Experimental Gerontology 99 (2017) 35–45

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Changes in Zn homeostasis during long term culture of primary endothelial cells and effects of Zn on endothelial cell senescence

Marco Malavolta[a,*], Laura Costarelli[a], Robertina Giacconi[a], Andrea Basso[a], Francesco Piacenza[a], Elisa Pierpaoli[a], Mauro Provinciali[a], Ogo A. Ogo[b], Dianne Ford[c]

[a] Advanced Technology Center for Aging Research, Scientific and Technological Pole, Italian National Institute of Health and Science on Aging (INRCA), Ancona, Italy
[b] Institute for Cell and Molecular Biosciences, University of Newcastle, Newcastle upon Tyne, UK
[c] Faculty of Health and Life Sciences, Northumbria University, Newcastle upon Tyne, UK

ARTICLE INFO

Section Editor: Borg Holly M Brown-

Keywords:
Cellular senescence
Zn homeostasis
p16
Zn transporters
Labile Zn
Aging

ABSTRACT

Endothelial cell senescence and Zn nutritional status influence cardiovascular disease. The influence of Zn appears dichotomous, hence it is imperative to understand the relationship with cellular senescence to improve knowledge about the molecular and cellular basis of the disease. Here we aimed to determine: 1) the impact of chronic exposure to a moderately high dose of Zn on senescence of endothelial cells; 2) the changes in Zn homeostasis during the lifespan of primary cultured endothelial cells; and 3) the susceptibility of proliferating and senescent endothelial cells to cell death after short term exposure to increasing doses of Zn and of the Zn chelator TPEN. Chronic exposure to Zn accelerated senescence and untreated cells at later passages, where doubling time had increased, displayed relocation of labile Zn and altered expression of genes involved in the response to Zn toxicity, including SLC30A1, SLC39A6, SLC30A5, SLC30A10 and metallothioneins, indicating that senescent cells have altered zinc homeostasis. Most Zn-dependent genes that were expressed differently between early and late passages were correlated with changes in the expression of anti-apoptotic genes. Short-term treatment with a high dose of Zn leads to cell death, but only in the population of cells at both earlier and later passages that had already entered senescence. In contrast, Zn depletion led to death of cells at earlier but not later passages, which suggests that there are sub-populations of senescent cells that are resistant to Zn depletion. This resistant senescent cell population may accumulate under conditions of Zn deficiency and contribute to vascular pathology.

1. Introduction

Endothelial cell senescence plays a role in human aging and age-related vascular disorders. Senescent endothelial cells are present in human atherosclerotic lesions (Minamino et al., 2002), where they are likely to promote pathology (Childs et al., 2016; Fyhrquist et al., 2013; Krouwer et al., 2012). Both transgenic and pharmacological approaches to clear senescent cells have provided preclinical evidence that selective ablation could contribute to the development of new therapeutic approaches to treating cardiovascular disease (Childs et al., 2016; Soto-Gamez and Demaria, 2017). However, to date few compounds able to selectively ablate senescent cells (senolytic drugs) are known, and they are not effective in all senescent cell types (Soto-Gamez and Demaria, 2017).

Why senescent cells accumulate in tissues and organs with age is still a key open question. Accumulation of senescent cells in aged tissues may be driven by several factors including an increased rate of formation of senescent cells, loss of senescence immunosurveillance, bystander effects caused by secretory phenotype on neighbouring cells and upregulation of intrinsic anti-apoptotic factors (van Deursen, 2014). Since Zn ions are important mediators of apoptotic pathways, and can both enhance or repress functional apoptosis (Garufi et al., 2016; McCabe et al., 1993; Perry et al., 1997; Truong-Tran et al., 2000; Zalewski et al., 1994), manipulating the exposure of cells to Zn may be an effective strategy to modulate cell senescence or to promote the death of senescent cells. In support of this idea, quercetin, a natural polyphenol and Zn ionophore (Dabbagh-Bazarbachi et al., 2014), induced selective death of senescent endothelial cells over proliferating cells (Zhu et al., 2015). Moreover, dysregulated Zn balance has been shown to induce senescence in dermal fibroblasts (Rudolf and Cervinka, 2011) and the Zn ionophore pyrithione was identified as a senescence-inducing compound in a screen of 4160 compounds (Ewald et al., 2009). Zn deficiency is considered a risk factor for the development of atherosclerosis (Beattie and Kwun, 2004), but the cellular and

