eNOS Activity Is Reduced in Senescent Human Endothelial Cells
Preservation by hTERT Immortalization

Hidetsugu Matsushita, Edwin Chang, Alexander J. Glassford, John P. Cooke, Choy-Pik Chiu, Philip S. Tsao

Abstract—Advanced age is associated with endothelial dysfunction and increased risk for atherosclerosis. However, the mechanisms for these observed effects are not clear. To clarify the association between aging and loss of endothelial function, young human aortic endothelial cells (HAECs), senescent HAECs transfected with control vector, and immortalized HAECs containing human telomerase reverse transcriptase (hTERT) were compared for expression of endothelial nitric oxide synthase (eNOS) and production of NO. To investigate a specific function modulated by endothelial NO, adhesion of monocytes under basal conditions as well as after exposure to TNF-α was assessed. A decrease in eNOS mRNA, protein, and activity was observed in endothelial cells at senescence as compared with young HAEC; this effect was blunted in hTERT cells. In all cells, shear stress induced a greater increase in the expression of eNOS protein with the final result being higher levels in hTERT compared with senescent cells. Basal monocyte binding was significantly elevated on aged endothelial cells compared with parental and hTERT cells. Exposure of TNF-α resulted in a 2-fold increase in monocyte adhesion in senescent cells, whereas this effect was reduced in cells transfected with hTERT. Prior exposure to fluid flow significantly reduced subsequent monocyte adhesion in all groups. These studies demonstrate that replicative aging results in decreased endothelial expression of eNOS accompanied by enhanced monocyte binding. Stable expression of hTERT results in endothelial cells with a younger phenotype with greater amount of eNOS and NO activity. Thus, telomerase transfection may have important functional consequences on endothelial cells. (Circ Res. 2001;89:793-798.)

Key Words: aging ■ telomerase ■ atherosclerosis ■ nitric oxide ■ shear stress

Vascular endothelial cells play an important role in maintaining normal vascular homeostasis by producing a balance of paracrine factors such as nitric oxide (NO) and angiotensin II. Many of these factors are known not only to play an important role in the regulation of vessel tone, but also have dramatic effects on vascular structure. Nitric oxide, for example, is not only a potent vasodilator, but it also inhibits platelet adhesion and reactivity, smooth muscle proliferation, and leukocyte adhesion.¹ Because each of these processes is thought to be important in the development of atherosclerosis, nitric oxide is hypothesized to be an important endogenous antiatherogenic molecule.

Nitric oxide is produced from the conversion of l-arginine to l-citrulline by the enzyme nitric oxide synthase (NOS).² In endothelial cells, the constitutive isoform, eNOS (NOSIII) is responsible for endothelium-derived NO production. In vivo, the predominant physiological stimulus for NO is wall shear stress.³ NO is produced in response to not only the specific level of shear but also to acute changes in blood flow. Interestingly, eNOS expression is also shear responsive with increasing levels of laminar shear stress resulting in enhanced eNOS mRNA and protein.⁴ Because NO has important regulatory function in atherogenesis, increased eNOS expression by laminar shear stress has been hypothesized to be a factor in the resistance of specific vascular segments to atherosclerosis, as compared with areas with more turbulent or complicated flow patterns.

Several risk factors for atherosclerosis are associated with reduced endothelial function characterized by diminished NO activity. Because atherosclerosis is thought to be an age-related disease, it is not surprising that increasing age is an independent risk factor for atherosclerosis.⁵ Older animals develop more atherosclerosis than younger counterparts even with the same cholesterol burden, thus implicating an age-dependent, endogenous alteration of the vessel wall as a factor in atherogenesis.⁶ Furthermore, aging is associated with other key processes in atherogenesis, such as increased susceptibility of LDL to oxidation, reduced circulating levels

References


Original received May 8, 2001; revision received August 29, 2001; accepted August 29, 2001.
From the Division of Cardiovascular Medicine, Department of Medicine (H.M., A.J.G., J.P.C., P.S.T.), Stanford University School of Medicine, Stanford, Calif; and the Geron Corp (E.C., C-P.C.), Menlo Park, Calif.
Correspondence to Philip S. Tsao, PhD, Stanford University School of Medicine, 300 Pasteur Dr, Stanford, CA 94305-5246. E-mail ptsao@stanford.edu
© 2001 American Heart Association, Inc.
Circulation Research is available at http://www.circresaha.org

DOI: 10.1161/01.HH2101.098443
of antioxidant enzymes, and increased endothelial expression of adhesion molecules such as vascular cell adhesion molecule-1.7,9

Although several animal and human studies have demonstrated reduced NO activity with aging, the exact mechanism for this effect remains controversial. Chou et al10 observed a significant reduction in eNOS expression with increasing age in the spontaneously hypertensive rat (SHR) but not in the normotensive Wistar Kyoto (WKY) rat. On the other hand, Cernadas et al11 documented a paradoxical increase in eNOS expression associated in older rats associated with reduced endothelium-dependent relaxation.

