Telomere attrition and accumulation of senescent cells in cultured human endothelial cells

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Received 2 February 2004; revision accepted 5 April 2004

Abstract. The human umbilical vein endothelial cell (HUVEC) is an important model of the human endothelium that is widely used in vascular research. HUVECs and the adult endothelium share many characteristics including progression into senescence as the cells age. Despite this, the shortening of telomeres and its relationship to the progression into senescence are poorly defined in HUVECs. In this study of several HUVEC lines we show notable consistency in their growth curves. There is a steady decline in the growth rate of HUVECs grown continually in culture and we estimate complete cessation of growth after approximately 70 population doublings. The HUVECs lose telomeric DNA at a consistent rate of 90 base pairs/population doubling and show a progressive accumulation of shortened telomeres (below 5 kilobases). This telomeric loss correlates with the accumulation of senescent HUVECs in culture as assessed by staining for β -galactosidase activity at pH 6. Although the telomere length of a large population of cells is a relatively crude measure, we suggest that in HUVECs a mean telomere length (as measured by terminal restriction fragment length) of 5 kilobases is associated with entry into senescence. These data demonstrate the strong relationship between telomere attrition and cell senescence in HUVECs. They suggest that DNA damage and subsequent telomere attrition are likely to be key mechanisms driving the development of endothelial senescence in the pathogenesis of vascular disease.

INTRODUCTION

Telomeres, the protective cap structures at the end of chromosomes, are long repeat sequences that become shortened as cells age. Cells, both *in vitro* and *in vivo*, enter a non-dividing state termed senescence after a variable number of cell divisions. The critical shortening of telomeres has been strongly implicated as a factor that directs cells into this senescent phenotype (Allsopp *et al.* 1992).

Telomeres shorten with each cell division as a result of the end-replication problem (Olovnikov 1973) as well as because of DNA damage caused by reactive oxygen species.

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Telomeres in young cells are approximately 12 kilobases (kb) in length and in fibroblasts approximately 50–100 base pairs (bp) of telomere are lost with each population doubling (von Zglinicki 2002). To counteract the loss of telomeres some cells express the telomerase complex, a DNA polymerase that extends the telomere sequence. In most somatic cells, telomerase becomes inactive at birth.

Human umbilical vein endothelial cells (HUVECs) are widely used as an *in vitro* model of the vascular endothelium. They share many characteristics with endothelial cells *in vivo* including the development of the senescent phenotype. Senescent endothelial cells accumulate in atherosclerotic lesions in human coronary arteries (Minamino *et al.* 2002). Telomere loss has been associated with the senescent phenotype in endothelial cells and the expression of telomerase delays the onset of senescence in these cells. Despite this, data examining telomere length and senescence in cultured endothelial cells are somewhat limited.

In this study we have grown human endothelial cells in culture and examined their growth kinetics. We demonstrate a loss of telomeric DNA as the cells age as measured by both median terminal restriction fragment (TRF) length and the percentage of TRFs below 5 kb. This telomere attrition is associated with an accumulation of senescent cells. This study helps define the relationship between critical telomere shortening and the onset of HUVEC senescence.

MATERIALS AND METHODS

Cell culture

Unless otherwise stated, chemicals were from Sigma (Poole, UK) and cell culture reagents were from Invitrogen (Paisley, UK). HUVECs were isolated from umbilical veins using standard techniques. Cells were grown on flasks coated with 1% (W/V) gelatin in M199 media supplemented with 20% (V/V) foetal calf serum, 0.5% (V/V) endothelial cell growth supplement, 10 µg/ml heparin, 100 µg/ml streptomycin, 2 mM pyruvate, 20 mM HEPES at 37 °C, 5% (V/V) CO₂. Cells were maintained under subconfluent conditions at all times and were passaged every 3–4 days. During passaging, cells were lifted using 0.1% (W/V) trypsin and an aliquot was removed for cell counting and stained with 0.1% (W/V) trypan blue; cell viability was > 95% throughout the culturing. Between 0.6×10^6 and 1×10^6 cells were plated onto fresh 75-cm² flasks. The number of population doublings (PD) was calculated as:

$$\text{PD} = \log(\text{cells harvested}/\text{cells seeded})/\log(2)$$

Estimation of telomere length

Genomic DNA was isolated using the QIAamp DNA extraction kit (Qiagen, Crawley UK) according to manufacturer's recommendations and 2 µg DNA was digested with 20 units each of *HinfI* and *RsaI* (New England Biolabs, Hitchin UK) for 4 h. Samples were separated by electrophoresis through 0.5% (W/V) agarose (1000 Vh) and a 1-kb ladder and high molecular weight markers (Invitrogen) were used to assess molecular weight. Gels were blotted onto positively charged nylon membrane (Amersham, Little Chalfont UK) using alkaline Southern blotting and were hybridized with a telomeric probe (TTAGGG)₄, ³²P-labelled using T4 polynucleotide kinase (Invitrogen). DNA markers were labelled using the Rediprime II kit (Amersham). Hybridization took place overnight at 50 °C (in 0.5 M Na₂HPO₄, 1 mM ethylenediaminetetraacetic acid, 7% (W/V) sodium dodecyl sulphate, 1% (W/V) bovine serum albumin) and blots were washed for 30 min in 15 mM sodium citrate, 150 mM NaCl pH 7 prior to autoradiography. Mean

