 (R&D Systems, Minneapolis, Minn., USA), CD34 (Beckman Coulter, Fullerton, Calif., USA) and CD133 (Miltenyi Biotech, Auburn, Calif., USA) were performed in selected samples.

Caspase-3 Activation and Cytochrome c

Apoptotic susceptibility of EPCs was assessed by determining intracellular active caspase-3 concentrations and cytochrome c levels. Activation of caspase-3 was induced by incubating EPCs with staurosporine for 3 h at 37°C. Cells were then modified with biotin-ZVKD-fmk and lysed in extraction buffer. Both active caspase-3 and cytochrome c concentrations were determined by enzyme immunoassay (R&D Systems). The rationale for using staurosporine to activate caspase-3 is several fold. Firstly, staurosporine is the most frequently used in vitro stimulus of active caspase-3, regardless of the cell line being studied. Secondly, a major advantage of using staurosporine as an apoptosis inducer is that all of the proteins required to execute staurosporine-induced cell death are constitutively expressed [20, 21]. As a result, important mechanisms of cellular apoptosis have resulted from using staurosporine [21, 22]. Thirdly, in addition to the reliability, reproducibility and specificity of action of staurosporine, we chose it to allow comparison of our results to other studies involving cellular apoptosis. Finally, staurosporine elicits similar increases in active caspase-3 compared with ox-LDL [23, 24].

Apoptotic Proteins

EPCs from 10 men (6 young and 4 older) were lysed in cell extraction buffer supplemented with protease inhibitors for determination of proteins in the apoptotic pathway. Proteins (30 μ g) were separated by SDS-PAGE on a 10.4–14% gradient gel (BioRad Criterion) and transferred onto a nitrocellulose membrane. Membranes were blocked overnight in 5% milk protein in phosphate-

buffered saline with 0.5% Tween (PBST). Primary and secondary antibodies were diluted in 5% bovine serum albumin in PBST per manufacturer's suggestions. Membranes were incubated with primary antibodies for 2 h. Thereafter, membranes were thoroughly washed with PBST and incubated with HRP-conjugated secondary antibodies for 2 h. Membranes were then washed with PBST prior to chemiluminescent imaging on a ChemiDoc-It Imaging System (UVP). Membranes were probed for total Akt, total Bad, Bcl-2, Bax, β -actin, 14-3-3 ϵ and p70 S6-kinase.

Telomerase Activity

A telomeric-repeat amplification protocol assay was used for quantitative analysis of telomerase activity (Telo TAGGG PCR ELISA^{PLUS}; Roche Molecular Biochemicals) in EPCs from 14 men (6 young and 8 older) as previously described by our laboratory [10].

Statistics

Group differences were determined by analysis of variance. Relations between variables of interest were assessed by linear and stepwise regression analysis. All data are expressed as mean \pm SEM. Statistical significance was set a priori at $p < 0.05$.

Results

Subject characteristics are displayed in table 1. None of the subjects were obese; however, the older men demonstrated significantly higher percentage of body fat, waist circumference and BMI compared with young men. Although within clinically normal ranges, systolic blood pressure, plasma concentrations of total cholesterol, low-density lipoprotein, triglycerides, glucose and insulin were higher in the older men.

There were no significant differences in basal concentrations of intracellular active caspase-3 (0.33 ± 0.09 vs. 0.44 ± 0.07 pg/ml) and cytochrome c (17.2 ± 0.7 vs. 20.4 ± 2.5 pg/ml) between the young and older men. However, staurosporine-induced intracellular active caspase-3 concentrations were approximately 35% higher ($p < 0.05$) in older (3.15 ± 0.29 pg/ml) compared with young (2.33 ± 0.24 pg/ml) men (fig. 1). There were no significant correlations between staurosporine-induced activation of caspase-3 and any of the measured anthropometric, hemodynamic or metabolic variables. Total Akt, p70 S6-kinase and Bcl-2 expression was significantly lower in the older than the young men (fig. 2). There were no age-related differences in the expression of 14-3-3 ϵ or Bax between groups (fig. 3). EPC telomerase activity was approximately 60% lower in older (0.16 ± 0.08 AU) compared with young (0.49 ± 0.10 AU) men (fig. 4).

