Glycated Collagen I Induces Premature Senescence-Like Phenotypic Changes in Endothelial Cells

Jun Chen, Sergey V. Brodsky, David M. Goligorsky, Dierk J. Hampel, Hong Li, Steven S. Gross, Michael S. Goligorsky

Abstract—Diabetic vasculopathy is central to the development of diverse cardiovascular, renal, retinal, and neurological complications of diabetes. We previously demonstrated that growth of endothelial cells on glycated extracellular matrix proteins (collagen and matrigel) results in a significant decrease in cell proliferation. In the present study, we show that early-passage human umbilical vein endothelial cells (HUVECs) grown on glycated collagen (GC) express hallmarks of premature cell senescence, ie, increase in the proportion of cells expressing senescence-associated \( \beta \)-galactosidase activity, apoptotic rate, and p53 and p14\(^{ARF} \) expression, but in contrast to replicative senescence, display neither attrition of telomeres nor decrease in telomerase activity. An increased frequency of prematurely senescent cells was similarly observed in vivo in aortae of young Zucker diabetic rats, compared with lean controls. NO production by HUVECs grown on GC was decreased, despite a 3-fold increase in eNOS expression and was associated with the increased nitrotyrosine-modified proteins. Development of premature senescence of HUVECs on GC could be prevented and reversed by treatments with the peroxynitrite scavenger, ebselen, eNOS intermediate \( \text{N}^\mathbf{\text{0}}\)-hydroxy-L-arginine (NOHA), or superoxide dismutase mimetic Mn-TBAP. Concomitant with the reversal of senescence, ebselen, and NOHA each restored NO production to levels observed with HUVECs grown on unmodified collagen. Our findings indicate that diabetes mellitus in vivo and GC exposure in vitro elicit premature senescence of the vascular endothelium, a process with distinct pathogenetic mechanisms. Premature senescence of the vascular endothelium is hypothesized to be an important contributor to diabetic vasculopathy and a consequence of reduced NO availability, peroxynitrite, and/or superoxide excess. (Circ Res. 2002;90:1290-1298.)

Key Words: telomerase \( \bullet \) nitric oxide \( \bullet \) superoxide \( \bullet \) p53 \( \bullet \) p14\(^{ARF} \)

Endothelial dysfunction is emerging as a key component in the pathophysiology of diverse cardiovascular abnormalities associated with atherosclerosis, diabetes, hypertension, and aging.\(^{1,2} \) Increased incidence of apoptotic cell death has been implicated as a hallmark of endothelial dysfunction.\(^{3-5} \) Molecular triggers of apoptosis in endothelial cells include oxidized LDL, TNF-\( \alpha \), bacterial lipopolysaccharide, oxidative stress, peroxynitrite, turbulent fluid shear stress, and high glucose, to name a few.\(^{6-8} \) Diabetes mellitus has been shown to be associated with a gradual increase in apoptotic death of endothelial cells,\(^{9} \) suggesting that some clues to vasculopathy may lie in the diabetic microenvironment.

In our previous studies of endothelial cells cultured in glycated collagen (GC)–containing gels, we demonstrated a defect in branching angiogenesis, accompanied by a time-dependent reduction in the rate of cell proliferation.\(^{10} \) This latter finding, consistent with the possibility of developing cell senescence,\(^{11} \) prompted us to explore the expression of other cellular markers of aging. Cellular aging has been extensively studied with reference to replicative aging, the process that is characterized by the appearance of senescence-associated \( \beta \)-galactosidase (SA \( \beta \)-gal) staining, expression of several regulators of the cell cycle, attrition of telomeres and suppression of telomerase activity, and irreversibility of the process.\(^{12,13} \) Hallmarks of the replication-independent cell senescence, however, remain scarcely examined.\(^{14} \) In the present study, we demonstrated that GC induces premature endothelial cell senescence, as judged by the appearance of SA \( \beta \)-gal staining, increased cell size, rate of apoptosis, and expression of p53 and p14\(^{ARF} \) in a dose- and time-dependent manner. However, telomerase activity and the length of telomeres did not show significant changes, suggesting that the pathogenesis of premature senescence is distinct from that of replicative senescence. Endothelial cell senescence was associated with a decreased calcium-dependent synthesis of nitric oxide (NO), despite an increased expression of eNOS, and with an increased abundance of nitrotyrosine-modified proteins. In contrast to the replicative senescence, premature senescence of human umbilical vein endothelial cells...

