

The Helix–Loop–Helix Protein Id-1 Delays Onset of Replicative Senescence in Human Endothelial Cells

Jun Tang, Gabriel M. Gordon, Brian J. Nickoloff, and Kimberly E. Foreman

Department of Pathology and Skin Cancer Research Laboratories, Cardinal Bernardin Cancer Center, Loyola University Medical Center, Maywood, Illinois

SUMMARY: Id proteins are negative regulators of basic helix–loop–helix transcription factors, which are critical for expression of genes associated with cellular differentiation. Previous studies have shown that overexpression of Id-1 delays cellular senescence in several cell types, including fibroblasts, mammary epithelial cells, and keratinocytes. Although previous studies have demonstrated the expression of Id-1 in endothelium, the regulation of Id-1 has not been studied in these cells. In this report, a retroviral vector was used to overexpress Id-1 in human endothelial cells. Sustained expression of Id-1 resulted in a 2- to 3-fold increase in the total number of population doublings (replicative capacity) of the cells compared with vector-treated controls, which correlated with low levels of p16, p21, and p27 expression. The cells, however, were not immortalized and did eventually undergo senescence despite elevated Id-1 levels. Senescence was characterized by a dramatic increase in p16, but not p21 and p27. Under these experimental conditions, telomerase activity was not detected and the telomeres became progressively shorter with time. These results demonstrate the importance of Id-1 in endothelial cell proliferation and indicate that Id-1 represses p16 expression, resulting in delayed senescence. These findings may have implications in the development of endothelial cell–derived tumors. (*Lab Invest* 2002, 82:1073–1079).

The basic helix–loop–helix (bHLH) family of transcription factors plays an important role in the normal proliferation and differentiation programs of various cell types. These proteins bind as homo- or heterodimers through the HLH domain, and the combination of the two basic regions creates a DNA binding site that is necessary for transcription of target sequences. Id proteins, including Id-1, Id-2, Id-3, and Id-4, function as negative regulators for the bHLH transcription factors (Benezra et al, 1990). Id proteins contain only the HLH domain and lack the basic amino acid region involved in DNA binding. Thus, Id proteins form heterodimers with bHLH proteins, but are unable to bind DNA and activate target gene expression (Benezra et al, 1990).

Id-1 was first identified in myoblasts where it was shown to prevent the E12/E47 bHLH protein from dimerizing with MyoD, a muscle-specific bHLH transcription factor (Benezra et al, 1990). Since then, investigators have shown that Id-1 expression is essential for cell proliferation and is repressed during cellular differentiation and senescence in several cell types, including fibroblasts, mammary epithelial cells, inflammatory cells, and keratinocytes (Alani et al, 1999; Desprez et al, 1998; Hara et al, 1994; Kreider et al, 1992; Nickoloff et al, 2000;

Sun, 1994). Much less is known, however, about Id protein expression in human endothelial cells (ECs). Studies by Benezra and colleagues demonstrated expression of Id proteins in ECs during mouse embryo development (Jen et al, 1997; Lyden et al, 1999). Using a knockout mouse model, they demonstrated that Id-1^{-/-} Id-3^{-/-} mice died during development from intracranial hemorrhage and displayed aberrant vessel formation in the brain. These studies were expanded to show that Id-1^{+/-} Id-3^{-/-} mice, although viable, resisted tumor growth and/or metastasis primarily due to poor angiogenesis and necrosis of the tumor (Lyden et al, 1999). Furthermore, studies by Outinen et al (1999) demonstrated that Id-1 mRNA expression is transiently increased in ECs treated with homocysteine, a reagent that causes endoplasmic reticulum stress and impairs protein processing, resulting in growth arrest. Although it is unclear if protein expression is altered under these experimental conditions, the short-term induction of Id-1 mRNA after homocysteine treatment may be the initial cellular response to this form of injury with the subsequent reduction related to eventual growth arrest.

In this report, the role of Id-1 in EC growth, proliferation, and senescence has been examined in both large and microvascular human ECs. Using a retroviral vector encoding for human Id-1, ECs expressing elevated, sustained levels of Id-1 (EC+Id-1) were created and showed a 2- to 3-fold increase in the total number of population doublings (PD; replicative capacity) compared with controls. These cells were not immortalized, and eventually underwent replicative senescence despite high levels of Id-1 expression. EC+Id-1 cells undergoing senescence demonstrated a molecular phenotype similar to naturally senescent ECs,

DOI: 10.1097/01.LAB.0000022223.65962.3A

Received April 22, 2002.