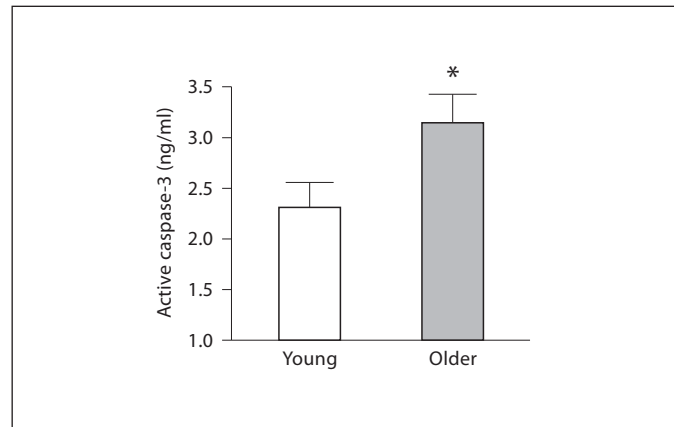


Fig. 1. Intracellular active caspase-3 concentrations in EPCs from young and older men in response to staurosporine stimulation. Values are means \pm SEM. * $p < 0.05$ vs. young men.

Table 1. Selected subject characteristics

Variables	Young (n = 16)	Older (n = 24)
Age, years	25 \pm 1	61 \pm 1*
Body mass, kg	79.7 \pm 2.2	87.3 \pm 2.5*
BMI	24.2 \pm 0.6	27.2 \pm 0.6*
Body fat, %	16.0 \pm 1.2	27.6 \pm 1.3*
Waist circumference, cm	84.0 \pm 1.8	96.8 \pm 1.8*
Systolic BP, mm Hg	113 \pm 3	127 \pm 2*
Diastolic BP, mm Hg	68 \pm 2	78 \pm 1*
Total cholesterol, mmol/l	4.2 \pm 0.2	5.2 \pm 0.2*
LDL cholesterol, mmol/l	2.5 \pm 0.1	3.3 \pm 0.1*
HDL cholesterol, mmol/l	1.3 \pm 0.1	1.2 \pm 0.1
Triglycerides, mmol/l	1.0 \pm 0.1	1.4 \pm 0.1*
Glucose, mmol/l	4.7 \pm 0.1	5.3 \pm 0.1*
Insulin, pmol/l	32.6 \pm 3.1	40.2 \pm 3.9

BMI = Body mass index; BP = blood pressure; LDL = low-density lipoprotein; HDL = high-density lipoprotein. Values are means \pm SEM. * $p < 0.05$ versus young.

Discussion

The primary new finding of the present study is that aging is associated with an increase in apoptotic susceptibility of EPCs in healthy men. Indeed, EPCs harvested from older healthy men demonstrated greater sensitivity to the proapoptotic stimulus staurosporine marked by significantly greater intracellular active caspase-3

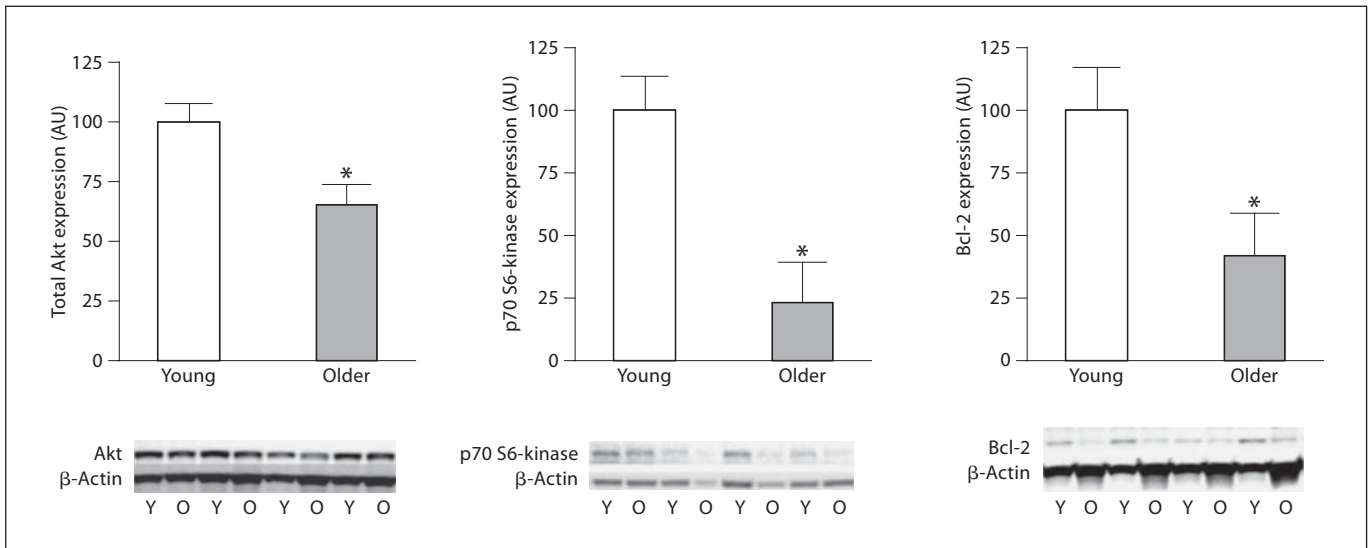


Fig. 2. Akt, p70 S6-kinase and Bcl-2 protein expression in EPCs from young (Y) and older (O) men. Protein abundance is expressed relative to β -actin to account for variations in protein loading and is shown normalized to the young mean. Values are means \pm SEM. * $p < 0.05$ vs. young men.