---

[*] Corresponding author.
E-mail address: m.malavolta@inrca.it (M. Malavolta).

http://dx.doi.org/10.1016/j.exger.2017.09.006
Received 23 June 2017; Received in revised form 5 September 2017; Accepted 8 September 2017

molecular basis of this association has not been investigated in depth. We propose that the influence of Zn on endothelial cell senescence makes an important contribution to this observation. Since impaired Zn homeostasis is a feature of older age, we also propose that altered Zn homeostasis in senescent compared with replicating endothelial cells is an interacting influence. Commensurate with this idea, experiments performed in a vascular smooth muscle cell model suggest that cellular senescence is a Zn-dependent mechanism driven by specific Zn transporters involved in the regulation of intracellular Zn ions (Patrushev et al., 2012). The complex homeostatic network to which these transporters belong includes members of two families of Zn transporters, which together comprise 24 genes (Cousins et al., 2006; Lichten and Cousins, 2009), metallothioneins (MTs) (Mocchegiani et al., 2013), and Zn sensors (ZNF658 and MTF-1) (Choi and Bird, 2014; Ogo et al., 2015).

The purpose of this study was to investigate: 1) the impact of chronic treatment with a moderate excess of Zn on Zn content and distribution and on replicative potential and senescence in human coronary artery endothelial cells (HCAECs); 2) changes in Zn homeostasis over the lifespan of endothelial cells, using passage number as a surrogate for senescence, using transcriptomic data from two endothelial cell models (HCAECs and Human Umbilical Vein Endothelial Cells-HUVECs); 3) the impact of Zn and passage number on the expression of major players in Zn homeostasis over the culture lifespan of HCAECs; 4) the susceptibility to cell death induced by increasing doses of Zn or of the Zn chelator TPEN at early and later passages of HCAECs.

2. Material and methods

2.1. Cell culture

HCAECs (Human coronary artery endothelial cells) were purchased from Clonetics Corporation (Lonza) and cultured in endothelial basal medium EBM, supplemented with EGM-2/MV SingleQuots with or without addition of 50 μM $ZnSO_4$. This concentration has been already defined by others as moderately excessive and does not have a significant effect on the growth or morphology of endothelial cells (Bobilya et al., 2008). HCAECs were plated at a seeding density of 2500 cells/cm^2 in T 25 flasks and passaged serially. Harvested cells were counted using the Trypan blue viability stain. Population doublings (PD) were calculated using the formula $(\log_{10}UCY - \log_{10}I) \times 3.32$ (where UCY is the number of cells at the end of the passage and I is the number of cells initially seeded). Cells were collected for immediate assessment by flow cytometry or for RNA extraction for subsequent analysis of gene expression. Total RNA was extracted from HCAECs using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. RNA concentration and purity were measured using a NanoDrop spectrometer and samples were stored at − 80 °C for measurement using RT-PCR.

2.2. Senescence biomarkers

HCAECs were grown for subsequent passages until two consecutive population doublings equal to or below 0 were achieved, associated with morphological changes indicating senescence revealed by microscopy and flow cytometry. Cumulative population doubling (CPD) was calculated as the sum of all population doublings (PDs). Senescence associated beta-galactosidase activity (SA-β-gal) was measured by flow cytometry as described previously (Noppe et al., 2009). As an additional biomarker of senescence, p16 mRNA levels were measured by real-time RT-PCR. Positive staining with propidium iodide was used as the marker of cell viability.

