FEBS Letters 470 (2000) 20–24 FEBS 23419

Homocysteine accelerates endothelial cell senescence

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Received 27 January 2000; received in revised form 8 February 2000

Edited by Barry Halliwell

Abstract In this study we demonstrate that exposure of cultured endothelial cells to homocysteine significantly accelerates the rate of endothelial senescence. Examination of telomere length demonstrates that homocysteine increases the amount of telomere length lost per population doubling. The effects of homocysteine on both senescence and telomere length are inhibited by treatment with the peroxide scavenger catalase. Chronic exposure of endothelial cells to homocysteine also increases the expression of two surface molecules linked to vascular disease, intracellular adhesion molecule-1 (ICAM-1) and plasminogen activator inhibitor-1 (PAI-1). Interestingly, the level of expression of both ICAM-1 and PAI-1 correlates with the degree of endothelial senescence. Taken together, these results suggest that homocysteine accelerates the rate of cellular senescence through a redox-dependent pathway. In addition, it suggests that chronic oxidative stress in the vessel wall may hasten the rate of senescence and that the senescent endothelial cell may in turn be pro-atherogenic.

Key words: Atherosclerosis; Intracellular adhesion molecule-1; Redox; Telomere; Aging

1. Introduction

The incidence of atherosclerotic disease dramatically increases as a function of age, however there is presently no defined mechanistic link between aging and vascular disease. On a cellular level, aging is often defined as replicative senescence. This is a process that occurs in primary cells in culture wherein after a certain number of population doublings, cells enter a viable but growth-arrested state. Phenotypically, the senescent state is characterized by an unresponsiveness to serum stimulation, the development of an enlarged and flattened morphology and the emergence of an endogenous senescence associated β-galactosidase activity [1].

The entry of cells into the senescent state is poorly understood. Of potential relevance is the observation that expression of either oncogenic forms of the small GTPase ras or over expression of the tumor suppressor gene products p53 or Rb appear capable of triggering the senescent phenotype in certain cells [2–4]. Interestingly, both ras and p53 can regulate the intracellular redox state [5–7]. As such, these studies are consistent with a 40 year old hypothesis linking the level of intracellular ROS to aging [8,9].

Another factor that appears to limit the life span of human cells in culture is telomere length. Telomeres are composed of a repeated nucleotide sequence on the ends of chromosomes. Each cell division results in the shortening of telomere length. It is generally believed that when telomeres reach a critical length, a signal is activated that initiates the senescent program. This has given rise to the hypothesis that telomeres act as mitotic clocks to regulate lifespan [10]. It is presently unclear how, if at all, ROS interact with telomere length to regulate lifespan.

In this report, we have attempted to begin to understand the connection between atherosclerosis and aging. In particular, we have sought to understand whether agents such as homocysteine that accelerate atherosclerosis also accelerate senescence and whether, in turn, the phenotype of the senescent cell may be pro-atherogenic.

2. Materials and methods

2.1. Cell culture and senescence associated β-galactosidase (SA-β-gal) activity

Human umbilical venous endothelial cells (HUVECS) were obtained from Clonetics and used beginning at passage 3. Cells were maintained in endothelial growth media (EGM, Clonetics) supplemented with 20% fetal calf serum. Cells were plated at a density of 2×10^5 and fresh media provided every 48 h. DL-Homocysteine was obtained from Sigma and purified catalase from Calbiochem. Since previous studies have indicated that many of the biological effects of homocysteine require the addition of copper [11], 4 μM $CuSO_4$ was added to all homocysteine treated dishes. The addition of copper alone had no effect on the rate of endothelial senescence (data not shown). SA-β-gal activity was detected according to the procedure of Dimri et al. [1]. Quantification of SA-β-gal cells was obtained by counting four random fields per dish and assessing the percentage of SA-β-gal positive cells from at least 150 cells per field.

2.2. Measurement of telomere terminal restriction fragment (TRF) length

Genomic DNA was isolated by standard means (Genomic DNA Purification Kit, Promega) and 3–5 μg digested overnight at 37°C with 30 U of *Rsa*1 and *Hin*f1 (Gibco/BRL). Telomere repeat oligonucleotides were labeled with [γ-P-32]ATP and hybridization was carried out at 60°C for 2 h in QuikHyb (Stratagene) followed by two washes with 5×SSPE for 30 min at 60°C and then with 0.1×SSPE for three 10 min washes at room temperature. Mean TRF length was determined by 1D Image Analysis Software from Kodak. This software allows quantification of the hybridization signal and subsequent determination of the mean signal intensity.