This study was supported in part by Public Health Service Grants CA76951 (KEF) and AR47307 (BJN).

Address reprint requests to: Dr. Kimberly E. Foreman, Department of Pathology, Skin Cancer Research Laboratories, Cardinal Bernardin Cancer Center, Room 302, Loyola University Medical Center, 2160 South First Avenue, Maywood, IL 60153–5385. E-mail: kforema@lumc.edu

including elevated levels of p16 and a lack of telomerase activity; however, p21 and p27 did not appear to be significantly involved. These findings indicate Id-1 delays the onset of senescence in ECs and may play a potentially important role in the development of EC-derived tumors.

Results

Expression of Id-1 in Normal Human ECs

Previous studies have demonstrated that Id-1 is repressed in many cell types as they differentiate; therefore, experiments were designed to determine the pattern of Id-1 expression, as well as several other proteins involved in cell cycle regulation, in cultured ECs. The cells divided every 3 to 4 days before becoming senescent (approximately PD17). Western blot analysis demonstrated high levels of Id-1 expression in early passage human dermal microvascular endothelial cells (HDMECs) with a gradual decrease in expression (Fig. 1). In contrast, expression of the cyclin-dependent kinase inhibitors, p16, p21, and p27, was low in early passage cells and gradually increased over time (Fig. 1). However, although p16 expression continued to increase, levels of both p21 and p27 appeared to decrease again at the onset of senescence, similar to previous reports in fibroblasts (Alcorta et al, 1996). At senescence, the HDMECs underwent a change in morphology from their classic small, cobblestone shape to enlarged, flattened cells with numerous cytoplasmic vacuoles (data not shown). At this point, the HDMECs failed to divide, but remained attached to the culture dish for several weeks before rounding up and detaching from the surface. The cells could not be stimulated to proliferate with either frequent media changes or replating. Virtually identical results were found in similar experiments using HUVECs (not shown).

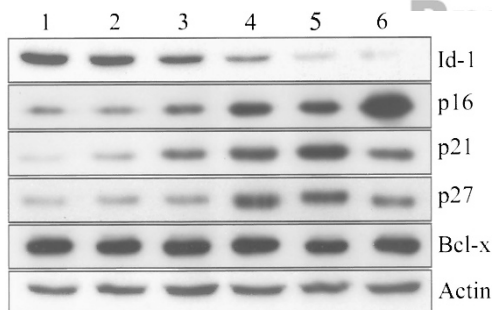


Figure 1.

Molecular phenotype of normal human dermal microvascular endothelial cells (HDMECs). Whole-cell protein extracts were prepared from HDMECs after various times in culture and analyzed by Western blot analysis for the expression of the indicated proteins. Lane 1, HDMECs population doubling (PD)5; Lane 2, PD8; Lane 3, PD10; Lane 4, PD13; Lane 5, PD15; Lane 6, PD17 (senescent HDMECs). Results are presented as a representative figure from three independent experiments using cells from different donors that showed similar results.

Overexpression of Id-1 in ECs Delays Onset of Replicative Senescence

To determine if sustained Id-1 expression would alter the normal EC lifespan in culture, HDMECs were transduced with a retroviral vector encoding for human Id-1 (HDMEC+Id-1). Preliminary experiments demonstrated that transduced ECs expressed high levels of Id-1 protein for approximately nine passages in culture before abruptly decreasing expression, presumably due to inactivation of the retroviral long terminal repeat (not shown), as previously reported (Challita and Kohn, 1994; Niwa et al, 1983; Palmer et al, 1991; Stewart et al, 1982). Therefore, in subsequent experiments, HDMECs were reinfected with LZRS-Id-1 (or LZRS-empty vector as a control) every 5 to 7 passages to sustain expression. HDMECs transduced with LZRS-empty vector (HDMEC+vector) showed a virtually identical molecular phenotype to untreated cells with decreasing Id-1 expression and increasing p16, p21, and p27 expression over time (Fig. 2A and not shown). The cells became senescent at approximately PD17 and demonstrated the enlarged, flattened morphology seen in the untreated cells. In contrast, HDMEC+Id-1 expressed elevated, sustained Id-1 levels (Fig. 2B). In early passage cells (PD5 or 6), Id-1 expression was increased approximately 50% compared with HDMEC+vector or untreated cells. The HDMEC+Id-1 cells demonstrated a significant increase in replicative capacity and continued to divide until PD38 (a 2- to 3-fold increase in three of four independently generated Id-1 overexpressing EC cell lines; Fig. 3), although the cells did eventually become senescent. No significant difference in growth rates was noted in any of the HDMEC+Id-1 cell lines compared with controls. The molecular phenotype of these cells was unique with elevated Id-1 expression