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Materials and Methods

Cell Culture
HUVECs were used between passages 3 to 5 and cultured in EGM-2 medium (Clonetics, San Diego, Calif) containing 2% fetal bovine serum. To generate “aged” HUVECs, cells were consecutively passaged 22 times until cell replication became near-arrested. Cells were passaged by trypsinization after reaching 80% to 90% confluency and seeded at about 4000 to 5000 cells/cm² in native or GC-precoated dishes. Matrix-coated dishes were prepared by incubation with collagen I (400 to 500 μg/mL, per 20 cm²) at 37 °C for 1 to 2 hours, dried, sterilized under UV light, and washed once with PBS. GC was prepared according to a previously detailed protocol. Concentration of glycated epitopes on collagen I equaled 94% of the AGE-BSA standard. This GC was dissolved 1:3 (vol:vol) in native collagen (NC) to generate samples used in this study. The carboxymethyllysine content averaged 6.5 μmol/g and fructosamine content was 30 μmol/g of collagen.

Cytocchemical and Histochemical Staining for Senescence-Associated β-Galactosidase and Apoptosis
SA β-gal expression by HUVECs was analyzed at pH 6, according to Dimri et al. Stained cells were viewed under an inverted microscope at ×200. The percentage of SA β-gal–positive cells was determined by counting the number of blue cells under bright field illumination, and the total number of cells in the same field under phase contrast. At least 8 random fields were counted for each culture dish. Detection of SA β-gal in the en face aortic preparations was performed using a previously described protocol. Quantification of apoptotic cells was performed using the following techniques: Hoechst and Annexin-V in situ staining and FACS analysis. The data presented were obtained by counting the number of apoptotic cells per 500 cells using fluorescence microscopy (in each experiment at least 15 to 20 randomly chosen fields were examined). FACS analysis of HUVECs stained with Annexin-V-FITC was performed using FACSscan flow cytometer (Beckton-Dickinson).

Immunoblot Analysis
Cell lysates were prepared in a buffer containing 50 mmol/L Tris, pH 7.4, 100 mmol/L NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, and protease inhibitor cocktail (Roche). Lysates were assayed for total protein concentration (BCA assay, Pierce), and 20 μg of clarified extract was resolved on a 4% to 20% SDS-polyacrylamide gel. Proteins were transferred to Immobilon-P membranes (Millipore) and incubated at 4 °C overnight with primary antibodies. HRP-conjugated anti-mouse or -rabbit secondary antibody was used for detection using enhanced chemiluminescence. Primary antibodies used were as follows: monoclonal anti-p53, polyclonal anti-p14ARF (Santa Cruz), and monoclonal anti-ENOS (Transduction Laboratories). Membranes were re-probed with monoclonal anti-β-tubulin (Sigma) to confirm equivalent loading of cell extracts. Nitrotyrosine-modified proteins were analyzed using immunoprecipitation with an anti-nitrotyrosine monoclonal antibody, followed by immunoblotting using an anti-nitrotyrosine polyclonal antibody (Upstate Biotechnology). Results were normalized by equalizing the protein concentration of each cell extract before immunoprecipitation and equivalent protein loading was confirmed by results of β-tubulin immunodetection on a separate gel.

