Contribution of p16<sup>INK4a</sup> and p21<sup>CIP1</sup> pathways to induction of premature senescence of human endothelial cells: permissive role of p53

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Increased formation of advanced glycation end-products (AGEs) (15, 40, 56, 72). AGE precursors arise from the nonenzymatic reaction between glucose/glucose-derived dicarbonyls and cellular proteins, known collectively as the Maillard reaction (1). The potential importance of AGEs is indicated by the in vivo observations that administration of AGE inhibitors or receptor for AGE (RAGE) blockers can prevent various functional and structural manifestations of diabetic microvascular disease in animal models, as well as in diabetic patients (30, 51, 53, 73). In endothelial cells exposed to high glucose, intracellular AGE formation can occur within a week. During the course of diabetes, AGEs are formed at an accelerated rate (76) and accumulate rapidly to reach a very high level in tissue proteins, especially in long-lived proteins like collagens (11, 34). Therefore, the consequences of AGE-induced cellular and tissue damage in diabetes are profound and persistent (22a, 27).

In our previous study (17) of human umbilical vein endothelial cells (HUVECs) cultured on glycated collagen I (GC), we found that the early passage cells expressed features of cellular senescence already after 3–5 days of incubation. The finding was confirmed in vivo in fa/fa diabetic rats (7). This process was accompanied by the increased expression of p14 and p53 levels. Telomere dysfunction was not apparent in GC-induced endothelial cell senescence, which rather was a consequence of excessive oxidative stress. It was found, both in vitro and in vivo, that normal functions of the endothelium were perturbed, as judged from the dysfunctional endothelial nitric oxide-nitrite oxide system, defective acetylcholine-induced vasorelaxation, disbalanced fibrinolysis, and impaired angiogenesis (7, 16, 17). It was thus surmised that the many changes accompanying the senescence process may contribute to the manifestation of endothelial dysfunction and hence diabetic complications.

Cellular senescence has been extensively studied mostly in fibroblasts and, to a lesser extent, epithelial cells with reference to replicative aging. It was first recognized by Hayflick and Moorhead (31) as a process that prevents normal fibroblasts from dividing indefinitely in culture. Replicative senescence has been found to be tightly linked to telomere attrition (3, 39, 64) and has been related to organism aging in vivo (19). Stimuli such as oxidative stress, DNA damage, chromatin remodeling, and intense mitogenic signaling have also been shown to induce growth arrest in primary cell cultures. This stress-induced premature senescence (PS) shares many com-
mon features with the replicative senescence but can be induced long before the Hayflick limit has been reached (18, 66).

Studies have assigned to several tumor suppressors a critical role in the underlying mechanisms of both replicative and stress-induced senescence engaging the cell cycle check point machinery. Prominent examples include p53, retinoblastoma (Rb), and the two products encoded by the cyclin-dependent kinase N2A (CDKN2A) locus. The CDKN2A locus has the transcript, p16, which binds directly to CDK4 and CDK6 and inhibits their activity to initiate the phosphorylation and functional inactivation of Rb (59). In contrast, the product of the β transcript, p14, interacts directly with MDM2, a protein that opposes the function of p53 by blocking its transcriptional activation, facilitating its nuclear export and catalyzing its ubiquitylation and proteasome-mediated destruction (47, 65). Ectopic expression of p14 therefore stabilizes p53 and increases the expression of p53-regulated genes, such as p21 (61), which in turn inhibit CDK4/6 as well as CDK2 activity. Confusion exists in understanding the roles of the p16/Rb and p53/p21 signaling pathways in mediating the senescence process in general and premature senescence in particular. Little is known about the interaction between these two pathways, especially at the senescence-initiation stage. In the present study, we investigated the causative roles of these two pathways in GC-induced PS. By taking advantage of the green fluorescent protein (GFP) construct and the small interference RNA (siRNA) strategy, we evaluated further the engagement of these two regulatory pathways in initiating endothelial cell senescence.

MATERIALS AND METHODS

Cell culture. HUVECs were acquired from Clonetics (San Diego, CA) and maintained in GEM-2 medium (Clonetics) with the supplement of 2% fetal bovine serum. Cells between passages 3 to 5 were used for experiments. After trypsinization, cells were seeded onto dishes at varied density depending on the duration of incubation and the purpose of an experiment. In general, 3,500 to 4,000 cells were seeded onto 1-cm² bottom culture area for experiments with 3-day incubation period. For 5-day long experiments, 1,500 to 2,000 cells were seeded onto 1 cm² of dish surface area.