Somewhat cells have a limited lifespan in vivo as well as in vitro and retire into senescence after a predictable number of cellular divisions. Telomeres, specialized end portions of eukaryotic chromosomes, shorten as a linear function of increasing cellular division, and a critically short telomere length triggers the onset of senescence. Differential expression of telomerase, a ribonucleoprotein complex that adds hexamer DNA repeats (TTAGGG) to the ends of chromosomes, has been implicated in cellular senescence of normal cells and immortalization of tumor cells.12 Expression of the catalytic component of human telomerase, human telomerase reverse transcriptase (hTERT), extends the lifespan of human fibroblasts and retinal pigmented epithelial cells beyond senescence without causing neoplastic transformation.12,13 Previously we have reported that both human large vessel and microvascular endothelial cells also bypass replicative senescence after introduction of hTERT. Indeed, relative to cells at senescence, hTERT-expressing endothelial cells exhibit resistance to induction of apoptosis by a variety of different conditions.14

Reparative aging in vitro of vascular cells involves alterations in gene expression,15,16 protein expression,16,17 and endothelial function.14,18,19 A number of these alterations occur with aged vasculature in vivo.10,20 Enhanced reparative turnover of vascular cells in vivo, as assessed by increased attrition in telomere length with respect to donor age, occurs in regions susceptible to atherosclerosis, such as the iliac artery and the abdominal aorta.21,22 These findings suggest that age-dependent dysfunction seen in vascular disorders may partially be attributed to enhanced turnover in focal regions of vascular tissue in vivo. However, the precise mechanisms whereby replicatively aged cells would contribute to vascular disorders are uncertain.

We designed this study to investigate the effect of aging on endothelial nitric oxide activity in human aortic endothelial cells (HAECs). Furthermore, we hypothesized that immortalizing cells by introduction of hTERT would induce a younger phenotype in HAECs and, therefore, enhance NOS expression. We provide evidence that senescent HAECs express lower levels of eNOS mRNA and protein and produce less NO compared with early passage cells. This effect was abrogated by stable expression of hTERT. In addition, an important function of NO, namely inhibition of monocyte adhesion, was found to be reduced in older endothelial cells by stable integration of hTERT.

**Materials and Methods**

**Cell Culture**

HAECs were obtained from Clonetics Corp (San Diego, Calif) and cultured in modified MCDB131 medium containing epidermal growth factor (10 ng/mL), hydrocortisone (1 μmol/L), VEGF (0.5 mL), hFGF-B (2 ng/mL), R3-IGF (0.5 mL), ascorbic acid (0.5 mL), heparin (0.5 mL), gentamycin, and supplemented with 15% FBS. Human monocyted cells (THP-1) were maintained in M199+10% FCS. Cells were incubated at 37°C in a humidified atmosphere of 95% air/5% CO2, with medium changes every 2 days.

HAEC-hTERT cells were generated by gene transfer of pBabe-hTERT (a generous gift from Dr Woody Wright, University of Texas Southwestern Medical Center, Dallas, Tex) by retrovirus, as previously described.12 Stable expression of hTERT was verified by reverse-transcriptase polymerase chain reaction. In addition, two different assays were used to demonstrate increased telomerase activity after gene transduction. Previous studies indicate that although transfection of empty retrovirus vector has no effect on endothelial cell morphology or function, transfection of hTERT extends the lifespan beyond senescence without causing neoplastic transformation.14 The population doubling ranges for the cells studied were 20 for low-passage parental cells, 58 to 60 for senescent (vector control) cells, and 100 to 140 for hTERT-containing HAECs.