2.3. FACS analysis and Western blot analysis

Endothelial cells were detached by 5 mM EDTA in PBS and washed once with PBS containing 1% BSA. To detect intracellular adhesion molecule-1 (ICAM-1) expression, the suspended cells were incubated with fluorescently-labeled human ICAM-1 antibody (Beckman-Coulter) on ice for 30 min and washed twice with PBS containing 1% BSA. After washing, the cells were resuspended in PBS–BSA and analyzed by FACS. To assess levels of plasminogen activator inhibitor-1 (PAI-1), the suspended cells were analyzed by indirect immunofluorescence by first incubating with mouse monoclonal antibody to human PAI-1 (America Diagnostica) and then with an anti-

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PII: S0014-5793(00)01278-3

mouse fluorescein conjugated second antibody (Boehringer Mannheim). For detection of PAI-1 levels, 20 μg protein cell lysate per lane were analyzed by 12% SDS–PAGE gel and transferred to Protran membrane (Schleicher and Schuell). The membrane was incubated with 5 μg/ml mouse antibody to human PAI-1 at room temperature for 1 h. The signal was detected by enhanced chemiluminescence (Amersham) as previously described [12].

3. Results

To identify whether homocysteine treatment altered the rate of senescence, primary cultures of endothelial cells were grown in the presence or absence of homocysteine and subsequently evaluated for evidence of senescence using neutral pH endogenous senescence associated β-galactosidase activity (SA-β-gal). As demonstrated in Fig. 1, the effects of homocysteine on SA-β-gal activity were seen with doses as low as 50 μM, although the results were more pronounced at higher concentrations. No effects on endothelial senescence were observed when equal concentrations of cysteine were used as a control (data not shown). Over the 25 day period in culture, homocysteine produced as much as a 4–5-fold increase in the percentage of senescent endothelial cells.

We next examined whether homocysteine altered the rate at which endothelial cells shorten their telomeres. Genomic DNA was harvested from endothelial cells grown in the presence or absence of homocysteine and telomere length measured by Southern blotting. As demonstrated in Fig. 2A, in control cells, as expected, the mean telomere length decreased as a function of days in culture. The apparent rate of telomere shortening was increased in the homocysteine treated cells. Since homocysteine treated cells grew at a slower rate then untreated cells, the difference in telomere shortening was even more apparent when telomere length was plotted as a function of population doublings (Fig. 2B).

Many of the atherogenic effects of homocysteine have been ascribed to its ability to increase hydrogen peroxide generation [11]. To understand whether the effects of homocysteine on endothelial senescence were mediated by increasing hydrogen peroxide levels we analyzed the ability of the peroxide-scavenging protein catalase to rescue cells. As demonstrated in Fig. 3, the increase in SA-β-gal positive cells induced by homocysteine could be attenuated in a dose-dependent manner by catalase treatment. Similarly, as seen in Fig. 4A, the telomere length of endothelial cells grown for 15 days in the presence of both catalase and homocysteine appeared intermediate in length between untreated cells and those treated with homocysteine treatment alone. A mean rate of telomere loss per population doubling was obtained by dividing the calculated loss in telomere length over a 15 day period by the number of population doublings over that same period. As demonstrated in Fig. 4B, it was observed that homocysteine treatment resulted in a nearly three-fold increase in the rate of telomere shortening and that this effect was attenuated by catalase addition.

We next attempted to more fully characterize the senescent state of homocysteine-treated endothelial cells. In particular we attempted to analyze the expression of surface molecules

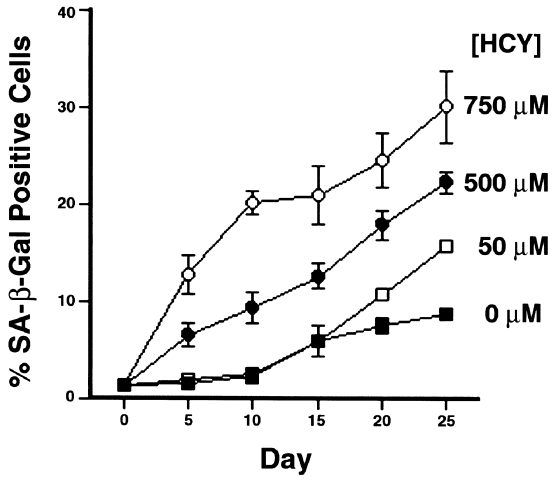

Fig. 1. Homocysteine accelerates endothelial senescence. Levels of SA-β-gal positive cells as a function of homocysteine concentration. Closed square, no treatment; open square, 50 μM homocysteine; closed circle, 500 μM homocysteine; open circle, 750 μM homocysteine. All results are the mean ± S.D. of triplicate cultures.