Preparation of GC and collagen-coated dishes. GC- and native collagen (NC)-coated dishes were prepared as previously described (16). In brief, 500 μg/ml collagen (Vitrogen; Cohesion, Palo Alto, CA) was prepared in 2X PBS (pH 7.4) containing 500 mM d-glucose. After the pH value was checked and adjusted to neutral (7.4), the solution was sterilized by using a 2.2-

Relative quantitative RT-PCR. HUVEC RNA was isolated by using Trizol reagent (Invitrogen, Carlsbad, CA). Relative quantitative RT-PCR was performed by using 18S as an internal standard, following the manufacturer’s instructions (Ambion). In brief, the linear range of the amplification efficiency and the optimal ratio of 18S primer: competitor (classic II) were first determined for each individual gene to be detected. Approximately 1 μg total RNA was used in a 50-μl Titan one-step RT-PCR system (Roche Applied Science, Indianapolis, IN). Products were separated on 2% agarose gel, stained with ethidium bromide, and analyzed with an Alphalmager image system. Primer sequences used were the following: p16 sense, CAA CGG ACC GAA TAC GTA CG; antisense, AGC ACC ACC AGC GTG TC; p21 sense, GCG CCA TTA TCG AAC CGG CT; antisense, GCA GCC TTC CTG TGG GCGGA; p53 sense, CTCACCATCATCACA CACTGG; antisense, TCTGAGTCAGGCCCTTCTG.

Immunoblot analysis. Cell lysates were prepared in a buffer containing 50 mM Tris, pH 7.4, 100 mM 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 20–60 μg of clarified extract were resolved on a 16% SDS-polyacrylamide gel. Proteins were transferred to Immobilon-P membranes (Millipore) and incubated at 4°C overnight with primary antibodies. Horseradish peroxide-conjugated anti-mouse or -rabbit secondary antibody was used for detection by using enhanced chemiluminescence. The primary antibodies used were the following: monoclonal anti-p53 (Do-1, Santa Cruz), polyclonal anti-p21 (c-19, Santa Cruz), polyclonal anti-p16 (c-20, Santa Cruz), and monoclonal anti-caspase-3 (8G10, Cell Signaling). Membranes were reprobed with monoclonal anti-β-actin (Sigma) to confirm equivalent loading of cell extracts.

Enhanced GFP recombinant vector construction. The full-length wild-type p16, p21, and p53 genes were cloned into enhanced GFP (EGFP)-expressing vector by using RT-PCR approach. The primer sequences used were the following: for p16 sense, CGA TAA GCT TGC CAT CAT GAC GGC GGC GGC GAC G; antisense, TCTGAGTCAGGCCCTTC.

Glucomeritroglycan isolation. GC and collagen-coated dishes were prepared as previously described (16). Briefly, 500 mg/ml collagen (Vitrogen; Cohesion, Palo Alto, CA) was prepared in 2X PBS (pH 7.4) containing 500 mM d-glucose. After the pH value was checked and adjusted to neutral (7.4), the solution was sterilized by using a 2.2-

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for 15 s, 55°C for 30 s, and 68°C for 1 min. The PCR product was then purified with QIAquick RCP Purification Kit (Qiagen, Santa Clarita, CA), digested by BamHI and HindIII, and analyzed by agarose gel electrophoresis. The DNA fragment with the predicted size was excised from the gel, recovered with QIAquick Gel Extraction Kit (Qiagen), inserted into pEGFP-N1-expressing vector predigested with the same enzymes, and transformed into Top 10 chemically competent Escherichia coli (Invitrogen) for amplification. The identity of the inserted sequence was validated. The encoded gene product was evaluated by its subcellular localization (monitored by GFP expression) and immunoblotting analysis before being used in the study.

**siRNA design and synthesis.** siRNA were synthesized through an available commercial source (Dharmacon, Lafayette, CO). To ensure the efficiency and specificity, all the sequences of the siRNA used in this study were chosen according to published references. Their targeted sequences were as follows: si-p53, AAG ACU CCA GUG GUA AUC UAC (12); si-p16, AAC GCA CCG AAA AGU AGC GGU (4); si-p21, AAG CUC UAC CUU CCC AGG GGG (67); si-Rb, AAA UGG AAA AUG AU (23) G UU (69). All three si-RNA duplexes were transfected into HUVECs by using Fugene6 transfection reagent (Roche Applied Bioscience) at a final concentration of 100 nM following similar procedures described in Transfection. The efficiency and specificity of each siRNA duplex were once again tested and confirmed by using relative quantitative RT-PCR approach.