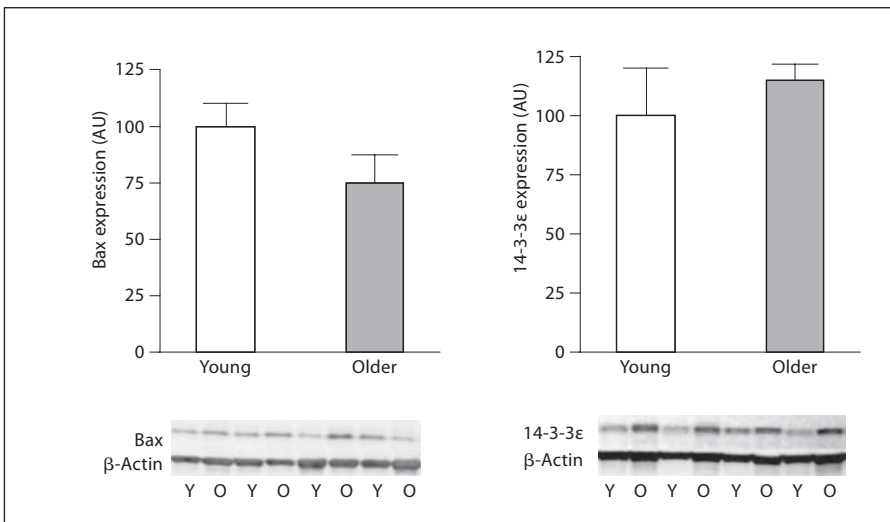


Fig. 3. Bax and 14-3-3 ϵ protein expression in EPCs from young (Y) and older (O) men. Protein abundance is expressed relative to β -actin to account for variations in protein loading and is shown normalized to the young mean. Values are means \pm SEM.

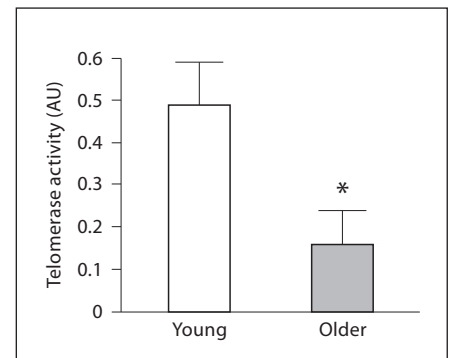


Fig. 4. Telomerase activity in EPCs from young and older men. Values are means \pm SEM. * $p < 0.05$ vs. young men.

concentrations compared with EPCs harvested from young men. To our knowledge, we are the first to demonstrate that EPCs from older adult humans have greater apoptotic sensitivity. Additionally, EPCs from older men have unfavorable alterations in the expression of key PI-3-kinase/Akt signaling pathway-associated pro-

teins. Specifically, we observed attenuated expression of antiapoptotic proteins Akt, p70 S6-kinase and Bcl-2, as well as reduced telomerase activity. Collectively, these data suggest that aging is associated with a proapoptotic EPC phenotype.

Intracellular concentrations of active caspase-3 and cytochrome c provide biological insight into the apoptotic tendency of a cell. Activation of caspase-3, the so-called 'executioner molecule', initiates a myriad of cysteine-aspartic acid proteases resulting in cellular instability, degradation and death. Functional deficits and impaired viability at both the cell and tissue levels have been related to elevations in proapoptotic signaling [25–27]. Hoffmann et al. [28] reported that aging is linked with enhanced sensitivity towards apoptosis via increased caspase-3 activity and reduced activation of the prosurvival enzyme endothelial nitric oxide synthase. Higami and Shimokama [29] reported that apoptosis signaling programs are elevated in multiple tissue depots (such as neurons, cardiac monocytes and hepatocytes) in aged mammals. The novel finding of the present study is that EPCs harvested from older men demonstrated markedly greater active intracellular caspase-3 concentrations in response to staurosporine stimulation compared with EPCs from young men. Heightened sensitivity of EPCs towards apoptosis may be one potential mechanism contributing to reduced EPC bioavailability and function with age [18]. Indeed, accelerated apoptosis and enhanced susceptibility to apoptotic factors are thought to underlie numerical and functional impairments in EPCs and contribute to endothelial dysfunction and atherogenesis [30]. Thus, an apoptotic EPC phenotype can limit bioavailability and reparative capacity, ultimately contributing to the increased risk of atherosclerosis and the prolonged and often complicated recovery from acute vascular events in older adults [18, 30].