Measurement of Telomere Terminal Restriction Fragment (TRF) Length and Telomerase Activity
Genomic DNA was prepared using a QiaGen DNA isolation kit. For this purpose, 5 μg DNA was digested overnight at 37 °C with 20 U of restriction enzymes Rsal and Hinfl (Roche), followed by precipitation with ethanol. After washing with 70% ethanol, samples were dissolved in 20 μL of TE buffer and resolved on a 0.6% agarose gel at 40 V and 4 °C overnight, together with a DNA ladder marker (1-Kb DNA ladder and DNA/HindIII, Gibco). Gels were denatured for 30 minutes in 0.5 mol/L NaOH/1.5 mol/L NaCl and neutralized for 30 minutes in 0.5 mol/L Tris, pH 7.4/1.5 mol/L NaCl. DNA was transferred to a nylon membrane, hybridized with digoxigenin 3′-end tailing-labeled (Roche) (TTAGGG)_n oligonucleotides overnight at 50 °C in PerfectHyb plus solution (Sigma). Membranes were washed twice in 2×SSC, 0.1 SDS for 15 minutes at room temperature and twice in 0.1××SSC, 0.1 SDS for 15 minutes at 50°C. The probe was detected by the digoxigenin luminescence detection (Roche). The optical density (OD) of each lane between 5 to 20 Kβ was analyzed with the NIH Scion Image software, and the length of TRFs was calculated as follows: TRF = 2OD/i/ODmax. Determination of telomerase activity (RTA) was derived by comparing the absorbance of samples to that of a control template. The PCR products were also resolved on a 12% nondenaturing polyacrylamide gel and visualized using biotin-luminescence detection protocol, which shows a series of ladder-like bands (TRAP ladder) with 6 bases difference between adjacent bands.

NO-Selective Microelectrode Measurements
NO concentration was monitored using porphyrin-electroplated, Nafion-coated, carbon-fiber electrodes, employing constant potential amperometry (0.7 mV) using a highly sensitive potentiostat, as previously detailed. The resulting signal was low-pass filtered at 0.5 Hz and sampled every 2 seconds. The microelectrode was mounted on a micromanipulator and positioned 5 to 10 μm away from cultured cells on the stage of an inverted microscope. After obtaining a stable baseline, test agents were added to the medium and responses were continuously recorded. At the completion of experiments, electrode function was confirmed using various dilutions of NO.

Results

Signs of Premature Senescence of Endothelial Cells In Vitro
When HUVECs (passage 3 to 4) were grown on GC-coated dishes for 3 or 5 days, the proportion of SA β-gal–positive cells showed a 2-fold increase compared with cells grown on NC (Figure 1). This effect was attenuated in a concentration-dependent manner when dishes were coated with 1:3, 1:6, and 1:10 dilutions of GC (in NC) and was not observed when cells were cultured on NC in the presence of 30 mmol/L d-glucose (not shown). Still, the frequency of SA β-gal–positive HUVECs at passage 5, grown on GC, was significantly lower than that observed after 22 passages under nonglycated conditions (9.77% versus 49.56%). Planimetric analysis of cell size demonstrated that SA β-gal–positive cells (5th passage) were almost twice as large as SA β-gal–negative
cells (not shown). Collectively, these data demonstrate that GC potentiates the expression by HUVECs of SA β-galactosidase, a senescence marker, and an increase in cell size of those SA β-gal–positive cells.

The proportion of apoptotic HUVECs cultured on GC (1:3 dilution in NC, GC 1:3), as measured with Hoechst staining, increased 2-fold compared with the companion 5th passage cells grown on NC (Figure 2A). This rate of apoptosis corresponded to that observed in control cells between passages 10 to 15 (inset), but was lower than that observed in nondoning senescent cells at passages above 22. This trend was further confirmed with Annexin-V-FITC staining, measured by cell counting using either fluorescence microscopy of attached or FACS analysis of dissociated cells (Figures 2B and 2C).

The expression of p53 and p14ARF proteins, consensus markers of cell senescence, was examined next using immunoblotting detection (Figure 3). HUVECs (passage 5) cultured on GC for 3 to 5 days showed a marked and concentration-dependent increase in the abundance of both proteins, indicating the possible involvement of p53/p14ARF signal transduction pathway in this process.

Previous studies have demonstrated that replicative cell senescence is associated with the attrition of telomeres, although no direct correlation between two has been found. Southern blot analysis of HUVEC DNA showed that the average length of telomeres terminal restriction fragments (TRFs) was 11.86 Kb in control, and decreased by approximately 0.2 Kb (P>0.05) in cells cultured on GC (Figure 4A). Telomerase activity, expressed as RTA, showed a similar insignificant decrease compared with NC control. Cells at passage 22, however, showed low-to-undetectable levels of telomerase activity. This finding was further confirmed by the direct visualization of TRAP ladder (Figure 4B). Lysates of HEK cell, a transformed cell line, were included as a positive control of telomerase activity.