**Transfection.** Transfection was performed by using Fugene6 transfection reagent (Roche Applied Bioscience) following the manufacturer’s instructions with modifications to improve the transfection efficiency in HUVECs. For a 35-mm dish (bottom area of 8 cm²), transfection solution was prepared by adding 40 ng of recombinant GFP construct to 100 μl serum-free endothelial cell basal medium-2 (EBM-2) containing 6 μl premixed Fugene6 reagent. The culture medium was removed before the transfection to leave just enough to cover the cells. The transfection mixture was then added gently. After incubation at 37°C for 3–4 h, fresh culture medium was exchanged once and the transfected cells were then cultured in a CO₂ incubator for the period required.

**Cytotoxic and immunofluorescent staining.** Senescence-associated β-galactosidase (SA-β-Gal) was detected by cytochemical staining using a previous reported procedure. Cells were immediately fixed in 3% paraformaldehyde for 10 min. This change did not affect the staining results but was crucial for preserving GFP fluorescence. Stained cells were viewed under an inverted microscope at ×200 magnification. The percentage of SA-β-Gal-positive cells was determined by counting at least eight random fields for each sample. Apoptotic cells were detected by annexin V (Alexa Fluor-568 conjugated) staining following standard protocol. After being counterstained with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI, Molecular Probes) in PBS for 10 min, apoptotic cells were visualized using fluorescence microscopy. Cells in at least 15 random fields were counted for each sample. Apoptosis was also analyzed by detecting the presence of the nuclear cleaved (activated) caspase-3 with the use of laser scanning cytometry (LSC). At fixation in 3% paraformaldehyde for 10 min, cultured cells were first permeabilized with 0.5% Triton X-100 solution (in PBS containing 1% BSA) for 15 min at room temperature. Cells were then incubated at 4°C with 1:100 diluted rabbit polyclonal anticleaved caspase-3 antibody (Cell Signaling Technology, Beverly, MA) overnight. Slides were washed twice in PBS, followed by incubation in 1:1,000 diluted Alexa Fluor 633 conjugated F(ab)’2 fragment of anti-rabbit antibody (Molecular Probes, Eugene, OR) for 30 min. After being stained with DAPI for 10 min, cells were analyzed by LSC. For immunofluorescent detection of p21 and p16 expression in HUVECs cultured on GC, cells were fixed and stained following the same procedure as described above. The antibodies used were polyclonal anti-p21 (1:500 diluted, c-19; Santa Cruz Biotechnology, Santa Cruz, CA) and monoclonal anti-p16 (1:500 diluted, f-12, Santa Cruz Biotechnology). Secondary antibodies used were goat anti-rabbit FITC-conjugated IgG (1:1,000 dilution, Jackson Immunotechnology) and goat anti-mouse Alexa Fluor 594-conjugated IgG (Molecular Probes), respectively.

**RESULTS**

GC upregulates p21 and p16 expression in HUVECs. We have previously demonstrated that HUVECs cultured on GC for 3–5 days showed significantly higher p14 and p53 protein expression levels. This implicates activation and involvement of the p53 pathways in the GC-induced premature endothelial cell senescence and apoptosis. To investigate the role of the cell cycle check point regulators in controlling the GC-induced senescence, we measured the p21 and p16 expression of the HUVECs cultured on GC versus NC. The p21 is an effecter of p53, the activation of which is believed to be an important event in directing the downstream effects of p53 activation toward the replicative senescence pathway. In addition, the p16 has been studied as a pro-senescence mechanism paralyzing the p53/p21 pathway. To measure their expression levels in HUVECs cultured on GC versus NC, we first analyzed the mRNA level by utilizing the relative quantitative RT-PCR approach (Fig. 1A, left and middle). The results indicated that the mRNA for p16 and p21 was readily expressed in the control HUVECs cultured on NC but that GC culture conditions markedly increased their expression as early as 24 h after being plated. This augmented expression was sustained for at least 3 days for p21 and was maintained during the 5-day experimental period for p16. Immunoblotting analysis indicated marked increases of the p21 and p16 protein level in HUVECs cultured on GC (Fig. 1A, right). When compared with cells cultured on NC, this increase appeared in the first 24 h and showed an accumulating trend for p16 and sustained for p21 during the 5-day experiment. This observation was also confirmed by the immunofluorescent staining (Fig. 1B). cultured on GC for 3 days, HUVECs showed stronger fluorescent staining signal for p16 and p21 compared with the cells cultured on NC, reflecting a higher protein expression level for these regulators under GC culture condition. It is also interesting to note that the expression levels for p16 and p21 did not necessarily correlate. Though the fluorescent signal was generally enhanced in the cells cultured on GC, any given cell could have stronger p16 staining but comparably weaker p21 staining or vice versa. This suggests that under stress conditions, the augmented expression of p16 and p21 may occur in a sequential order or it may reflect differential regulation of p16 and p21 expression in individual cells dependent on their biological state. Taken together, these results indicate that in endothelial cells, GC can upregulate several CDK inhibitors that are crucial for turning on the proposed essential Senescence pathways.