**Fluid Flow**

Confluent monolayers were placed in serum-free medium for 1 hour and then exposed to static conditions or flow. Flow was induced by placement of confluent 60-mm culture dishes on a mixing table (Thermylene) rotating at 120 rpm for 6 or 24 hours. Compared with the well-defined cone-plate viscometer, this technique induces qualitatively similar changes in cell alignment, NO production, and NOS mRNA transcription.23,24

**Northern Blotting**

Total RNA was isolated using TRI reagent. RNA (20 μg) was separated on 1.3% agarose gels containing 2.2 mol/L formaldehyde and transferred to nylon membrane (Osmonics Inc). The cDNA probes were labeled with [32p]dCTP by random primer kit (Amersham). The blots were autoradiographed using Kodak Biomax MR film (Eastman Kodak) at −80°C.

**Western Blotting**

Western blot was performed for analysis of eNOS protein levels. Total protein was extracted by incubation of cells in lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 20 mmol/L EDTA, 1% SDS, 100 mmol/L NaCl). Samples containing 30 μg protein were run on 6% sodium dodecyl sulfate polyacrylamide gels. Proteins were separated by SDS/PAGE, transferred to nitrocellulose membrane (Hybond ECLTM, Amersham), and incubated overnight with a polyclonal antibody to eNOS (1:200). Antibodies were diluted in 4% nonfat dry milk and 0.1% Tween 20 in PBS. The membranes were then washed and incubated with a 1:2000 dilution of rabbit IgG horseradish peroxidase-conjugated antibody (Amersham). Bound antibodies were detected by enhanced chemiluminescence (ECL, Amersham) and HyperfilmTM-MP, Amersham). Western blotting of the structural protein α-tubulin (1:100, Calbiochem) was also performed to confirm the equal amounts of loaded proteins.

**Measurement of NO Production**

Nitrite (NO2), the stable breakdown product of NO, in conditioned medium from endothelial cells after static or shear-stress conditions was measured by the Griess reaction after 10X concentration with a Speed-Vac (Savant, Holbrook, NY).25 The cells were gently detached with 5 mmol/L EDTA, and cell protein was measured according to the Lowry method. NO2 data were expressed as picomole per milligram of cell protein.
Adhesion Assay

Monocyte adhesion assays were performed as previously described. Briefly, cells exposed to the above conditions were washed with HBSS (Irvine Scientific) containing (in mmol/L) CaCl$_2$ 2, MgCl$_2$ 2, and HEPES 20. In some cases, cells were subsequently stimulated with tumor necrosis factor (TNF-$\alpha$; 250U/mL) for an additional 4 hours in static conditions. Culture dishes were then placed on a rocking platform, and THP-1 cells were incubated with cells for 30 minutes, with dishes rotated 120° clockwise every 10 minutes to ensure even distribution of cells. Medium was aspirated and replaced with fresh HBSS to remove nonadherent monocytes. After a second washing, dishes were returned to the rocker platform for an additional 5 minutes. Medium was again aspirated and replaced with HBSS containing 2% glutaraldehyde. After overnight fixation, adherent cells were quantified by computer-assisted video microscopy.

Statistical Analysis

All values are expressed as mean±SEM of at least 4 separate experiments. Analysis of variance with subsequent Bonferroni’s test was used to determine significant differences in multiple comparisons. A value of $P<0.05$ was considered significant.

Results

Effect of Aging on eNOS mRNA and Protein Levels

The expression of eNOS mRNA was reduced in senescent vector control compared with low-passage parental HAECs. Densitometric analysis of autoradiographic bands showed a 50% to 75% reduction in eNOS expression in Vector Control endothelial cells. By contrast, cells stably transfected with hTERT demonstrated levels of eNOS mRNA similar to parental cells (Figure 1; $P<0.01$). Western blotting demonstrated that these changes in mRNA were mirrored by eNOS protein levels (Figure 2; $P<0.01$). In addition, production of NO by senescent cells was blunted compared with parental or hTERT endothelial cells (Figure 3; $P<0.05$). Similar values were found when NO production was normalized to cell number. To demonstrate that these results were not unique to the low-serum conditions, NO production was measured in cells exposed to growth medium containing 10% serum. These studies also indicated that NO production in senescent vector control endothelial cells during static or shear conditions was significantly lower than parental or hTERT cells (data not shown).