**In Vivo Senescence of Endothelial Cells in Zucker Diabetic Rats**

To test the relevance of the above in vitro findings to the course of diabetic angiopathy, the following experiments...
were performed in young and old diabetic Zucker rats (Charles River Genetic Models, Indianapolis, Ind). En face SA \( \beta \)-galactosidase staining of aortas derived from age-matched Zucker diabetic and Zucker lean rats revealed that the former exhibited a uniform enrichment in senescent endothelial cells, especially at the branching points of daughter vessels. Indeed, SA \( \beta \)-galactosidase staining of aortas obtained from 35 week-old Zucker diabetic or lean rats showed an almost invariant SA \( \beta \)-galactosidase staining of tributary arteries in both groups of animals, which was associated with the developing vasculopathy in both strains (not shown).

**Figure 4.** Length of terminal restriction fragments (TRF) and relative telomerase activity (RTA) in the 5th passage HUVECs cultured on native or glycated collagen, compared with the 22nd passage HUVECs. A, Representative gel showing TRF length. Results of 3 independent experiments showed minor, statistically nonsignificant differences in TFR length. B, RTA shows no significant differences from native collagen control. Note that cells at passage 22 show very low-to-undetectable relative telomerase activity (RTA). This finding was further confirmed by the direct visualization of the TRAP ladder shown on the left. HEK cell lysates were included as a positive control, whose telomerase activity is too high to measure with ELISA in the same experiment. IS indicates internal standard. \( *P<0.05 \) compared with p5 HUVECs.

**NO Generation by Senescent Endothelial Cells**

It has been demonstrated that NO exerts an antiapoptotic effect through S-nitrosylation of caspases\(^ {18} \) and protects endothelial cells from senescence.\(^ {19} \) Therefore, in the next series of experiments, we measured NO production by HUVECs using NO-selective microelectrodes. NO generation was stimulated by calcium ionophore A23187 (5 \( \mu \)g/mL), but was significantly diminished in early passages of HUVECs cultured on GC or glycated matrigel, compared with nonglycated native matrices (Figure 6A). Paradoxically, the expression of eNOS was enhanced rather than suppressed in cells grown on glycated matrices (Figure 6B). A somewhat similar situation has been described in aging endothelium,\(^2 \) where NO deficiency in the face of enhanced eNOS protein has been attributed to the generation of superoxide by eNOS and consequent production of peroxynitrite.

**Effects of Peroxynitrite Scavenger, Ebselen, Intermediate eNOS Substrate, NOHA, and Cell-Permeable SOD Mimetic MnTBAP on Endothelial Cell Senescence**

Nitrotyrosine-modified proteins, reflecting peroxynitrite formation, were examined using immunoprecipitation and immunoblotting analyses. As shown in Figure 6C, growth of HUVECs on GC elicited a concentration-dependent increase in immunodetectable nitrotyrosine-modified proteins. To elucidate the possible significance of enhanced peroxynitrite formation as an initiator of premature senescence of HUVECs, cells after 4 passages were plated on GC with and without the addition of the peroxynitrite scavenger, ebselen (1 \( \mu \)mol/L), an intermediate in NO synthesis, 0.1 mmol/L NOHA (added to the same concentration of l-arginine), or a SOD mimetic MnTBAP (2.5 \( \mu \)mol/L). These concentrations of compounds were chosen based on preliminary experiments testing effects of a range of concentrations for each compound and selecting the lowest one, which was efficient but nontoxic. Ebselen, NOHA, and MnTBAP were each applied on day 1, 2 hours after HUVEC plating, added daily, and cells were studied on day 3. Using this protocol, HUVECs grown on both glycated and native matrix were examined for the extent of SA \( \beta \)-galactosidase staining. Addition of ebselen, NOHA, or MnTBAP to the culture medium completely abolished the development of premature senescence in HUVECs grown on GC (data not shown). In addition, to test for possible reversibility of senescence by ebselen, NOHA, or MnTBAP, HUVECs were plated on GC for 3 days (time sufficient to induce premature senescence) and thereafter ebselen, NOHA, or MnTBAP were added every 12 hours, until SA \( \beta \)-galactosidase staining on day 5. As shown in Figure 7, growth on GC elicited a concentration-dependent increase in the proportion of SA \( \beta \)-gal–positive cells by 3 days in culture. Ebselen, NOHA, and MnTBAP (Figures 7A through 7C) were each able to reverse premature senescence at all dilutions of GC, in contrast to l-arginine (LA) alone (Figure 7D), which reversed senescence at low but not high concentrations of GC.