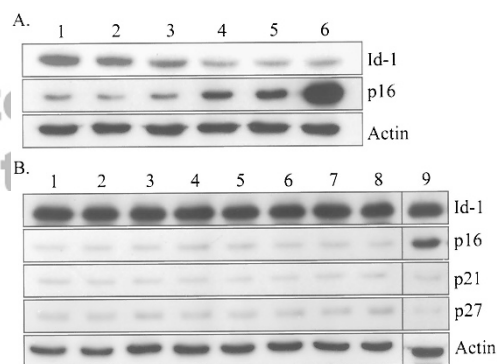


Figure 2.

Molecular phenotype of HDMECs transduced with the LZRS retroviral expression vector. Proliferating HDMECs (PD3) were transduced with an Id-1-encoding retrovirus or the empty retroviral vector as a control. Whole-cell protein extracts were prepared after various times in culture and analyzed by Western blot analysis for the expression of the indicated proteins. A, HDMEC+vector. Lane 1, PD5; Lane 2, PD8; Lane 3, PD10; Lane 4, PD13; Lane 5, PD15; Lane 6, PD17 (senescent). B, HDMEC+Id-1. Lane 1, PD6; Lane 2, PD8; Lane 3, PD10; Lane 4, PD13; Lane 5, PD16; Lane 6, PD20; Lane 7, PD23; Lane 8, PD31; Lane 9, PD38 (senescent). Results are presented as a representative figure from three independent experiments using cells from different donors that showed similar results. Experiments performed with HUVECs showed virtually identical results.

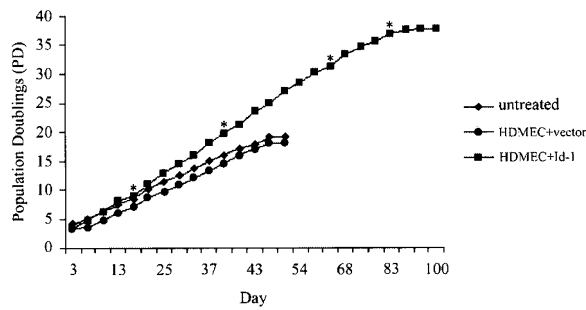


Figure 3. Overexpression of Id-1 extends the replicative capacity of HDMECs. Normal HDMECs (◆), HDMEC+vector (●), and HDMEC+Id-1 (■) were grown under standard conditions in tissue culture until senescent. PD was calculated as described in "Materials and Methods." Both HDMECs and HDMEC+vector cells became senescent at PD17, whereas HDMEC+Id-1 continued to proliferate until PD38 (a 2.2-fold increase, range was 2- to 3-fold increase in three of four independent experiments using cells from different donors). * Indicates where cells were reinfected with either LZRS-Id-1 or LZRS-empty vector to sustain protein expression. A representative experiment of three independent experiments is shown.

in all tested samples, including PD38 when the cells were senescent (Fig. 2B). Levels of p16, p21, and p27 were low before senescence when, despite high Id-1 expression, p16 levels increased dramatically. Interestingly, p21 and p27 levels did not increase before senescence and appeared similar or slightly decreased at senescence. Therefore, under these experimental conditions, induction of senescence does not appear to be related to expression of p21 and p27.

The results were extended to evaluate the relative levels and phosphorylation status of Rb in the transduced HDMECs. Both active (pRb) and inactive (ppRb) forms are expressed in midpassage HDMEC+vector (Fig. 4, Lane 1) and HDMEC+Id-1 (Fig. 4, Lane 3). However, at senescence, HDMEC+vector have dramatically reduced expression of both pRb and ppRb (Fig. 4, Lane 2). In contrast, HDMECs+Id-1 showed slightly lower, but sustained levels of both Rb forms (Fig. 4, Lanes 4 and 5) until senescence, when levels of pRb and ppRb were barely detectable (not shown).