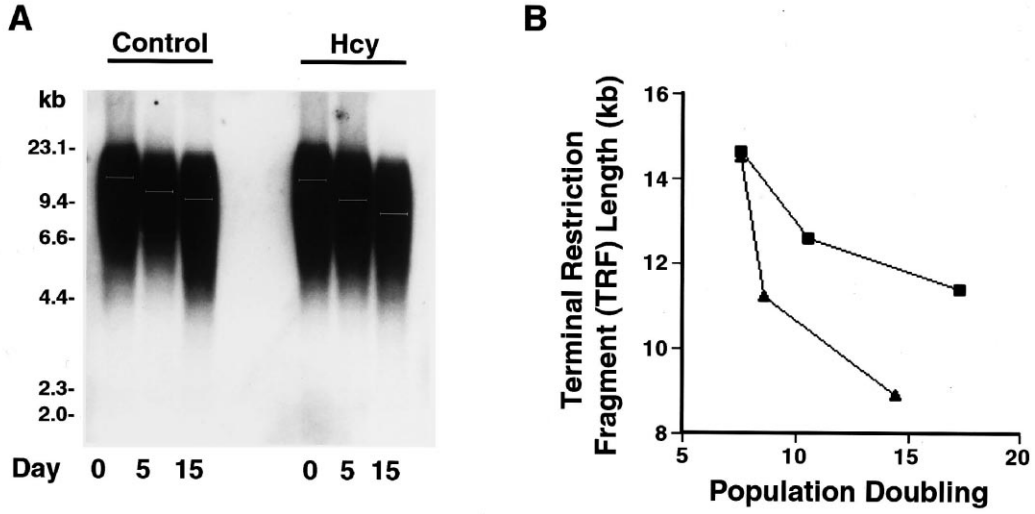

Fig. 2. Homocysteine accelerates the rate of telomere shortening. A: Southern blot analysis for telomeric length. The calculated mean telomere length, computed by densometric scanning as described in Section 2, is denoted by a white line. B: Mean telomere length from duplicate samples are plotted as a function of population doublings in untreated (square) and homocysteine-treated cells (triangle).

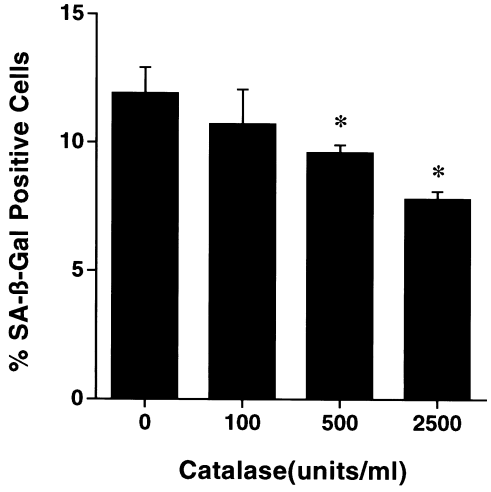

Fig. 3. Catalase inhibits homocysteine-induced senescence. Levels of SA-β-gal positive cells after 5 days in the presence of homocysteine (750 μM) and indicated amounts of catalase (U/ml). Results are mean ± S.D. of triplicate cultures. * indicates $P < 0.05$ compared to cells not treated with catalase.

which appear to be over expressed on senescent cells and that may contribute to atherosclerosis. As shown in Fig. 5A, FACS analysis of control and homocysteine treated cells demonstrated that surface expression of ICAM-1 was increased following chronic homocysteine treatment. To further extend the relationship between homocysteine treatment, senescence and ICAM-1 expression, we sorted homocysteine-treated cells based solely on their level of ICAM-1 expression. As demonstrated in Fig. 5B, the level of surface ICAM-1 expression was a good predictor of the degree of senescence. In particular, homocysteine-treated cells with the highest ICAM-1 (top 10% level of expression) expression had approximately 5-fold higher rates of senescence then homocysteine treated cells with the lowest (bottom 10%) ICAM-1 expression.