Exogenous overexpression of p53, p21, and p16 initiates HUVEC apoptosis and senescent features. To address the question whether the increased expression levels in HUVECs of these cell cycle regulators, alone or in combination, are
causative factors in initiating the premature endothelial cell senescence under GC culture condition, we prepared the recombinant EGFP plasmid constructs for wild-type p53, p21, and p16. By using these constructs, we were enabled to precisely monitor the PS and apoptotic changes among the transfected cell population (GFP signal-bearing cells). The recombinant genes were first validated by nucleic acid sequencing, followed by the immunoprecipitation by using anti-GFP antibody and immunoblotting analysis by using antibodies against the corresponding regulators (Fig. 2B, top). In addition, the time-dependent expression of these recombinant proteins in transfected HUVECs was also analyzed by immunoblotting using anti-GFP antibody (Fig. 2B, bottom). The results indicated gradually decreased expression of p16 and p21-GFP fusion protein level in transfected HUVECs over time at a degree that was comparable to the empty EGFP vector transfection control. The p53/GFP fusion protein was apparently detected at a lower level at days 1 and 3 and became undetectable at day 5. As will be discussed below, this may have resulted from its strong apoptosis promoting activity, that is noticeable generally as early as 12 h after transfection (data not shown). After being expressed in HUVECs, the recombinant p53 and p21 fusion GFP proteins were mostly localized in the nuclei, and p16 fusion protein was localized in both nuclei and cytoplasm (data not shown), as previously reported by other investigators (71). The results of DNA content analysis using LSC indicated that, on transfection of each of these recombinant regulatory genes, HUVECs entered G1 cell cycle arrest as reflected from the decreased S phase DNA content and the concomitant increase in G1 phase DNA content (Fig. 2A). This was readily detectable on the histograms of the whole cell population but was more pronounced when only the GFP-positive transfected cell population was used as a denominator. Previously we showed that the proportion of apoptotic HUVECs was also elevated after exposure to GC, though to a lesser degree than premature senescent cells (17). Thus we
analyzed apoptotic rate in transfected HUVECs (Fig. 3). The results of annexin V staining indicated a significant increase in apoptosis in cells transfected with p53 for 1–3 days compared with the empty EGFP vector transfection control. By days 5–6, the difference disappeared. The HUVECs transfected by p21 showed elevated apoptotic rate on day 1, and the difference from control disappeared on days 3–6. This phenomenon could not be explained only on the basis of increased apoptosis because, unlike p53, there were p21-positive transfected cells surviving at 6 days after transfection. In contrast to p53 and p21, HUVECs transfected with p16 did not show any change in apoptotic activity compared with the control during the entire period of observation. To confirm these results, we analyzed the cleaved caspase-3 activity in HUVECs 24 h after transfection by using LSC (Fig. 3D). In agreement with annexin V analysis, the percentage of the cleaved caspase-3-positive cells among GFP-positive cell population was increased in p53 and p21 transfection group and remained unchanged in p16 transfected HUVECs. Furthermore, immunoblotting analysis indicated the elevated presence of cleaved caspase-3 large fragments (17/19 kDa) in cells overexpressing exogenous p53 and p21 at 24 and 72 h after transfection (Fig. 3E).

We next analyzed the proportion of senescent cells after exogenous overexpression of p53, p21, and p16 (Fig. 4) in HUVECs. When compared with the results of empty vector control, HUVECs transfected with p16 exhibited a rapid increase in the proportion of senescent cells, as assessed by SA-β-Gal staining on days 3 and 5. Cells transfected by p21 also showed accelerated senescence, but this occurred only after 5 days of incubation and was milder than the effect of p16 transfection. In contrast, HUVECs transfected with p53 showed no PS.