In an attempt to distinguish between the potential contribution of increased NO levels versus diminished superoxide anion/peroxynitrite levels in preventing premature cell sene-
cence, HUVECs were cultured on NC in the presence of either 1 mmol/L L-NAME or 1 μmol/L peroxynitrite. SAβ-Gal staining was analyzed on days 3 and 5 of treatment. As summarized in Figure 8, inhibition of the basal NOS activity with daily additions of L-NAME did not increase the proportion of senescent cells until day 5 of treatment. In contrast, daily treatments with peroxynitrite resulted in a 2- to 3-fold increase in the proportion of senescent cells already by day 3 of such treatment, and the extent of this response was similar to that seen in cells grown on GC.

**Discussion**

Vasculopathy underlies the development of macrovascular and microvascular complications of diabetes mellitus, and...
stems mainly from endothelial dysfunction, typically associated with reduced NO bioactivity in vivo. Increased frequency of apoptosis has been documented in the vascular endothelium of diabetic animals and humans. Our finding of the premature senescence of endothelial cells in Zucker diabetic rats and in HUVECs cultured on glycated extracellular matrices, together with the established propensity of senescent cells to undergo apoptotic death, suggests that premature senescence may contribute to diabetic vasculopathy. The preferential localization of senescent cells at the orifices of branching daughter vessels is consistent with the known sites of accelerated development of atherosclerotic lesions. In fact, recent demonstration of SAβ-gal–positive senescent endothelial cells in atherosclerotic plaques of human aortae supports this conclusion. In addition, the observed novel marker of endothelial cell senescence, β-thymosin, has been identified in atherosclerotic plaques by differential display studies of endothelial cells after exposure to GC.

Figure 7. Proportion of SAβ-gal–positive HUVECs cultured on glycated collagen and treated with ebselen, NOHA, MnTBAP, and l-arginine. Cells were cultured on GC for 3 days (time sufficient to induce cell senescence), followed by addition with every 12 hours (arrows). A, B, C, and D, Treatments with ebselen, NOHA, MnTBAP, or l-arginine, respectively, and the dynamics of SAβ-gal–positive HUVECs cultured on different dilutions of GC (1:3, 1:6, and 1:10, respectively, mixed with the native collagen). Dashed lines show the data obtained in HUVECs cultured on native collagen. Note that ebselen, NOHA, and Mn-TBAP resulted in a complete reversal of the number of SAβ-gal–positive cells, whereas l-arginine treatment was effective only in cells grown on lower concentrations of GC, but not on higher concentration.
Despite the fact that several consensus markers of cell senescence were markedly upregulated in HUVECs when grown on glycated collagen, telomerase activity was unimpaired, and telomere length was decreased only marginally in early passage cells. This single deviation from the typical senescence phenotype should not disavow the bulk of evidence supporting a role for glycated proteins in triggering premature endothelial cell senescence. Indeed, these statistically insignificant changes may have resulted from a moderate proportion of senescent cells seen in 5th passage HUVECs grown on glycated matrix compared with 22nd passage cells (9.77% versus 49.56%). On the other hand, it has recently become appreciated that chromosome instability is determined by the shortest telomere rather than average telomere length. In this context, it is not surprising that the relatively minimal differences among cultures were not detectable.24 Furthermore, the view that attrition of telomeres is a marker for the oxidative stress–induced senescence phenotype has recently been questioned.12,25,26 Although it has been demonstrated that proatherogenic factors inactivate telomerase in HUVECs, whereas NO activates telomerase,19,27 it is generally true that human cells express rather low levels of telomerase activity.23 Collectively, our findings suggest that additional mechanism(s) are operant in inducing premature endothelial cell senescence and/or that premature senescence is mechanistically distinct from replicative endothelial cell senescence. Attenuated NO generation by endothelial cells appears to be one mechanism that can trigger senescence: inhibition of eNOS has been shown to induce cell senescence as detected by the higher proportion of SA β-gal–positive cells, whereas addition of NO donors to the culture medium was associated with a decreased proportion of SA β-gal–positive HUVECs.19