We performed a similar analysis with another surface molecule PAI-1, whose expression is increased in certain senescent cells and whose expression might be viewed as pro-atherogenic [13,14]. As demonstrated in Fig. 6A, by FACS analysis homocysteine treatment resulted in a significant increase in PAI-1 surface expression. Similarly, by Western blot analysis, homocysteine resulted in an increase in total PAI-1 protein levels (Fig. 6B). When homocysteine-treated cells were sorted based on PAI-1 expression and subsequently assayed for the degree of senescence, we again observed a significant correlation between the degree of PAI-1 expression and the percentage of SA-β-gal positive cells (Fig. 6C).

4. Discussion

In this report we demonstrate that homocysteine treatment results in an increase in the rate of endothelial senescence. Interestingly, the rate of telomere shortening in homocysteine-treated cells is significantly increased when compared to control endothelial cells. These results argue that the amount of telomere loss is not strictly a function of the number of cellular divisions, but can also be modulated by various intracellular processes. The observation that catalase reverses, in part, the effects of homocysteine on the rate of telomere loss/population doubling suggests that the intracellular redox state is one such intracellular variable. As such, these results provide one potential mechanism linking increased ROS levels and telomere length to the development of senescence. It should be noted that we saw no effects on endothelial senescence when homocysteine was used in the absence of copper (unpublished observations). Although serum levels of free copper are low, a variety of evidence suggests that in the vessel wall, copper bound primarily to ceruloplasmin can contribute to local redox-dependent reactions [15–17].

It is presently unclear what role cellular aging has in the atherosclerotic processes. Previous studies have examined telomere length from human blood vessels obtained at autopsy [18]. Interestingly, genomic DNA isolated from areas prone to atherosclerosis had shorter telomere length when compared to adjacent non-diseased areas. Another potential link between aging and atherosclerosis comes from patients with Werner's

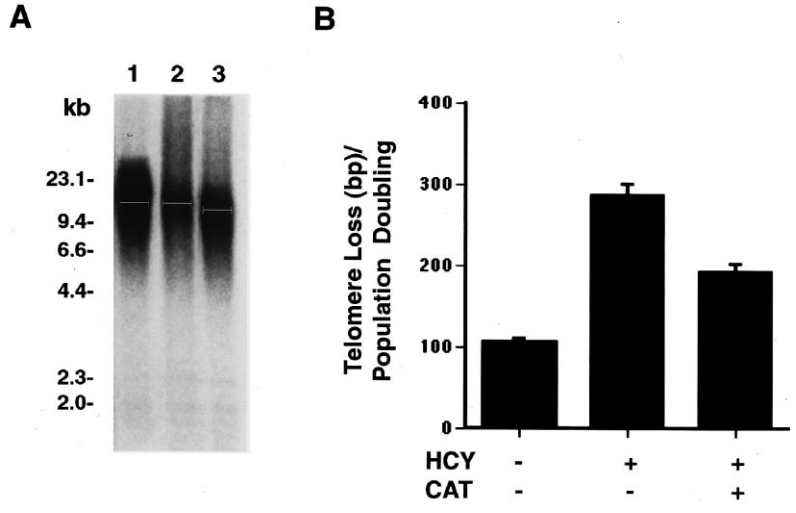

Fig. 4. Catalase inhibits homocysteine-induced changes in telomere length. A: Southern blot analysis for telomere length in endothelial cells maintained in culture for 15 days with either no treatment (lane 1), homocysteine treatment only (lane 3)or homocysteine plus catalase (lane 2). B: Averaged calculated telomere shortening per population doubling in untreated, homocysteine-treated (750 μM), or cells treated with homocysteine (750 μM) and catalase (7500 U/ml). Results are the mean quotient of three separate determinations for telomere length and population doublings.