To examine a possible interaction between p16 and p21 overexpression, HUVECs were simultaneously cotransfected with both constructs (Fig. 5). Similar to the results discussed above, HUVECs transfected with p16 alone exhibited a rapid induction of senescence at days 3 and 5 and with p21 only at day 5 (Fig. 5A, left). The cotransfection with p16/p21 did not show additive effect on senescence of HUVECs and was identical to p21 transfection characterized by noticeable eleva-

Fig. 2. Cell cycle analysis of HUVECs transfected with p53-enhanced green fluorescent protein (p53EGFP), p21EGFP, and p16EGFP. A: DAPI staining histogram (top, dark histogram, and middle) of laser scanning cytometry (LSC) analysis showed decreased S phase but increased G1 phase nuclear DNA content in HUVECs 24 h after transfection with indicated cell cycle regulators. These noticeable changes were even more prominent in HUVECs bearing GFP signal (top, gray histogram, and bottom). B, top: immunoprecipitation (IP) and blotting (IB) analysis of transfected HUVECs showed expression of corresponding regulator-GFP fusion protein (indicated by arrows). Each of these regulators was first immunoprecipitated by using the anti-GFP antibody and then detected by using corresponding antibody on immunoblotting. B, bottom: immunoblotting analysis using anti-GFP antibody showed prolonged expression of p16 and p21 fusion proteins, though with a decreasing level similar to EGFP empty vector transfection control over indicated experimental period. In contrast, the p53 fusion protein was detected at a lower level and became hard to detect at day 5. This may result from the strong apoptotic promoting activity of p53 rather than differences in transfection ratio. The studies were repeated once with the same results.
tion of senescence by day 5 and at a smaller magnitude compared with p16. In addition, we analyzed the proportion of PS cells among the GFP-positive cell population and found that it was similar to the results obtained for the entire cell population but at a much higher level (Fig. 5A, right). By day 3, ~48% of the p16-GFP-positive cells were SA-β-Gal positive, and this number increased to ~75% by day 5. Both of these numbers were significantly higher than the corresponding results of the control group remaining at ~15% for both experimental time points. In p21-GFP-positive cells, ~40% were SA-β-Gal positive by day 5, which is significantly higher than control but lower than p16 transfection result on day 5. At the same time point, ~50% of GFP-positive cells were SA-β-gal-positive in the p21/p16 cotransfection group.

p53 expression is permissive for the exogenous p16 overexpression-induced senescence. To gain additional information on the interactions between the p53/p21 and p16 senescence pathways and the necessity of individual components, we studied the effect of suppressing the endogenous p53, p21, or p16 expression on the senescence induced by p16 or p21 transfection. We employed the RNA interference strategy to knock down p53, p21, or p16 expression in HUVECs at the time when the cells were transfected with p16 or p21 recombinant. The efficiency and specificity of siRNAs used in the study were first evaluated by using RT-PCR. Twenty-four hours after transfection of the corresponding siRNA, the endogenous p53 expression level was decreased nearly 95% compared with the siGL3 control, the p21 more than 80%, and p16 nearly 82% (Fig. 6A). We then analyzed the extent of PS in the p16- or p21-transfected HUVECs at the time when the endogenous p53, p21, or p16 expression was suppressed (Fig. 6, B and C). The results indicated that the p16-transfection-induced PS (10% for p16/siGL3 by day 5) could be effectively prevented when the endogenous p53 level was suppressed. In contrast, suppression of endogenous p21 level did not interfere with the senescence induced by p16 transfection (10% for p16/si-p21), nor did the suppression of endogenous p16 have any influence on the PS outcome of p21 transfection (8% for p21/siGL3; 7% for p21/si-p16 cotransfection). In these experiments, siGL3 was used as a control siRNA, and the results indicated that it did not have any pro-senescent activity, nor did it interfere with the senescence-promoting effect of p16 and p21 transfection. In addition, we also analyzed the extent of PS in the GFP-positive cell population and found an identical result. In the p16/siGL3 group, ~77% cells were SA-β-Gal positive. When siRNA to p53 was used along with p16GFP, the level of PS was lowered significantly to 33%, which is similar to the previous EGFP control results. The use of siRNA to p21 did not interfere with PS induced by p16GFP (at 70% level). The use of siRNA to p16 also did not change the proportion of senescent cells induced by p21GFP transfection (44% for p21/siGL3 and 46% p21/si-p16 cotransfection).