Prior studies have shown that laminar shear stress induces both morphological and biochemical changes in endothelial cells. After exposure to 24 hours of fluid flow, all endothelial cells demonstrated an elongated phenotype with the long axis aligned with the vector of flow. Interestingly, though not quantified, the morphological response of senescent endothelial cells appeared blunted relative to parental and hTERT cells (data not shown). In addition, shear stress induced an
increase in eNOS mRNA (Figure 1) and protein (Figure 2) \( (P<0.01) \). However levels in senescent cells after shear stress remained significantly lower than parental or hTERT cells.

**Monocyte Binding**

Because NO is known to regulate leukocyte adhesion, monocyte-binding assays were performed to monitor an important physiological function of endothelium-derived NO. Senescent cells display increased monocyte adhesion compared with low-passage parental and hTERT cells at baseline conditions (Figure 4). Increased expression of eNOS in hTERT transfected cells was associated with normalization of monocyte binding. As we have previously demonstrated, exposure of HAECs to 4 hours fluid flow reduces endothelial adhesiveness for monocytes.24,26 In the present study, shear stress also inhibited endothelial adhesiveness in hTERT cells to a level similar to parental cells. Although reduced endothelial adhesiveness for monocytes after shear stress was observed in senescent cells, the level of binding was significantly higher than parental and hTERT cells.

**Monocyte Binding After TNF-α Stimulation**

Inflammatory cytokines such as TNF-α have been shown to increase the adhesive properties of vascular cells. To assess the effect of inflammatory stimulus in cells transfected with hTERT, cells were exposed to TNF-α for 4 hours under static conditions. Exposure to TNF-α resulted in a significant increase in endothelial adhesiveness in all cells tested. However, the resultant number of cells bound was greater in senescent cells compared with parental or hTERT cells. (Figure 5).

**Discussion**

Vascular endothelial cells play an important role in maintaining normal vascular homeostasis. With increasing age, however, endothelial cell function may be compromised. Endothelial dysfunction is a hallmark of a number of vascular disorders, such as changes in vascular compliance, increased monocyte binding, and atherogenesis.1 The age-dependent disruption in vascular function could be because of focal portions of the vasculature reaching replicative senescence and elaborating a significantly different profile of gene expression compared with replicitively young cells.15,21 Several of these changes may have important implications for atherogenesis.

Replicative senescence is associated with the endpoint indicated by a critical shortening of chromosomal ends due to the “end-replication problem” of DNA synthesis.27 The function of telomerase is implicated in cellular senescence and immortalization of cells.12 Expression of the catalytic component of human telomerase, human telomerase reverse transcriptase (hTERT) extends the lifespan of human fibroblasts, retinal pigment epithelial cells, and dermal endothelial cells beyond senescence without causing neoplastic transformation.12,14 The data presented in this study demonstrate that expression of hTERT also preserves several functions normally associated with endothelial cells.

Our data indicate that endothelial cell aging reduces the expression and function of eNOS. This is in concordance with data from other groups indicating that NO activity decreases.
Endothelial cells are exquisitely sensitive to the biomechanical force of laminar shear stress, responding with altered intracellular signaling and gene expression. One of the earliest described endothelial responses to shear was an increase in NO activity. Moreover, shear stress is a potent inducer of eNOS expression at the mRNA and protein levels. Here we showed that this response appears to be preserved with endothelial cell age. However, the basal level of NO activity, as well as the level attained after shear, is significantly lower than in younger cells. In comparison, shear stress caused a greater response in hTERT cells with eNOS upregulation similar to that of younger HAECs. This is true despite the fact that the hTERT cells underwent nearly twice as many doublings as senescent cells.

The mechanism responsible for enhanced atherosclerosis with increasing age is still controversial and most probably multifactorial. The earliest observable abnormality of the vessel wall in animal models of atherosclerosis is enhanced monocyte adherence to the endothelium. This phenomenon is thought to be mediated by induction of adhesion molecules and chemotactic proteins such as vascular cell adhesion molecule (VCAM)-1 and monocyte chemotactic proteins. Liao and colleagues have shown that NO can reduce cytokine-stimulated expression of VCAM-1. Moreover, the enhanced activity of eNOS is known to have important stimulatory effects on endothelial cell proliferation. Furthermore, the angiogenic activity of endothelial cells display higher levels of eNOS than confluent cells, all experiments in the recent studies were performed using confluent cells. In addition, normal telomerase activity in replicating endothelial cells is repressed in quiescent, confluent monolayers. Importantly, the enhanced activity of NO is known to have important stimulatory effects on endothelial cell proliferation. Moreover, the angiogenic effects of various growth factors are dependent on the action of NO. It is interesting to note that Dimmeler and colleagues have recently demonstrated that exogenous NO can also activate endothelial telomerase activity and delay passage-induced senescence.