The mechanisms responsible for the apparent proapoptotic EPC phenotype with aging are unclear. Factors such as inflammation and oxidative stress that are known to increase with age may play a role [31, 32]. Indeed, the pro-inflammatory cytokine C-reactive protein, that generally increases with age, has been shown to induce EPC apoptosis *in vitro* [33]. To elucidate possible signaling mechanisms that may be responsible for the greater apoptotic susceptibility of EPCs in older adults, we determined the relative expression of several upstream proteins in the PI-3-kinase/Akt signaling pathway known to effect caspase-3 activation. This pathway plays a vital role in cell survival by initiating downstream activation of antiapoptotic proteins and suppression of cell death signals [34]. Perturbations in PI-3-kinase/Akt signaling may lead to either excessive cell death or a number of disease processes, such as in cancer [34, 35]. In the present study, we demonstrated that aging is associated with decreased expression of antiapoptotic proteins involved in the PI-3-kinase/Akt signaling pathway. Specifically, older men had lower Akt,

p70 S6-kinase and Bcl-2 expression compared with young men. Previous studies have shown that constitutive activation of Akt and p70 S6-kinase promotes cell survival, while pharmacological inhibition of these proteins increases apoptosis [36]. Similarly, suppression of Bcl-2 expression results in greater cell death in multiple cell types [37, 38]. Thus, the greater apoptotic susceptibility in EPCs from older men may be due, at least in part, to overall lower expression of antiapoptotic proteins associated with the PI-3-kinase/Akt signaling pathway. In addition, telomerase activity has been shown to be inversely associated with intracellular caspase-3 activation [39]. Considering we observed approximately 60% less telomerase activity in EPCs from older men compared with younger men, it is possible that age-related reductions in telomerase activity may contribute to enhanced caspase-3 activation.

A number of experimental considerations regarding the present study should be mentioned. Firstly, there is no clear consensus on what criteria should be used to isolate, culture or quantify EPCs, creating ambiguity when comparing and interpreting investigations. In the present study, we isolated putative EPCs with minimal time spent in culture. These cells demonstrate several endothelial phenotypic characteristics including DiI-ac-LDL uptake, lectin binding and expression of von Willebrand factor, VE-cadherin, VEGFR-2 and CD31 [40, 41]. Additionally, it has been shown that these cells participate in vasculogenesis and repair [5] and are related to both endothelial dysfunction and cardiovascular risk [42, 43]. Other studies involving EPCs have employed different isolation procedures and *in vitro* conditions, such as expanding cells on culture dishes coated with different extracellular matrix proteins (collagen, gelatin) and exposure to extended endothelial specific *in vitro* culture conditions (2–4 weeks) [44]. These techniques may yield different results and thus the findings of the present study should be viewed within the context of our isolation, culture and characterization methodology. Secondly, as with all cross-sectional experimental designs, we must acknowledge the possibility that genetic and/or lifestyle behaviors influenced our results. Moreover, numerous pathologic, pharmacologic and physiologic factors have been reported to influence EPC number and function. We attempted to minimize these potential influences by studying strictly screened healthy adults who were free of overt disease, nonmedicated, non-smokers and not physically active. Thirdly, reduced telomerase activity has been linked to cellular senescence [45, 46]; we did not address the possible influence of age-related senescence on our results. Fourthly, although we observed significant age-related differences in the protein

expression of Akt, p70 S6-kinase and Bcl-2, we are unable to comment on the activity status of these proteins. Finally, the results reported herein pertain only to men; considering differences in EPC function have been reported between men and women [9] extrapolation of our findings to women should be done with caution.

In summary, both experimental [47, 48] and clinical studies [49, 50] have shown that EPCs are essential to vascular homeostasis. Their capacity to maintain, repair and/or regenerate the endothelial monolayer and restore functional activity is fundamentally important to prevent atherosclerotic lesion development and thrombus formation [7, 51]. Herein, we demonstrate that EPCs harvested from older men have elevated apoptotic suscepti-

bility. Additionally, our results suggest that aging tips the balance between pro- and antiapoptotic signaling proteins to a more proapoptotic EPC state. The proapoptotic phenotype in EPCs harvested from older men may contribute, at least in part, to the age-related decline in EPC number and function observed in this population.

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