2.3. Measurement of labile Zn and metallothionein protein

Labile Zn was measured by flow cytometry using the membrane permeable Zn specific probe Fluozin-3-AM (Haase et al., 2006). Localization of fluorescence after staining endothelial cells with the membrane permeable Zn probe and Lysotracker red (Thermofischer Scientific) was detected by imaging flow cytometry (Flowsight, Amnis Corporation, Seattle, WA, USA), as described previously for brain endothelial cells (Lopes Pinheiro et al., 2016). Briefly, cells were acquired on the basis of their area. Analysis was performed with a 488 nm laser line on single cells after compensation. The relative level of co-localization was calculated using the bright detail similarity feature R3 in the Ideas v6.0 software (Amnis-Merck Millipore). This feature corresponds to the logarithmic transformation of Pearson's correlation coefficient of the localized bright spots with a radius of 3 pixels or less within the whole cell area in the two input images. Labile Zn spots marked by Fluozin-3 were calculated using the spot count feature of the Ideas software.

Metallothionein protein expression was measured by flow cytometry following a previously-established protocol (Malavolta et al., 2008; Malavolta et al., 2007; Malavolta et al., 2006). Briefly: cells were incubated with the MT-specific monoclonal antibody (1 μg/10^5 cells/ 200 μl) (monoclonal mouse anti-horse metallotionein clone E9,from DAKOCYTOMATION, Denmark) for 30 min at room temperature. Following treatment with the primary antibody, the cells were washed with staining buffer and incubated for 1 h at room temperature with a FITC-conjugated secondary antibody (goat anti mouse Ig, Becton Dickinson, USA). MT-specific fluorescence (MFI) of the sample was determined by subtracting the mean fluorescence intensity obtained with the isotypic antibody clone MOPC21, IgG1k, (Sigma-Aldrich, St. Louis, USA) from the one obtained with the MT-specific antibody.

2.4. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

cDNA synthesis from total RNA was performed using i-Script reverse transcriptase (Biorad, Hercules, CA) according to the manufacturer's guidelines. Messenger RNA for β-actin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) reference genes and for p16, SLC30A1, SLC39A6, SLC39A1, SLC39A2, SLC39A3, SLC30A5, SLC30A10, CBWD, ZNF658, MT2A, MT1X and MT1F was then measured by real-time PCR on a BioRad iQ5 optical realtime thermal cycler (Biorad, Hercules, CA) using 1 μg of cDNA in a total volume of 25 μl containing iQ SYBR Green Supermix (Biorad, Hercules, CA). Primer sequences and thermal cycling parameters for SLC30A5, SLC30A10, CBWD, and GAPDH were as specified previously (Coneyworth et al., 2012). The other primers used are listed in Supplementary Table 1. Primer concentrations of 200 nM were used for MT1X, MT2A, SLC30A1, ZNF658, SLC39A3 and SLC309A6 genes; 150 nM for p16 and β-ACTIN, and 300 nM for MT1F and SLC39A2 genes. Assays for each transcript were carried out as duplicates. Fold changes relative to the control condition at the earliest passage were calculated using the ΔΔCt method. β-Actin was used as the reference gene for p16. GAPDH was used as the reference gene for the other mRNAs measured. Data are compared to the control condition at the earliest passage.

2.5. Whole genome expression analysis

Data were obtained using the GeneChip 3′ IVT Express kit (Affymetrix, Santa Clara, CA, USA). An integrated meta-analysis of the original *.CEL files was performed using Partek Genomic Suite Software v6.6 (Partek Inc.) and data from 3 replicates each of HuVECs and HCAECs harvested at early and late passages (passages 4 and 18 for HuVECs; passages 6 and 12 for HCAECs). For statistical analysis, we first identified genes significantly ($p < 0.05$ and $FDR < 0.05$) upregulated and downregulated in late compared with early-passage cells (both HUVEC and HCAEC) using the ANOVA tool integrated in the Partek Genomic Suite. Those genes in this list known to regulate Zn homeostasis were selected for cluster analysis. We also imported