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The senescent hepatocyte gene signature in chronic liver disease

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ABSTRACT

Hepatocyte senescence is associated closely with fibrosis stage and an adverse outcome in chronic liver disease, but it is uncertain whether there is a causal relation with clinical manifestations of chronic liver disease, which was the subject of this study of the senescent hepatocyte gene signature. Senescence was induced in HepG2 cells using sub-lethal concentrations of H_2O_2. Gene expression of control and senescent HepG2 cells were studied. Comparison was made with patients with cirrhosis and three public microarray datasets. H_2O_2-treated HepG2 cells demonstrated characteristic cellular senescence. There was differential expression of 354 genes in senescence. Up-regulated genes in HepG2 senescence were also up regulated in patients with cirrhosis. The senescent hepatocyte gene signature distinguished liver disease from normal by unsupervised clustering in the public chronic liver disease microarray datasets, with enrichment of the senescence gene signature in all three datasets. The senescent hepatocyte gene signature included changes in cell cycle regulation, morphology, inflammation, signal transduction, metabolism and stellate cell activation, which alongside impaired synthetic function in senescence *in vitro* were consistent with manifestations of clinical liver disease, suggesting a close relation between hepatocyte senescence and manifestations of chronic liver disease including fibrosis and impaired synthetic function.

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1. Introduction

Cellular senescence, a stable form of cell cycle arrest that limits the proliferative potential of cells (Campisi and d'Adda di Fagagna, 2007), is triggered in normal cells in response to a variety of cellular stresses, including telomere uncapping, DNA damage and oncogene activation (Campisi and d'Adda di Fagagna, 2007; Ben-Porath and Weinberg, 2005; Collado et al., 2007). The senescent phenotype is extremely stable and resistant to apoptosis (Campisi and d'Adda di Fagagna, 2007). A senescent cell is unresponsive to mitogenic stimuli but remains metabolically active (Narita, 2007; Serrano and Blasco, 2001), although to what extent the metabolic activity resembles that of a mature normal cell is less clear. Senescent cells accumulate within organs (Campisi and d'Adda di Fagagna, 2007; Collado et al., 2007) and the decline in organ function with ageing has been attributed to such accumulation (Campisi, 1998).

Senescent cells have well defined features, which include cell cycle arrest, morphological change such that cells are enlarged and flattened with enlarged nuclei (Narita, 2007; Mehta et al., 2007), expression of senescence-associated β-galactosidase (SA-β-GAL) (Dimri et al., 1995), accumulation of senescence-associated heterochromatic foci (SAHF) (Narita et al., 2003, 2006), accumulation of DNA damage foci (d'Adda di Fagagna et al., 2003; Wang et al., 2009) and acquisition of the senescence-associated secretary phenotype (SASP) (Coppe et al., 2008; Davalos et al., 2010). These changes are known collectively as the 'cellular senescence signature' (Sikora et al., 2011). All senescence-inducing signals involve p53 and/or the p16-retinoblastoma protein and encompass complex stress-signal integration and processing pathways to induce and then maintain senescence (Campisi and d'Adda di Fagagna, 2007; Ben-Porath and Weinberg, 2005). p21, a transcriptional target of p53 and a potent cell cycle inhibitor, plays a critical role in linking those two pathways and maintaining senescence (Campisi and d'Adda di Fagagna, 2007; Ben-Porath and Weinberg, 2005).

The role of cellular senescence in chronic liver disease has come under recent scrutiny. Accelerated hepatocyte ageing and the accumulation of senescent hepatocytes have been demonstrated in diverse chronic liver disorders (Nakajima et al., 2006, 2010; Richardson et al., 2007; Aravinthan et al., 2013a, 2013b; Marshall et al., 2005; Wood et al., 2014). Further, these studies show that the increased proportion of senescent hepatocytes is associated independently with increased fibrosis stage, impaired hepatic function and an adverse liver-related outcome, including liver-related death (Nakajima et al., 2006, 2010; Richardson et al., 2007; Aravinthan et al., 2013a, 2013b; Marshall et al., 2005; Wood et al., 2014; Gonzalez-Reimers et al., 1988). However,

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whether hepatocyte senescence contributes causally to fibrosis progression, declining hepatic function or the increased risk of an adverse liver-related outcome is less clear.

The aim of the present study was to characterise alterations in gene expression in senescent hepatocytes and to determine whether any changes in the hepatocyte senescence gene signature might explain the observed links between hepatocyte senescence and clinical features of disease progression.

2. Materials and methods

2.1. Cell culture and induction of senescence

The human liver cell line HepG2 (ATCC HB-8065) was grown in Dulbecco's Modified Eagle Medium containing 10% foetal calf serum and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). Cells were seeded in 6-well plates at a density of 5×10^5 per well and allowed to adhere overnight before treatment. Cells were incubated with 0.5 mM H_2O_2 in culture media for 60 min to induce senescence (the optimal conditions which were determined for induction of senescence rather than apoptosis or death). Control HepG2 cells were incubated in culture media alone. Cells were then washed three times with PBS and incubated at 37 °C, 5% CO_2 for 5 days before observing cell morphology and assessing the senescent phenotype.

The senescence phenotype was assessed using cell morphology, SA-β-GAL activity, expression of both cell cycle phase markers and cell cycle inhibitors, the presence of senescence-associated heterochromatic foci and senescence-associated secretary phenotype.