HDMEC+Id-1 Do Not Express Telomerase Activity

Because reports have indicated that sustained telomerase activity in human ECs results in bypass of normal cellular senescence (Yang et al, 1999), studies

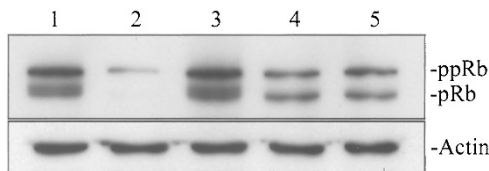


Figure 4. Expression of Rb in HDMEC+Id-1. Whole-cell protein extracts were prepared from samples and analyzed by Western blot analysis for the expression of pRb (active form) and ppRb (inactive form). Lane 1, HDMEC+vector, PD8; Lane 2, HDMEC+vector, PD17; Lane 3, HDMEC+Id-1, PD8; Lane 4, HDMEC+Id-1, PD17; Lane 5, HDMEC+Id-1, PD31. A representative experiment from two independent experiments is shown.

were performed to determine if telomerase activity was altered in HDMEC+Id-1. The characteristic ladder of the PCR-based telomerase repeat amplification protocol (TRAP) assay was readily detected in a positive control, immortalized cell line and, as expected, heat inactivation of the sample resulted in a loss of telomerase activity (Fig. 5A). There was, however, no evidence of telomerase activity in normal ECs (PD11; Fig. 5A), although low levels of telomerase could be detected in early passage cells (not shown), as previously reported (Hsiao et al, 1997; Yang et al, 1999). Similarly, HDMEC+Id-1 and HDMEC+vector demonstrated no telomerase activity (P11, Fig. 5A). To confirm and extend this result, telomere length of HDMEC, HDMEC+Id-1, and HDMEC+vector cells was determined. The results, as shown in Figure 5B, demonstrated progressive telomere shortening in the Id-1 overexpressing cell line. At PD9, telomere lengths were approximately 6 kb; they were reduced to approximately 5 kb at PD15; and at PD38, the telomeres had been reduced to approximately 2 kb. HeLa cells, used as a control, had the shortest telomeres at approximately 1.6 kb.

Altered Morphology of HDMEC+Id-1

As shown in Figure 6, the morphology of the HDMEC+Id-1 changed from the typical cobblestone-shaped cells seen in normal HDMECs to elongated, spindle-shaped cells. In contrast, HDMEC+vector and untreated cells maintained the traditional EC morphology throughout the experiment until senescence. These changes did not occur immediately on infection with the LZRS-Id-1 retrovirus, but instead appeared after PD20, when the cells had surpassed the normal lifespan of the control cells. Interestingly, the spindle-shaped HDMEC+Id-1 continued to express the EC marker CD31 (PECAM) at levels similar to

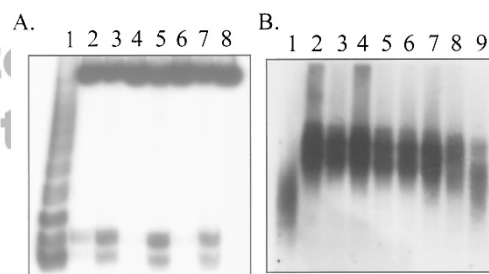


Figure 5. A, Telomerase activity in HDMECs. The PCR-based telomerase repeat amplification protocol (TRAP) assay was used to evaluate telomerase activity in HDMECs (PD11). Lane 1, Positive control, immortalized cell line; Lane 2, Heat-inactivated positive control; Lane 3, HDMECs+Id-1; Lane 4, Heat-inactivated HDMEC+Id-1; Lane 5, HDMEC+vector; Lane 6, Heat-inactivated HDMEC+vector; Lane 7, normal HDMEC; Lane 8, Heat-inactivated HDMEC. Representative data from three independent experiments is shown. B, Estimation of telomere length in HDMECs. Telomere lengths were shown to be progressively shortening in HDMEC+Id-1 cells until senescence. Estimated telomere lengths are indicated for each sample. Lane 1, HeLa cells as a control (1.6 kb); Lane 2, HDMEC (PD9, 6 kb); Lane 3, HDMEC+vector (PD9, 6 kb); Lane 4, HDMEC+Id-1 (PD9, 6 kb); Lane 5, HDMEC (PD15, 5 kb); Lane 6, HDMEC+vector (PD15, 5 kb); Lane 7, HDMEC+Id-1 (PD15, 5 kb); Lane 8, HDMEC+Id-1 (PD28, 4 kb); Lane 9, HDMEC+Id-1 (PD38, 2 kb).