TRF length, an approximation of telomere length, was defined by analysis of the resulting telomeric smears with the TELOMETRIC software (Grant *et al.* 2001). Densitometry was used to calculate the percentage of telomeric smears below 5 kb: the ALPHAIMAGER 1220 gel documentation system (Alpha Innotech, Braintree UK) was used to measure the optical density of the telomeric smear below 5 kb and this was calculated as a percentage of the total density in each lane. On reproduced images the extremes of telomere smears are not always clearly visible but they can be measured by densitometry on autoradiographs.

Senescence-associated β -galactosidase staining

The cellular accumulation of β -galactosidase activity, which is detectable histochemically at pH 6, is an established marker of senescence in many cell lines, including HUVECs (Dimri *et al.* 1995; Kalashnik *et al.* 2000).

Sub-confluent HUVECs on 25 cm² flasks were washed twice with warmed phosphate-buffered saline (PBS) and fixed for 200 s at room temperature with 4 ml 1% (V/V) formalin in PBS. After two further washes with 6 ml warmed PBS, cultures were incubated for 24 h at 37 °C in 2.5 ml warmed senescence-associated β -galactosidase staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, 2 mM MgCl₂ and 40 mM NaH₂PO₄, pH 6.0).

β -Galactosidase activity was microscopically revealed by the presence of a blue, insoluble precipitate within the cell. The percentage of senescence-associated β -galactosidase-positive cells was determined by counting at least 700 cells from randomly selected fields in each sample.

RESULTS

Human endothelial cells were grown in culture for an extended period of time. Growth curve data from six separate HUVEC lines cultured for varying lengths of time are shown in Fig. 1(a). Visual examination does not reveal a significant slowing of growth rate until in excess of 50 population doublings are reached. However upon closer examination the growth rate, expressed as number of population doublings per day, shows a steady decline as the cells are continually passaged (Fig. 1b). Extrapolation, solving for population doubling level (PDL), suggests that endothelial cells reach the Hayflick limit (Hayflick 2003) at approximately 69 population doublings.

Telomere length, as assessed by the TRF length, was measured in these growing populations (Fig. 2a). Freshly isolated HUVECs had an average TRF length of 11.5 kb, this fell to 8 kb in cells with a PDL of approximately 35. This represents an average loss to the telomeres of 89 bp per cell doubling. Extrapolation suggests that these cells enter senescence with a TRF length of approximately 5 kb (Fig. 2b). It has been suggested that the relative amount of shortened telomeres is a better measure of telomere loss than the mean (or median) TRF length. Furthermore, the prevalence of shortened telomeres is more likely to be associated with cell senescence than the median telomere length. With this in mind, the relative amount of TRF below 5 kb was measured in these cells; data are presented in Fig. 2(c). In freshly isolated endothelial cell cultures 7% of TRFs were below 5 kb in length. This becomes progressively larger and at 40 population doublings approximately 20% of TRFs are below 5 kb in length. These values correlate with the number of senescent cells in these cultures as assessed by staining for a well-established marker of cellular senescence, β -galactosidase activity at pH 6 (Dimri *et al.* 1995, Fig. 3).

Figure 1. HUVECs divide at a consistent growth rate but in culture this slows during continued passaging. (a) Endothelial cells were cultured from six independently isolated umbilical veins and were maintained in culture for varying lengths of time. Population doubling level (PDL) was plotted against time. HUVEC growth rate in culture slows during continued passaging. Growth rate/day was calculated at each passage and is plotted with PDL. Linear regression, solving for PDL, gives: $PDL = -59.6 \times \text{Growth rate} + 68.7$. Data from all six cell lines were included.

Figure 2. Telomere attrition is evident in growing cultures of endothelial cells. (a) Representative blot showing the terminal restriction fragment attrition in HUVECs. Selected size markers and population doubling levels are indicated. (b) Cultured endothelial cells show a consistent attrition of telomeric DNA. Telomere length, as estimated by terminal restriction fragment length, is plotted against PDL. Data from three cell lines were examined and standard deviation are shown. (c) Shortened telomeres accumulate in populations of continuously cultured endothelial cells. Densitometry was used to analyse autoradiographs of the TRF blots used in (b). The percentage of telomeric smears below 5 kb is plotted against PDL. Standard deviation is shown ($n = 3$).