Senescence induced by p16 and p21 overexpression is prevented by suppression of endogenous Rb expression. To further test the hypothesis that Rb is a critical downstream mediator of senescence induced by p16 and p21 overexpression in HUVECs, we studied the effects of downregulation of the endogenous Rb expression by using RNA interference approach. We first analyzed the efficiency of the synthesized siRNA in knocking down the Rb. The relative quantitative RT-PCR indicated that endogenous Rb expression level was decreased nearly 90% by siRb compared with the siGL3 control 24 h after transfection (Fig. 7A). After confirming its effect, we then cotransfected siRNA to Rb with p16 and p21 GFP constructs. The extent of PS was analyzed 5 days post-transfection and showed an increased percentage of senescent HUVECs transfected by p16 and p21 GFP constructs (Fig. 7B), similar to the results described above. This senescence-promot-
Cellular senescence has long been recognized as a mechanism to avoid immortalization of somatic cells. It is believed that both life span-based senescence and oncogene-mediated senescence are potentially important for tumor suppression (25). In parallel with the induction of apoptosis, many conventional anti-cancer therapies have proved to contribute to the outcome of the treatment through induction of cell senescence (57, 62). Senescence is characterized to be a permanent growth arrest, in which cells remain metabolically active but are fully refractory to mitogenic stimuli (31). A broad range of cellular changes and functional alterations accompany senescence process (19). The consequences of senescence and senescence-associated changes have been suggested to contribute to the loss of normal organ function (13, 14, 26). By inference, senescence may be mechanistically related to the development of some chronic diseases. Our recent studies and studies by other investigators have provided some supporting evidence for this hypothesis (7, 17, 20, 38, 44, 45).

Though the underlying mechanism(s) of senescence is still far from clear, it has been widely attested that the decelerated cell cycle progression gated by several tumor suppressors is one of the central mechanisms (5, 43). Among them, the regulators constituting the p53/p21 and p16/Rb pathways are best studied, despite the scarcity of data on the initiating upstream cascades for their activation. From our previous studies (17) as well as the results of the present study, we concluded that both of these pathways are activated in GC-induced premature HUVEC senescence as illustrated in Fig. 8. It is reflected by the upregulation of p14, p53, and p21 expression, comprising the p53/p21 pathway, and upregulation of p16 expression, exemplifying the p16/Rb pathway. The activation occurs relatively quickly. A significant upregulation of these factors can be readily documented 24 h after being plated on GC and is generally maintained at an augmented level.

Senescent cells are believed to survive for years under appropriate tissue culture environment, and they are resistant to apoptosis. On the basis of this observation, it would appear that senescence and apoptosis are two independent outcomes in cells subjected to overthreshold stresses. Even though the same stressor is often capable of inducing either senescence or apoptosis, and these two outcomes do share some important elements in their signaling cascades, as exemplified by the activation of p53 tumor suppressor, it is generally thought that in a given cell, only one pathway will be activated. In our previous study, we documented that, in parallel with the replicative senescence in HUVECs, the frequency of apoptosis was also elevated (17). In the GC-induced HUVEC premature senescence, an increased apoptosis was also noticeable. The present study suggests that the enhanced apoptosis, seen in senescent endothelial cells, may be controlled through the p53-dependent and -independent pathways. Our results show that overexpression of wild-type p53, as well as p21, can induce apoptosis in HUVECs (detected with two techniques: annexin V staining and cleaved caspase-3 analysis). This may indicate that, in contrast to fibroblasts, apoptotic pathway can be activated in senescent endothelial cells. This view is in agreement with the studies performed in porcine pulmonary artery endothelial cells (78) as well as in HUVECs (68, 70). An augmented apoptosis program has been also reported in the aged vascular system in vivo (32).

Overexpression of p16 and p21 in HUVECs is capable of rapidly initiating senescent phenotype, as judged by the presence of SA-β-Gal and the senescence morphology, in a matter of only 3 or 5 days, respectively. This matches the time frame of GC-induced PS in HUVECs. In comparison, the senescence-promoting effect of p16 in HUVECs is much more prominent than that of p21. This observation supports the hypothesis that GC can induce PS in endothelial cells by activating the p53/p21 and p16 pathways.
Fig. 5. Analysis of premature senescence of HUVECs transfected/cotransfected by p16 and p21. 

A: when compared with results of empty vector transfection control (left), HUVECs transfected with p16 showed an increased proportion of senescent cells as assessed by SA-β-Gal staining at days 3 and 5. Cells transfected by p21 also showed increased senescent activity, but this only occurred after 5 days incubation. There was also an increased senescence in HUVECs cotransfected with p16 and p21, but like p21 single transfection, this change only occurred after 5 days incubation and at a smaller magnitude compared with p16 transfection. The same patterns were observed when the proportion of senescent cells was calculated only among the GFP signal-bearing HUVECs (right) but with much more robust outcome. In p16-positive-transfected cells (GFP*), nearly 48% cells were SA-β-Gal positive at day 3 and nearly 75% at day 5. 

B: representative images showing the SA-β-Gal-positive HUVECs 5 days after transfection (left), the GFP signal-bearing HUVECs (middle), as well as their merged images (right). Experiments were repeated 3 times and presented as means ± SE. *P < 0.05 compared with control.

Fig. 6. Analysis of senescence in HUVECs cotransfected by p16 or p21 EGFP recombinant with small interference RNAs against p53, p21, or p16. 