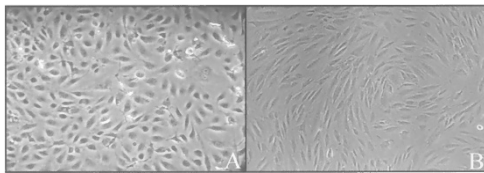


Figure 6.

Overexpression of Id-1 alters EC morphology. A, Typical cobblestone EC morphology of untreated ECs. B, After approximately 20 PDs, HDMEC+Id-1 demonstrated a spindle-shaped cell morphology. A representative picture is shown from three independent experiments performed with ECs from different donors.

those seen in both early and late passage ECs (Fig. 7). This unique, spindled morphology was present until the cells became senescent, at which point they had the typical flattened appearance of senescent ECs.

Discussion

Normal human ECs, like most somatic cells, have a limited life span in culture, and after a certain number of divisions, the cells no longer replicate, although they are still metabolically active. These senescent cells exhibit a number of characteristics, including changes in morphology, senescence-associated (SA)- β -galactosidase positivity, and increased expression of particular cell cycle-related proteins, such as p16, p21, or p27. Cellular senescence has been the focus of intense research in recent years, because it is suspected to play a critical role in both aging and tumor suppression.

In this study, we demonstrate that sustained Id-1 expression in human ECs results in a 2- to 3-fold increase in replicative capacity beyond that seen in normal or control-treated cells. However, despite increased levels of Id-1, the cells eventually undergo replicative senescence, which is characterized by in-

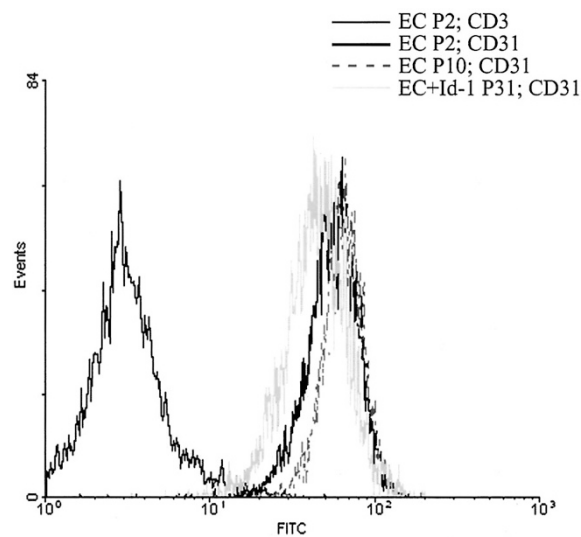


Figure 7.

Expression of the EC marker CD31 (PECAM) in HDMEC+Id-1. HDMEC+Id-1 (PD31) continue to express high levels of CD31 similar to those seen in both early and later passage HDMECs.

creased p16, but not p21 or p27, expression. This is the first report to our knowledge evaluating Id-1 expression in small and large vessel human ECs, and evaluating the effect of Id-1 overexpression on EC proliferation and senescence. Our results are consistent with those of Prabhu et al (1997), who reported that overexpression of Id-1 in NIH3T3 cells resulted in a modest increase in growth rate in culture, which corresponded with a 2- to 3-fold reduction in p21 mRNA. In addition, studies published by one of us (BJN) using human primary keratinocytes demonstrated that Id-1 overexpression increased the normal replicative capacity of the cells by 2- to 3-fold (Nickoloff et al, 2000). These cells were not immortalized, but eventually underwent senescence, which was characterized by increased p16 expression. It is, however, important to remember that different cell types express unique combinations of the four Id protein family members. Id-1 and Id-3 are commonly expressed in a broad number of cell types, whereas Id-2 and Id-4 have restricted expression patterns (Riechmann et al, 1994). Although the four Id proteins are clearly related, studies have demonstrated that they each possess some unique biologic activities (Norton, 2000). Therefore, the expression of a particular repertoire of Id proteins in any given cell type (HDMECs express Id-1, Id-2, and Id-3) may alter the cellular response to different stimuli (Norton, 2000), making it important to consider each cell type individually.

