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Quantitative and integrated proteome and microRNA analysis of endothelial replicative senescence

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ABSTRACT

Age-related changes in vascular functioning are a harbinger of cardiovascular disease but the biological mechanisms during the progression of endothelial senescence have not been studied. We investigated alterations in the proteome and miRNA profiles in the course of replicative senescence using primary human umbilical vein endothelial cells as an in vitro vascular model. Quantitative proteomic profiling from early growth stage to senescence was performed by isotope-coded protein label coupled to LC-ESI-MS/MS analysis. Some proteins consistently changed their expression during the senescence whereas others appeared as deregulated only during the late senescence. The latter was accompanied by alterations in morphology of senescent endothelial cells. MicroRNA expression profiling revealed transient changes in the level of miR-16-5p, miR-28-3p and miR-886-5p in the early senescence, decrease in the level of miR-106b-3p at the late stage, and continuous changes in the expression of miR-181a-5p and miR-376a-3p during the whole senescence process. Integrating data on proteomic and microRNA changes indicated potential crosstalk between specific proteins and non-coding RNAs in the regulation of metabolism, cell cycle progression and cytoskeletal organization in the endothelial senescence. The knowledge of molecular targets that change during the senescence can ultimately contribute to a better understanding and prevention of age-related vascular diseases.

1. Introduction

Replicative senescence was first described more than 50 years ago by Leonard Hayflick and Paul Moorhead [1]. Since then, much effort has been made to understand the nature of senescent cells both in vitro and in vivo [2]. Cellular senescence has been associated with both cancer and age-related pathologies [3]. In cultured cells replicative senescence occurs as an irreversible growth arrest after multiple population doublings.

The attrition of telomeres accompanying repeated cell divisions has been postulated to be the molecular trigger for cellular senescence. However, shortening of telomeres may also be a consequence and not a cause of the senescence process [4]. In vivo, growth- and age-dependent cellular senescence in the rat kidney occurs despite an absence of critical telomere shortening [5]. The data from epithelial cells also suggest the existence of a proliferative life span barrier that is unrelated to telomere erosion [6]. Furthermore, the presence of telomerase activity is unable to prevent premature senescence, indicating that the senescence process can be activated by other signals than shortened telomeres [7]. A wide variety of conditions, including oxidative stress and the mutational activation of a cellular oncogene (e.g. Myc, Ras and Raf) induce premature senescence [8].

Endothelial cell senescence is assumed as an underlying mechanism for age-related cardiovascular disease [9]. Senescent endothelial cells have been observed in atherosclerotic lesions and diseases caused by various stimuli [2]. Senescent cells retain some metabolic activity and are viable despite displaying typical phenotypic changes such as senescence-associated beta-galactosidase activity [10]. Recently, a number of deregulated genes and proteins have been suggested as potential biomarkers for replicative senescence [11–13]. However, these molecules do not exclusively hallmark the senescence state as

Abbreviations: HUVEC, human umbilical vein endothelial cell; IPA, Ingenuity Pathway Analysis; ICPL, isotope-coded protein labeling; ESI, electrospray ionization; LC, liquid chromatography; MS/MS, tandem mass spectrometry; miRNA, microRNA; mRNA, messenger RNA; MVS, MicroVigene signal-intensity; PCR, polymerase chain reaction; RPPA, reverse phase protein array.

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their differential regulation has been associated with several pathologies such as cancer and aging [14].

MicroRNAs (miRNAs) are evolutionary conserved non-coding RNAs that suppress gene expression either by promoting mRNA degradation or repressing protein translation [15]. Regulation of miRNA expression has been reported in several pathophysiological diseases such as atherosclerosis and inflammatory diseases [16]. Several miRNAs such miR-34a and miR-125a-5p are known to be involved in endothelial dysfunction and to promote senescence [17,18].

Primary human umbilical vein endothelial cells (HUVECs) are widely used as an in vitro endothelial cell model to study aging vasculature, partly due to their ability to form capillary-like structures on matrix in response to angiogenic stimulus [19]. Cellular and molecular studies have provided some insight into the endothelial senescence but due to the complexity of underlying pathways during this process, senescence is still considered as an enigmatic event.

In this study we focused on alterations in both the proteome and miRNAome during the progression of replicative senescence in HUVECs. The integration of the two data sets strongly suggests that there is intensive crosstalk between deregulated proteins and miRNAs in endothelial replicative senescence. This study has identified potential factors that contribute to the mechanisms of endothelial cell senescence.

2. Materials and methods

2.1. Cell culture conditions and cell growth kinetics

HUVECs (Invitrogen, Paisley, UK) were obtained from a single donor and cultured in Media 200 (Invitrogen, Paisley, UK) supplemented with low serum growth supplement containing 2% fetal bovine serum, 1 μg/μl hydrocortisone, 10 ng/ml epidermal growth factor, 10 μg/ml heparin, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37 °C in a 95% air/5% CO_2 humidified atmosphere. HUVECs (passage 2) were serially sub-cultured until they reached senescence. Cells were passaged using accutase (Invitrogen, Paisley, UK) every seven days (5000 cells/cm^2) with culture medium changed every two days. The growth rate kinetics was calculated using the equation:

$$\text{population doublings (PD)} = \ln(n1/n0)/\ln 2$$

where n0 is the number of the cells seeded and n1 is number of the cells that were counted after one week.