A: relative quantitative RT-RCR analysis demonstrated ~82, 80, and 95% decrease of the endogenous p16, p21, and p53 mRNA expression level in HUVECs 24 h after transfection with the respective siRNA duplex. HUVECs transfected with siGL3 served as a control. 

B: SA-β-Gal staining revealed a significant increase in senescent HUVECs after transfection with p16 and p21 for 5 days. Cotransfection of HUVECs with si-p53 eliminated the senescence-inducing activity of the p16 construct. Interestingly, this blocking effect was not observed when the p16 construct was cotransfected with siRNA against p21. Cotransfection of HUVECs with si-p16 did not affect the senescence-inducing activity of the p21 construct. 

C: in a much more robust way, analysis of the percentage of senescent HUVECs among the GFP signal-bearing cells revealed a very similar blocking effect when p16 construct was cotransfected with si-p53 but not si-p21. Experiments were repeated 3 times and presented as means ± SE. *P < 0.05 compared with siGL3 control; #P < 0.05 compared with p16GFP/siGL3 cotransfection.
During recent years, significant efforts have been made in dissecting the contribution of the p53/p21 and p16/Rb pathways to the replicative senescence, as well as stress-induced senescence (9, 24, 29, 37, 75). The p53/p21 pathway has been generally accepted as the crucial controller of the replicative senescence program (8, 48, 74), even though there are reports that suggest an important role of the p16 pathway (2). In the stress-induced senescence, the p16/Rb pathway is considered essential, at least as reported in the case of Ras- and histone deacetylase inhibitor-induced senescence (8, 50, 60). On the other hand, though the p53/p21 pathway is considered necessary for the stress-induced senescence in rodent fibroblasts, it appears to be dispensable in human fibroblasts (8, 60). This conclusion is challenged in the case of HUVECs as indicated by our present results. When the endogenous p53 expression was suppressed by siRNA, the overexpression of p16 was unable to initiate senescence in HUVECs. Interestingly, this result is not observed when the endogenous p21 expression is suppressed instead of p53. The inhibition of endogenous p16 expression has also no effect on PS induced by p21 transfection. Two tentative conclusions can be made from these observations. First, the p53/p21 and p16/Rb are both essential to the PS program in HUVECs; second, the integrity of p53 pathway appears to be permissive for p16-mediated initiation of senescence in HUVECs, and this cooperation occurs at some point proximal to p21.

Previous studies (46) have shown that the induction of p21 and its inhibition of CDK2 are necessary contributors to the cell cycle arrest imposed by p16 in an osteogenic sarcoma cell line and hence implied that p21 serves as a potential point of cooperation between the p16/Rb and p53/p21 tumor suppressor pathway. Our study also indicates the existence of cooperation between these two pathways in HUVECs; however, the potential crossing point is p53 but not p21. This suggests that the crucial gating effect mediated by p53 on the p16-induced endothelial cell senescence may be dependent on factors other than p21, as has been also suggested by another group (77). Nonetheless, on the basis of our results and referenced study, we speculate that upregulation of p21 is important for GC-induced endothelial cell senescence as reflected in its three potential contributions. First, it may be an important step in directing the effect of p53 activation toward senescence rather than apoptosis; second, it is capable of directly inducing senescence; third, it may provide an opportunity for senescent cells to become apoptotic.

To test the hypothesis that the endothelial cell senescence induced by ectopic overexpression of p16 and p21 is dependent on the action of Rb protein on cell proliferation, the endogenous Rb expression level was suppressed through the siRNA strategy, whereas the exogenous p16 or p21 was introduced into HUVECs. After 5 days of incubation, we found that none of these two CDK inhibitors was still capable of initiating a senescent phenotype in HUVECs. This indicates that the in-

Fig. 7. Inhibition of premature senescence-inducing activity of p16 and p21 EGFP recombinants when cotransfected with siRNA duplex against retinoblastoma (Rb) protein. A: relative quantitative RT-RCR analysis demonstrated a nearly 28-fold decrease for Rb mRNA expression level in HUVECs 24 h after transfection with siRb duplex. HUVECs transfected with siGL3 served as a control. B: SA-β-Gal staining revealed a significant increase in senescent HUVECs 5 days after p16 and p21 transfection. Cotransfection of HUVECs with siRb blocked the senescence-inducing activity for both of these CDK inhibitors. C: in a much more robust way, analysis of the percentage of senescent HUVECs among the GFP signal-bearing cells revealed a very similar inhibitory effect when p16 construct was cotransfected with siRb. Experiments were repeated 3 times and presented as means ± SE. *P < 0.05 compared with siGL3 control; #P < 0.05 compared with the respected p16GFP/siGL3 and p21GFP/siGL3 cotransfection groups.