Our results are also consistent with previous studies evaluating cell cycle regulatory proteins in ECs. Watanabe et al (1997) reported that HDMECs grown in the presence of vascular endothelial growth factor (VEGF) could be cultured for 15 to 20 more PD than controls. The VEGF-treated cells expressed lower levels of p16, p21, and p27 than untreated cells, and at senescence (or withdrawal of VEGF), p16 levels were increased along with a modest increase in p21 and p27. In contrast, other reports have indicated a more crucial role for p27 and/or p21 in inhibiting EC proliferation and inducing growth arrest (Ashton et al, 1999; Yang et al, 1996; Zezula et al, 1997). Zezula et al (1997) described p21 as a critical mediator of phorbol 12,13 dibutyrate-induced growth arrest in ECs. The authors demonstrated that phorbol esters mediate induction of p21, but not p27, in HUVECs. However, Ashton et al (1999) demonstrated increased p27 expression, but not p16 or p21, in serum-stimulated rat capillary ECs overexpressing protein kinase C δ . The difference between these reports is likely due to the use of different mechanisms to induce growth arrest or the use of ECs from different species and sources.

Although it is clear that Id-1 expression decreases as p16 levels increase, the mechanisms by which these cell cycle-related proteins are regulated is not well understood. Recently, Ohtani et al (2001) reported the binding of the Ets1 and Ets2 transcription factors to the p16 promoter, which resulted in activation. Ets2 was also found to bind Id-1, and the authors proposed that in young cells, where Ets2 levels are high, Ets2 is sequestered by Id-1 and prevents induction of p16. As the cells age, Ets1 levels increase and are available to

activate the p16 promoter, resulting in increased protein expression and contributing to cellular senescence. Alani et al (2001) also described repression of the p16 promoter in the presence of Id-1; however, they showed that Id-1 binds directly to the p16 promoter to repress transcription without the involvement of Ets transcription factors. The difference between these studies may be due to different experimental conditions (as described) (Alani et al, 2001).

Tumor cells need to escape replicative senescence and proliferate indefinitely. As Id-1 expression promotes cellular proliferation and inhibits differentiation in many cell types, it is a natural focus of studies examining the molecular mechanisms of tumor development. Although not comprehensively studied, reports indicate that Id-1 is overexpressed in cells derived from several types of tumors, including pancreatic cancer, lung cancer, breast cancer, neuroblastoma, neuroepithelioma, astrocyte tumors, and endometrial cancers (Andres-Barquin et al, 1997; Biggs et al, 1992; Lin et al, 2000; Maruyama et al, 1999; Takai et al, 2001; Zhu et al, 1995). Indeed, recent studies have indicated that higher levels of Id-1 expression may be related to more aggressive tumors (Lin et al, 2000; Takai et al, 2001). Studies have not yet been reported if Id-1 expression is altered in hemangiomas, pyogenic granulomas, Kaposi's sarcoma, or other EC-derived tumors. However, our results indicating that Id-1 delays EC senescence may indicate that Id-1 plays an important role in the initiation or progression of EC tumors.

Materials and Methods

EC Culture

HUVECs were isolated by collagenase treatment of freshly obtained human umbilical cords, as previously described (Foreman et al, 1996). The cells were plated on gelatin-coated tissue culture dishes and were maintained in EGM-2MV media (BioWhittaker, Walkersville, Maryland). HDMECs were purchased from BioWhittaker and cultured in EGM-2MV on plates coated with EC attachment factor (Cell Systems, Kirkland, Washington) as recommended by the manufacturer. PD was calculated as previously described (Nickoloff et al, 2000). In HDMECs, this calculated PD represents the replicative capacity in our experiments and does not include proliferation before passage 3 (the passage provided by the manufacturer). Therefore, the PD value appears slightly lower than that reported by other investigators (Yang et al, 1999).

HUVECs and HDMECs were characterized by specific staining for CD31 (PECAM) using flow cytometry (FACs), as previously described (Foreman et al, 1996). Briefly, single cell suspensions were washed twice in FA buffer (Difco, Detroit, Michigan), containing 0.1% sodium azide and 1% fetal bovine serum, and then incubated with unconjugated primary antibody (CD31, 1:100 dilution; R & D Systems, Minneapolis, Minnesota; or CD3, 1:10 dilution; Becton-Dickinson, San Jose, California) for 30 minutes on ice. After two

washes, the cells were incubated for an additional 30 minutes on ice with fluorescein isothiocyanate-conjugated goat anti-mouse IgG secondary antibody (1:40 dilution; BioSource International, Camarillo, California). After two more washes, the cells were fixed in 2% paraformaldehyde and analyzed on a Coulter Epics-MCL FACS. Ten thousand cells were analyzed per gated determination.