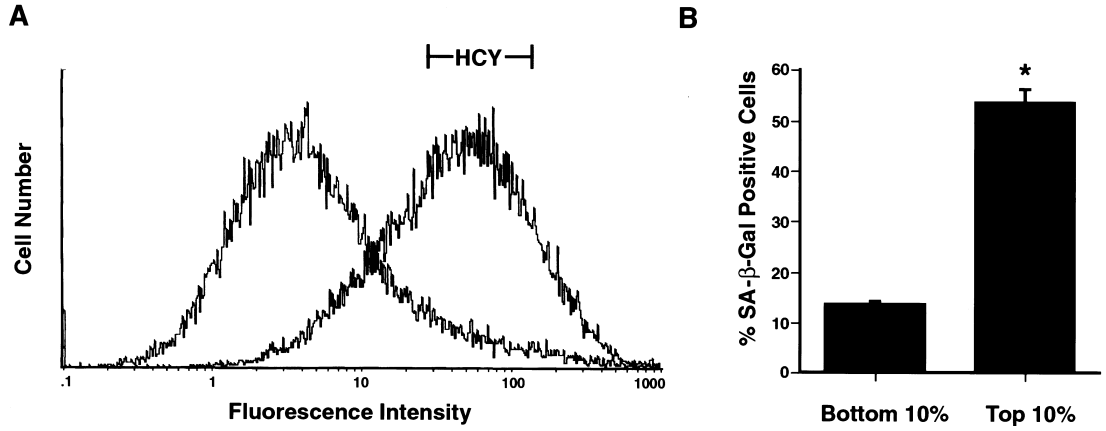

Fig. 5. ICAM-1 expression is increased on the surface of homocysteine-treated, senescent endothelial cells. A: Endothelial cells were assayed by indirect immunofluorescence followed by FACS analysis. Levels of ICAM-1 increased following 15 days of homocysteine exposure as evidenced by the rightward shift of the curve. B: Homocysteine treated cells expressing the highest levels of ICAM-1 (top 10%) and lowest levels of ICAM-1 were sorted by FACS. These cells were replated and 48 h later assessed for percentage of SA-β-gal positive cells.

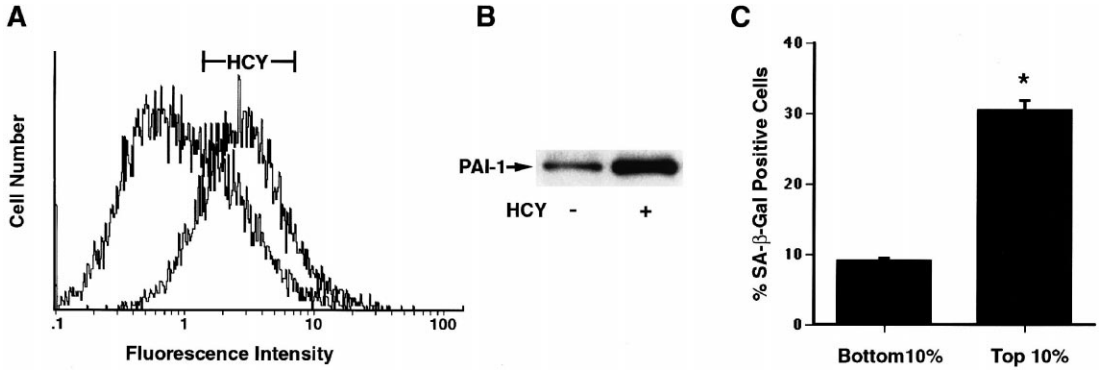

Fig. 6. Homocysteine treatment results in increased PAI-1 expression. A: Endothelial cells untreated or treated for 15 days with homocysteine were assessed by indirect immunofluorescence for PAI-1 followed by FACS analysis. Evidence for a homocysteine-induced increase in surface PAI-1 is demonstrated by a rightward shift of the curve. B: Western blot analysis for total PAI-1. C: Cells treated with homocysteine were sorted by FACS based on the level of PAI-1. Cells expressing the highest (top 10%) and lowest (bottom 10%) levels of PAI-1 were collected, replated and 48 h later assessed for β-gal positivity.

syndrome, a disease of accelerated aging. The disease is inherited in an autosomal recessive fashion and the disease gene has recently been identified as a DNA helicase [19]. On a clinical basis, although patients have a number of aging related disorders, the leading cause of death in affected individuals is atherosclerotic disease. Consistent with what we have observed in our in vitro studies, at least some individuals with Werner's contain high serum levels of ICAM-1 and PAI-1 [20]. It is tempting to speculate that accelerated aging of the vessel wall may play a primary role in the vascular disease observed.

In summary, our results demonstrate that a link exists between chronic exposure to homocysteine and the rate of senescence. In addition, the senescent endothelial cell exhibits a pro-atherogenic phenotype as exhibited by high ICAM-1 and PAI-1 expression. The ability of homocysteine to increase telomere shortening and senescence is significantly attenuated by the peroxide scavenger catalase. These results raise the possibility that one effect of chronic oxidative stress may be to accelerate the rate of vessel wall aging. The notion that a localized accumulation of senescent cells within the vessel wall may in turn be pro-atherogenic provides one potential explanation for the steep rise in atherosclerotic disease with advancing age.

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