2.2. Evaluation of SA-β-GAL activity

SA-β-GAL activity was evaluated using Senescence β-Galactosidase Staining Kit (Cell Signaling Technology™) according to the manufacturer's recommendations. Cells were examined under a light microscope for development of blue staining following overnight incubation at 37 °C.

2.3. Immunofluorescence

The following antibodies were used for immunofluorescence assays: (i) unconjugated rabbit anti-Mcm-2 (cell signaling; concentration 1:50, FITC fluorochrome-conjugated donkey anti-rabbit secondary antibody); (ii) Alexa Fluor® 647-conjugated rabbit anti-PH3 (cell signaling; concentration 1:50); (iii) Alexa Fluor® 488-conjugated rabbit anti-p21 (cell signaling; concentration 1:50); (iv) unconjugated rabbit anti-HMGI-C (Santa Cruz Biotechnology; concentration 1:500; FITC fluorochrome-conjugated donkey anti-rabbit secondary antibody); and (v) unconjugated mouse anti-HP1γ (Millipore; concentration 1:500; Cy3 fluorochrome-conjugated donkey anti-mouse secondary antibody).

In brief, HepG2 cells (H_2O_2-treated and control) were grown on coverslips, washed twice with PBS before being fixed with 4% PFA for 15 min at room temperature and permeabilised with 0.2% Triton X-100 for 5 min. Cells were washed again before incubation at room temperature for 45 min with specific antibody diluted in PBST (0.5% Tween-20 in PBS) containing 1% normal goat serum. Where required (for Mcm-2, HMGI-C and HP1γ), cells were washed with PBST before incubation with a species-specific fluorochrome-conjugated secondary antibody. 4′,6-Diamidino-2-phenylindole (DAPI; concentration 1:500) served as a nuclear stain. Coverslips mounted on glass slides with fluorescence mounting medium (Dako) were visualised by epifluorescent microscopy.

2.4. Determination of SASPs and secretory proteins in conditioned media

The levels of SASP-related cytokines (IL-6 and IL-8) and fibrinogen in conditioned media from H_2O_2-treated and control HepG2 cells were determined using the ELISA Ready-SET-Go kits (Human IL-6 and Human IL-8; eBioscience) and Fibrinogen Human ELISA Kit (Abcam) according to the manufacturers' recommendations. The levels of α-foetoprotein and retinol-binding protein were measured using time-resolved fluoroimmunoassay (AutoDELFIA™ hAFP) and 'Top-down' mass spectrometric analysis, respectively, by Clinical Biochemistry at Cambridge University Hospitals, Cambridge, UK.

2.5. RNA extraction and microarray analysis

RNA extraction was performed using Qiagen RNeasy Plus Mini Kit according to the manufacturer's recommendations. RNA quality and quantity were measured using spectrophotometry at 260 and 280 nm and on 2100 Bioanalyzer Eukaryote Total RNA Nano Series II chip (Agilent).

Genomics CoreLab-Cambridge NIHR Biomedical Research Centre, Cambridge, UK undertook the microarray experiments. RNA was processed using Affymetrix GeneChip® Human Gene 1.0 ST arrays. RNA integrity number values were between 9.7 and 10 for all samples. Labelling of the sample material, hybridisation and scanning of the microarrays were carried out according to Affymetrix standard protocols.

2.6. Generation of differentially expressed gene lists

Initial normalisation of the array data and generation of a differentially expressed gene list was carried out using R (version 12.14.2), and the following packages: limma, affy, affyPLM and affycoretools.

Normalised expression estimates from the raw intensity values were performed using the probe level linear model pre-processing algorithm available in the Bioconductor library AffyPLM (fitPLM) using default settings. To inspect arrays for potential artefacts, intra-array quality was visualised using image plots of raw, weights, residuals and the sign of residuals.

Sample quality and the effect of normalisation were visualised by inspection of plots of sample intensity and principal components before and after normalisation, relative log expression (RLE), normalised unscaled standard error (NUSE), and RNA degradation. MA plots were also generated comparing chips to a pseudo-median reference chip as well as within sample groups (cell line and treatment status).

Differential expression of genes between H_2O_2-treated and matched control HepG2 cells was assessed using an empirical Bayes' statistic and p-values were adjusted for multiple testing using the Bioconductor library limma. A linear model was fitted (lmFit) to compute estimated coefficients and standard errors between groups (contrasts.fit). Empirical Bayes shrinkage of standard errors was then used to rank genes in order of evidence for differential expression (eBayes). Principal component analysis plots were generated using the Bioconductor library affycoretools (plotPCA) to examine the relation between H_2O_2-treated and matched control HepG2 cells.

Genes showing up- or down-regulation during senescence were taken as those showing a differential expression of at least 2-fold change at adjusted $p < 0.01$ between H_2O_2-treated and control samples.

2.7. Functional annotation of genes

Genes present in the senescence gene list were annotated with gene ontology terms using DAVID (http://david.abcc.ncifcrf.gov/) to obtain probable functional roles of individual genes.

Significant enrichment of gene ontology terms in the up- and down-regulated gene lists were assessed using FuncAssociate 2.0 (http://llama.mshri.on.ca/funcassociate/), which performs Fisher's exact test analysis with empirical resampling to correct for multiple hypotheses.

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