2.2. Assay of senescence-associated-ß-galactosidase

SA-ß-gal activity was determined using a histochemical staining kit according to the manufacturer's instructions (Sigma-Aldrich). Cells were collected for senescence associated beta-galactosidase at time points 1, 3, 6, 10, 12 and 15 weeks. The percentage of SA-ß-gal blue stained HUVECs was determined by counting at least 1500 cells using light microscopy.

2.3. Proteome analysis of HUVECs

2.3.1. Whole cell protein extraction

The whole cell protein extraction was performed from harvested HUVECs at weeks 1, 6, and 12 as the protein content of cells at the very late state of senescence (week 15) was too low for a proteomics analysis. For cell lysis we used isotope-coded protein labeling (ICPL) lysis buffer (SERVA Electrophoresis GmbH, Germany). Protein concentration was determined by Bradford assay following the manufacturer's instructions. All experiments were performed with three biological replicates.

2.3.2. Isotopic labeling of proteins

The protein extracts from weeks 1, 6 and 12 were labeled with ICPL triplex reagents according to the manufacturer's instructions (SERVA Electrophoresis GmbH, Germany). Triplicate aliquots of 50 μg of proteins obtained from week 1, 6 and 12 samples were individually labeled using ICPL0 (light), ICPL4 (medium) and ICPL6 (heavy) labeled reagents, respectively, and the reaction was allowed to proceed for 2 h at pH 8.3 at room temperature. The labeling was quenched by using hydroxylamine. Esters which form during the labeling procedure were hydrolyzed by raising the pH to 11–12 for 20 min. Equal amounts of light, medium and heavy labeled samples for each replicate were combined and separated by 12% SDS gel electrophoresis [20]. A protein mixture with known ratios of heavy and light label was used as an internal standard for labeling efficiency and data acquisitions. The labeling was done using three biological replicates.

2.3.3. LC-ESI-MS/MS analysis

After staining with colloidal Coomassie solution each SDS gel lane corresponding to an individual replicate was sliced into 5 pieces. Proteins were de-stained with 50 mM NH_4HCO_3 in 30% acetonitrile (ACN) prior to overnight in-gel digestion with trypsin (sequencing grade, SERVA Electrophoresis GmbH, Germany) at a protein to enzyme ratio of 50:1 in 10 mM NH_4HCO_3. Peptides were extracted and acidified with 1% formic acid for subsequent mass spectrometry analysis. The initial liquid chromatography separation of proteins from each SDS gel slice was performed on a nano-scale liquid chromatography system (Ettan MDLC, GE Healthcare). Peptides were loaded onto an RPC trap column at a flow-rate of 6 μl/min [(loading buffer: 0.1% formic acid; trap column C18 PepMap 100, 5 μm bead size, 300 μm i.d., 5 nm length, LC Packings (Dionex, Netherlands))] and were subsequently separated on an analytical column (C18 PepMap 100, 3 μm bead size, 75 μm internal diameter., 15 cm length, LC Packings) with a 120 min linear gradient (A: 0.1% formic acid, B: 84% ACN and 0.1% formic acid) at a flow-rate of 260 nl/min. The gradient used was: 0–30% B in 80 min, 30–60% B in 30 min, 60–100% B in 10 min.

Mass spectrometry was performed on a linear ion trap mass spectrometer (Thermo LTQ Orbitrap, Thermo scientific) coupled directly to the nano-LC system. For electrospray ionization a distal coated Silica Tip (FS-360-50-15-D-20) with a needle voltage of 1.4 kV was used. The MS consisted of a cycle combining one full MS scan (mass range: 300–2000 m/z) with five data-dependent MS/MS events (35% collision energy) in parallel mode. The dynamic exclusion was set to 30 s.

The acquired MS/MS spectra were searched against the ENSEMBL Human database using MASCOT (Matrix Science, version 2.3.02) with the following parameters: MS/MS spectra were searched with a precursor mass tolerance of 10 ppm and a fragment tolerance of 0.8 Da. Trypsin was selected as the cleaving enzyme (SERVA Electrophoresis GmbH, Germany). One missed cleavage was allowed and carbamidomethylation was set as the fixed modification. Oxidized methionine and the heavy, medium and light ICPL labels of lysines, as well as labeled protein N-termini were set as variable modifications.

2.3.4. Quantification of labeled proteins

Data processing for protein identification and quantification of ICPL pairs was performed using Proteome Discoverer version 1.3 (Thermo Fisher) as described before [21,22]. Briefly, Proteome Discoverer software performs automated statistical analysis of the results and uses unique peptides to calculate accurate relative protein quantification. The complete peptide and protein profiles were filtered using high peptide confidence and top one peptide rank filters. The false discovery rate was calculated at the peptide level for all experimental runs using the Decoy option in MASCOT, and the significance threshold was set to 0.05. Proteins with a lower score were manually scrutinized and regarded as unequivocally identified if they fulfilled the following four criteria: (a) they had fragmentation spectra with a long, nearly complete y- and/or b-series; (b) all lysines were modified; (c) the numbers