Although HUVECs displaying signs of premature senescence showed an enhanced expression of eNOS, their ability to generate NO was markedly diminished. NO deficiency could result from either attenuated eNOS activity or decreased NO bioavailability. Recent studies on the switch of eNOS from NO-generating to superoxide-producing enzyme,21,28–32 together with the enhanced production of reactive oxygen species in diabetes,33 favor the latter possibility. Indeed, a diminished lifetime of NO is indicated by the finding of increased accumulation of nitrotyrosine-modified proteins in the lysates of senescent endothelial cells, an indication of enhanced production of reactive oxygen species that react at near-diffusion limited rate with NO to form peroxynitrite. Notably, a similar enhancement in nitrotyrosine formation has been observed in association with vascular aging. Interestingly, in endothelial cells undergoing replicative senescence, eNOS activity is also reduced, albeit as a result of decreased expression of the enzyme, thus suggesting that the mechanisms for reduced availability of NO are different in replicative and premature cell senescence. Under these circumstances, the therapeutic strategy of choice should conceivably involve scavenging of peroxynitrite, dismutation of superoxide, or shifting the balance of NO and superoxide toward the former compound. Using this strategy we were able to demonstrate that the bona fide peroxynitrite scavenger and glutathione peroxidase and SOD mimetic, respectively, were able to reverse premature senescence.

Similarly, NOHA, an intermediate in the synthesis of NO and a scavenger of superoxide,38–40 showed the ability to prevent premature senescence of HUVECs and reverse it with a potency superior to l-arginine, a substrate for eNOS. This effect of NOHA was associated with the restoration of NO-synthetic function and may have also been associated with diminishing oxidant stress in HUVECs. Hence, ebsele, MnTBAP, and NOHA shared the ability to reverse premature senescence of HUVECs cultured on GC.

In the past, the senescence process has been considered to be irreversible. The fact that premature senescence can be reversed by scavenging peroxynitrite, dismutation of superoxide, or acceleration of NO synthesis argues that this particular form of senescence involves the operation of redox-mediated mechanisms, perhaps, much more dominant than in the replicative cell senescence. Oxidants have been recognized as important initiators of senescence. Earlier studies revealed that cells grown in the presence of high oxygen concentration have reduced life span.41 Similarly, treatment of cultured fibroblasts with nonlethal doses of hydrogen peroxide activated a rapid, senescence-like growth arrest.42 Oxidant stress was responsible for the activated Ras-induced senescence in human diploid fibroblasts, and growth arrest could be reversed by reducing ambient oxygen or by antioxidants.43 This is in agreement with our observations that premature senescence-like phenotypic changes in HUVECs grown on GC are also reversible. Among the common stress signaling pathways, p53 and p14ARF activation is believed to be one of the main mechanisms mediating oxidative stress-induced senescence. This appears to be also true in the case of premature senescence-like changes of HUVECs cultured on GC. Although the initiating events leading to the activation of this pathway in response to GC remain to be unveiled, the elevated p53 expression may further propa-
gate oxidative stress and this positive feedback loop may be important in committing cells to apoptosis. The identity of a compound(s) directly responsible for premature senescence of HUVECs, although suggestive of peroxynitrite, remains uncertain, because pharmacological tools used in this study are not entirely selective and have overlapping antioxidant functions. The fact that inhibition of NOS did not increase the proportion of SA β-gal–positive cells, may argue against the role of reduced NO production per se in triggering premature cell senescence, at least under static cell culture conditions. The fact that SA β-gal–positive cells accumulated in HUVECs treated with peroxynitrite, together with the finding that ebselen and MnTBAP prevented and reverted this process, argue in favor of oxidative and nitrosative mechanism. This leaves the mode of NOHA action incompletely understood: does it act via an increase in NO generation by eNOS or through conversion to NO, and is it capable of restoring NO-generating function to the uncoupled eNOS? Notwithstanding these uncertainties, ebselen, MnTBAP, and NOHA are able to prevent and reverse GC-associated premature senescence of endothelial cells in vitro, revealing new potential opportunities for therapy of diabetic vascular complications.

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