Fig. 8. Pathways that induce cell apoptosis and senescence in HUVECs. A number of regulatory proteins transduce senescence-inducing signals or mediate cell entry into senescence through two cell cycle checkpoint pathways. Upregulation of p16 tumor suppressor can keep Rb protein (pRb) in hypo-phosphorylated growth-inhibitory form through its ability to quench CDK4/6 activity. Elevated p14 protein will bind and sequester MDM2, inhibiting the phosphoarginate growth-inhibitory form through its ability to quench CDK4/6 activity. Overexpression of p16 tumor suppressor can keep Rb protein (pRb) in hypophosphorylated growth-inhibitory form through its ability to quench CDK4/6 activity. Elevated p14 protein will bind and sequester MDM2, inhibiting the

Fig. 8. Pathways that induce cell apoptosis and senescence in HUVECs. A number of regulatory proteins transduce senescence-inducing signals or mediate cell entry into senescence through two cell cycle checkpoint pathways. Upregulation of p16 tumor suppressor can keep Rb protein (pRb) in hypophosphorylated growth-inhibitory form through its ability to quench CDK4/6 activity. Elevated p14 protein will bind and sequester MDM2, inhibiting the MDM-dependent degradation of p53. This in turn activates a number of genes, in which the activation of p21 will mediate cell growth arrest and senescence. Activation of p53/p21 pathway may also mediate a mild apoptosis program in HUVECs and, in addition, activation or integrity of p53 plays a permissive role for regulation of p16/Rb senescent pathway. Pathways described in this study are shown by solid bold arrows. ROS, reactive oxygen species.
tegrity of the Rb protein is essential for initiating the senescence program in HUVECs induced by exogenous p16 and p21 overexpression. Though it was the first tumor suppressor gene to be cloned (28), the functions of Rb protein are still not fully understood (22). It has been shown that Rb serves as the major target of cyclin D1-CDK4/6 cell regulation. The Rb protein regulates E2F-DP transcription factor complexes (E2F-1, -2, and -3; and DP-1, -2, and -3), which in turn regulate a number of genes required to initiate or propagate the S phase of the cell cycle. Phosphorylation of Rb by cyclin D1-CDK4 releases E2F-DP proteins from the Rb complex, relieving repression of these genes or activating their transcription (41). Studies have indicated that p16 can bind to the cyclin D-CDK4/6 complex and inhibit kinase activity (63). Therefore, p16 can regulate Rb function by inhibiting its phosphorylation (inactivation) via CDK and hence halt the mechanism for cell cycle progression. In the case of p21, the situation is even more complex. p21 can bind to a number of cyclin and CDK complexes: cyclin D1-CDK4, cyclin E-CDK2, cyclin A-CDK2, and cyclin A-Cdc2. One molecule of p21 per complex appears to permit CDK activity (and may even act as an assembly factor), whereas at higher stoichiometric ratios, p21 has been shown to inhibit kinase activity, prevent Rb phosphorylation, and block cell cycle progression (41). These data can help to explain the critical role of Rb protein in endothelial cell senescence initiated by the activation of p16 and p21 pathway.

The available evidence suggests that activation of cyclin D-CDK4/6 and cyclin E-CDK2 complexes can not only phosphorylate and inactivate Rb but also phosphorylate and inactivate the Rb-related proteins p107 and p103 (21, 49, 58). Though p107 and p103 have functions distinct from those of Rb, data exist that they share redundant functions with Rb in the regulation of E2Fs and can functionally compensate for the loss of Rb protein in cell cycle regulation (36). This apparently is not the case in the senescence program initiated in HUVECs by exogenous overexpression of p16 and p21, because suppression of endogenous Rb completely abolished their senescence-initiating effect. However, it is worth noting that the possible involvement of p107 and p103 in regulating HUVEC senescence in longer-term experiments cannot be totally ruled out. The shortcomings of the plasmid-based gene delivery system do not permit long-term experiments and hence limit our ability to verify this hypothesis.

Given the fact that Rb can interact with numerous cellular proteins, it is not excluded that some unknown targets for Rb-mediated transcriptional repression may exist that are distinct from E2F (42). This would complicate our attempts to explain the regulation of premature endothelial cell senescence. Nonetheless, the data presented herein suggest that p53 plays a permissive role in p16-driven PS and that both CDK inhibitors, p16 and p21, are capable of arresting the HUVECs in G1 phase and both require the critical downstream Rb-mediated action.

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