Figure 3. Senescent cells accumulate as the HUVEC population ages. Staining for β -galactosidase activity at pH 6 was used to measure percentage of senescent cells in the endothelial cell culture. Percentage of staining cells is plotted against PDL.

DISCUSSION

HUVECs are widely used as an *in vitro* model of the vascular endothelium. Both HUVECs and the endothelium exhibit characteristic changes during ageing, these include an increased expression of intercellular adhesion molecule-1 and loss of endothelial nitric oxide synthase activity leading to vascular dysfunction (Minamino *et al.* 2002). Here we have examined ageing HUVECs grown in culture. We found that endothelial cells isolated from different umbilical veins show remarkable consistency in their growth curves (Fig. 1a). Closer examination of these data shows a gradual slowing of the growth rate as these cells age. Figure 1(b) shows growth rate plotted with PDL. The slowing growth rate in these HUVECs is likely to arise from an accumulation of non-dividing cells rather than an increase in apoptosis or cell cycle time. Although the magnitude of apoptosis has not been quantified in this study, Kalashnik and colleagues, using a similar protocol to our study, have shown a progressive decline in the percentage of pKi67-positive cells in serially passaged HUVECs. Moreover, the same group also showed that the number of apoptotic cells, as assessed by the TUNEL technique, remains low and relatively constant in ageing HUVEC cultures (Kalashnik *et al.* 2000). Consequently, we conclude that the decreased growth rate of aged HUVECs is most likely accounted for by an increased accumulation of senescent cells. Linear regression, solving for PDL, predicts that these HUVECs will cease dividing at approximately 69 population doublings. This value fits well with the data presented in Fig. 1, and with the work of other groups (Yang *et al.* 1999). Using extrapolation is not without risks, as it assumes a linear relationship, however, it is also difficult to predict the point at which growth ceases because of long culture times in very late passages.

We observe an average loss of 90 bp of telomeric DNA with each population doubling. Other groups have measured a telomere attrition of 190 bp/population doubling in endothelial cells but these cells were examined over a narrower range of population doublings than those in our study. We have shown a consistent loss of 90 bp/population doubling that, by extrapolation, would be consistent with a 5-kb telomere length at senescence (Fig. 2b). Other workers have demonstrated a similar TRF length at senescence (Yang *et al.* 1999).

Telomerase activity has been detected in young cultures of HUVECs but this activity diminishes rapidly as the cells are passaged (Vasa *et al.* 2000). Although we have not examined telomerase in this study it is clear that there is a progressive loss of telomeric DNA even in very early passages. This suggests telomerase activity alone, is not enough to stabilize telomere length in young HUVEC cultures.

It has been suggested that the mean (or median) telomere length is not as useful a measure as the relative percentage of shortened telomeres (Cherif *et al.* 2003). Using 5 kb as a cut-off point, there is a steady increase in short telomeres as the endothelial cells divide (Fig. 2c). However, compared to median telomere length, the percentage of shortened telomeres shows more experimental variation and is also more affected by the length of exposure to autoradiography used. For this reason we would recommend caution when this approach is used. Nevertheless, increase in shortened telomeres does correlate well with the increase in the percentage of senescent cells in the culture as measured by staining for β -galactosidase activity at pH 6 (Fig. 3).

This is potentially important because senescence of endothelial cells prompts a phenotypic shift towards a pro-inflammatory cell type, typical of that seen in atherosclerotic plaques. Our data suggest that this phenotypic shift, resulting from cell senescence, is strongly related to telomere attrition. Thus, our data suggest that the study of mediators and mechanisms of telomere attrition in HUVECs could provide important insights into the pathogenesis of endothelial dysfunction *in vivo*.

Senescent cells are more prevalent in areas of the vasculature prone to atherosclerosis, such as the coronary arteries, than in vessels that do not commonly show plaque formation (Minamino *et al.* 2002). Additionally, limited studies show comparably shorter telomeres in the endothelia from vessels subject to high stress such as in bifurcations of the vasculature where haemodynamic turbulence is high (Chang & Harley 1995). Although measuring the terminal restriction fragment length of a large population of cells is a relatively crude approach, this work suggests that in HUVECs the senescent phenotype is associated with a TRF length of approximately 5 kb. These data and the recent introduction of a polymerase chain length-based method for measuring telomere lengths (Cawthon 2002) (allowing the analysis of small amounts of material) may be invaluable in uncovering the link between senescence and telomere length in endothelial cells. Although this study has focused on cell replication and telomere attrition, it is widely recognized that telomere loss can be accelerated by oxidative DNA damage (von Zglinicki 2002). This mechanism is likely to be important in the pathogenesis of vascular disease where oxidant stress has been identified as a key mediator of endothelial cell injury (Taniyama & Griendling 2003).

ACKNOWLEDGEMENTS

The authors wish to thank Mrs Carol Orme for her expert technical assistance. Dr Hastings is funded by the British Heart Foundation project grant PG/2001110.

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