Retroviral Vectors and Transduction of ECs

Id-1 cDNA was inserted between the *Bam*HI and *Not*I sites of the LZRS retroviral expression vector. The LZRS vector was kindly provided by Dr. Paul A. Khavari (Stanford University School of Medicine, Stanford, California) and used as previously described (Nickoloff et al, 2000; Qin et al, 1999). The Phoenix-Ampo retroviral packaging cells were obtained from American Type Culture Collection (Manassas, Virginia) with permission from Dr. Gary P. Nolan (Stanford University Medical Center, Stanford, California). The packaging cells were cultured in DMEM containing 10% fetal bovine serum and transfected with the LZRS vectors using standard CaCl_2 and $2\times$ HBSS methodologies. After overnight incubation, the cells were fed with fresh medium and incubated at 32° C for an additional 24 to 48 hours. The retroviral-containing supernatants were collected, filtered to remove contaminating cells, and used for infection of ECs.

ECs were seeded into 6-well plates and infected with 1 ml of viral supernatant in the presence of 4 $\mu\text{g/ml}$ polybrene for 1 hour at 32° C. After the infection, fresh media was added, and the cells incubated overnight at 37° C in 5% CO_2 . The cells were then washed and propagated using standard tissue culture techniques. The LZRS vector containing green fluorescent protein (LZRS-GFP, provided by Dr. Paul A. Khavari) was used as a control to determine infection efficiency. Studies showed that we could reproducibly infect more than 85% of ECs (range, 87.4% to 93%) with LZRS-GFP under these experimental conditions (not shown).

Western Blot Analysis

Whole-cell extracts were prepared by lysing cells in CHAPS buffer containing a mixture of protease inhibitors (Mini-Complete; Roche Molecular Biochemicals, Summerville, New Jersey), incubating the cells for 20 minutes on ice, and centrifuging to remove insoluble cellular debris (Liles et al, 1995). Protein concentration was determined using a Bradford Assay (Bio-Rad, Hercules, California). Fifteen to 30 μg of protein was loaded on a 12.5% SDS-polyacrylamide gel, transferred to an Immobilon-P (polyvinylidene difluoride) membrane, and blocked with 5% powdered milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.01% Tween 20). The membrane was then incubated with primary antibodies diluted in 2.5% powdered milk in TBST, washed extensively, and incubated with HRP-conjugated species specific secondary antibodies (Jackson Immunoresearch, West Grove, Pennsylvania).

nia). Proteins were visualized with ECL reagents (Amersham Pharmacia Biotech, Piscataway, New Jersey) according to manufacturer's instructions. Even loading of proteins in the wells was confirmed by Ponceau S staining. Antibodies against Id-1 (SC-488, 1:800 dilution), p21 (SC-397, 1:800 dilution), p16 (SC-468, 1:800 dilution), p27 (SC-528, 1:800 dilution), Bcl-x_{S/L} (SC-634, 1:500 dilution), Rb (SC-50, 1:4,000 dilution), and actin (SC-8432, 1:400 dilution) were purchased from Santa Cruz Biotechnology (Santa Cruz, California).

Telomerase Assay and Telomere Length

Telomerase activity was evaluated using the PCR-based TRAP assay, as previously described (Counter et al, 1994). Positive control samples included a telomerase-positive immortalized cell line (SLK cells) and negative controls consisted of heat-treating the samples for 10 minutes at 95° C to inactivate telomerase. Telomere length was estimated by hybridizing a radiolabeled telomeric (TTAGGG)₄ repeat probe to *HinfI* and *RsaI*-digested genomic DNA separated on 0.8% agarose gels (Kim and Wu, 1997; Nickoloff et al, 2000). Radioactive signal was quantified using a Fujifilm Fluorescent Analyzer (FLA-2000; Fujifilm Medical Systems USA, Stamford, Connecticut) and analyzed using NIH Image software (NIH, Bethesda, Maryland). Weighted averages of the distances of band migration were calculated using Microsoft Excel and compared with DNA molecular weight standard to determine the estimated telomere length.

Acknowledgements

The authors thank Ms. Jing Chen, Mr. Jeffery Panella, and Dr. Manuel O. Diaz for assistance with the TRAP assay and determining telomere length.

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