Matsushita et al have demonstrated that subconfluent, dividing endothelial cells display higher levels of eNOS than confluent cells, all experiments in the recent studies were performed using confluent cells. In addition, normal telomerase activity in replicating endothelial cells is repressed in quiescent, confluent monolayers. Importantly, the enhanced activity of NO is known to have important stimulatory effects on endothelial cell proliferation. Moreover, the angiogenic effects of various growth factors are dependent on the action of NO. It is interesting to note that Dimmeler and colleagues have recently demonstrated that exogenous NO can also activate endothelial telomerase activity and delay passage-induced senescence.

Endothelial cells are exquisitely sensitive to the biomechanical force of laminar shear stress, responding with altered intracellular signaling and gene expression. One of the earliest described endothelial responses to shear was an increase in NO activity. Moreover, shear stress is a potent inducer of eNOS expression at the mRNA and protein levels. Here we showed that this response appears to be preserved with endothelial cell age. However, the basal level of NO activity, as well as the level attained after shear, is significantly lower than in younger cells. In comparison, shear stress caused a greater response in hTERT cells with eNOS upregulation similar to that of younger HAECs. This is true despite the fact that the hTERT cells underwent nearly twice as many doublings as senescent cells.

The mechanism responsible for enhanced atherosclerosis with increasing age is still controversial and most probably multifactorial. The earliest observable abnormality of the vessel wall in animal models of atherosclerosis is enhanced monocyte adherence to the endothelium. This phenomenon is thought to be mediated by induction of adhesion molecules and chemotactic proteins such as vascular cell adhesion molecule (VCAM)-1 and monocyte chemotactic protein-1. Endothelium-derived NO is a potent inhibitor of endothelial interaction with monocytes in part by inhibiting expression of important adhesion molecules and chemokines. In addition, the physical stimulus of shear stress causes endothelial cells to produce greater amounts of NO and further inhibit leukocyte adherence. However, the effect of aging on endothelial adhesiveness for monocytes is not clear.

We have shown that basal and cytokine-stimulated monocyte adhesion is increased in senescent compared with younger endothelial cells. These effects were abrogated by increasing the lifespan of endothelial cells with stable expression of hTERT. The activity of endothelium-derived NO in aging cells in all likelihood plays a major role in this interaction. Monocyte adherence to endothelial cells in culture may be inhibited by administration of NO with a short time course that implies an effect of adhesion pathways on signal transduction. We have previously demonstrated that the adherence of monocytes to bovine aortic endothelial cells is inhibited by brief (ie, 15-minute) exposure to NO donors or increases in endogenous NO in the absence of any changes in VCAM-1 or intercellular adhesion molecule-1 expression. However, long-term exposure of endothelial cells to NO may have a persisting effect on their adhesiveness for monocytes by reducing the expression of adhesion molecules and chemokines. Liao and colleagues have shown that NO can reduce cytokine-stimulated expression of VCAM-1. This effect is thought to be because of the ability of NO to increase the expression and stability of iκBα. Recent studies indicate that these regulatory mechanisms are dysfunctional in older endothelial cells and that stable expression of hTERT may rescue these effects.

In summary, we have demonstrated that stable expression of hTERT reverses the deficit in eNOS expression with increasing age. This effect is associated with increased NO activity and reduced endothelial adhesiveness for monocytes. These results indicate that maintenance of a youthful phenotype for endothelial cells through hTERT expression may provide a rationale and strategy for therapeutic interventions to atherogenesis.

Acknowledgments
This work was supported by a grant from the National Institutes of Health (HL62889) and the Pacific Vascular Research Foundation. Dr Tsao is a recipient of a Scientist Development Grant from the American Heart Association.

References


17. Chang EB. Rigidity and aging: do we become more rigid in our ideas as we age? Perspectives. 1993;17:12–14.


eNOS Activity Is Reduced in Senescent Human Endothelial Cells: Preservation by hTERT Immortalization
Hidetsugu Matsushita, Edwin Chang, Alexander J. Glassford, John P. Cooke, Choy-Pik Chiu and Philip S. Tsao

Circ Res. 2001;89:793-798; originally published online September 13, 2001; doi: 10.1161/01.HHR.0000046588.69987.C7

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/89/9/793

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/