Research Article

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Hepatocyte senescence predicts progression in non-alcohol-related fatty liver disease

Aloysious Aravinthan, Cinzia Scarpini, Phaedra Tachtatzis, Suman Verma, Sue Penrhyn-Lowe, Rebecca Harvey, Susan E. Davies, Michael Allison, Nick Coleman, Graeme Alexander*

Division of Gastroenterology and Hepatology, Department of Medicine, University of Cambridge, Cambridge CB2 0QQ, UK

See Focus, pages 407–408

Background & Aims: Models of non-alcohol-related fatty liver disease (NAFLD) reveal features of accelerated ageing, such as impaired regeneration, and an increased risk of hepatocellular carcinoma. The relation between accelerated ageing, disease progression and clinical outcome has not been previously investigated and is the subject of the current study.

Methods: Liver sections from 70 patients with NAFLD (105 biopsies) and 60 controls were studied for telomere length, nuclear area, DNA damage and cell cycle phase markers, using quantitative fluorescent *in situ* hybridization and immunohistochemistry.

Results: Hepatocyte telomeres were shorter in NAFLD than controls (p <0.0001). Hepatocytes in NAFLD demonstrated lack of cell cycle progression beyond G1/S phase and high-level expression of p21, the universal cell cycle inhibitor (p = 0.001). γ-H_2AX expression increased with steatosis (p = 0.01), indicating DNA damage, and was associated with shorter hepatocyte telomeres (p <0.0001). Hepatocyte p21 expression correlated with fibrosis stage and diabetes mellitus, independently (p <0.001 and p = 0.002, respectively). Further analysis revealed that an adverse liver-related outcome was strongly associated with higher hepatocyte p21 expression and greater hepatocyte nuclear area (p = 0.02 and p = 0.006), but not with telomere length. In paired biopsies, changes in hepatocyte p21 expression and nuclear area mirrored changes in fibrosis stage (p = 0.01 and p = 0.006, respectively).

Conclusions: These findings are consistent with hepatocyte senescence and permanent cell cycle arrest in NAFLD. Hepatocyte senescence correlated closely with fibrosis stage, diabetes mellitus, and clinical outcome. Hepatocyte p21 expression could be used as a prognostic marker and for stratification in clinical studies.

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Introduction

Non-alcohol-related fatty liver disease (NAFLD) is a leading cause of chronic liver disease worldwide [1,2], encompassing a wide spectrum of liver disorders ranging from simple steatosis, through steatohepatitis with or without fibrosis, to cirrhosis and hepatocellular carcinoma [1,2].

In *ob/ob* mice, NAFLD is associated with increased expression of the cell cycle inhibitor p21 and impaired liver regeneration following partial hepatectomy [3]. In humans, fatty liver is linked to an increased risk of liver failure and death, following hepatic resection [4]. Delayed graft function and graft failure are more common in liver allografts with pre-existing fatty infiltration [5,6]. Furthermore, hepatocellular carcinoma may develop in fatty liver, even in the absence of significant fibrosis [7]. The combination of impaired regeneration and an increased incidence of cancer in NAFLD suggests accelerated ageing.

Cellular senescence, a stable form of cell cycle arrest block ing further proliferation, is triggered by multiple mechanisms, including DNA damage and telomere shortening [8]. Along with the rest of the chromosome, telomeres are damaged during oxidative stress, but telomere repair is less efficient than elsewhere in the chromosome, leading to accelerated telomere loss [9–12]. A critically short telomere, or irreparable DNA damage, triggers the signal amplification cascade known as the DNA damage response (DDR) [13], leading to irreversible cell cycle arrest mediated by p21 [14]. Local phosphorylation of the histone H_2AX (γ-H_2AX) is a key step in nucleation of the DDR [15]. The DDR signalling pathway contributes to the stability of cell cycle arrest, long after induction of senescence [16]. Senescent cells cease to respond to mitotic stimuli, undergo characteristic morphological changes, including

Keywords: Senescence; Cell cycle arrest; Telomere; Non-alcohol-related fatty liver disease.

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* Corresponding author. Address: Division of Gastroenterology and Hepatology, Department of Medicine, University of Cambridge, Box 157, Cambridge University Hospitals, Hills Road, Cambridge CB2 0QQ, UK. Tel.: +44 1223 586614; fax: +44 1223 216111.

E-mail address: gja1000@doctors.org.uk (G. Alexander).

Abbreviations: NAFLD, non-alcohol-related fatty liver; DDR, DNA damage response; γ-H_2AX, phosphorylated histone H_2AX; Q-FISH, quantitative fluorescent *in-situ* hybridization; MCM, minichromosome maintenance protein; PH3, phosphorylated histone 3; MFI, mean fluorescent intensity.

Genetic and Metabolic Diseases

increased nuclear size, and become resistant to apoptosis [17,18].

A study in 1988 on alcohol-related liver disease, before senescence had been described in liver disease, demonstrated a close relation between increased hepatocyte nuclear area, advanced fibrosis, and hepatic dysfunction [19]. Premature telomere shortening, increased nuclear area, and p21 expression, all markers of ageing or cellular senescence, have each been described separately in hepatocytes in NAFLD [20–22]. However, the relation between the DDR, accelerated ageing and cellular senescence, and clinical outcome in NAFLD has not been established and was investigated in this cross sectional and longitudinal study.

Materials and methods

Liver biopsy specimens

All liver biopsy specimens were obtained in accordance with local research ethics committee guidelines.

Patient tissue

One hundred and five archived formalin-fixed paraffin-embedded liver needle biopsy specimens were studied from 70 patients within the spectrum of NAFLD, including paired biopsy specimens from 35 subjects. All patients studied were hepatitis C virus antibody negative, HBsAg seronegative, antimitochondrial, antinuclear and anti-smooth muscle antibody seronegative, negative for *HFE* mutations C282Y and H63D, and had normal serum immunoglobulin, copper, caeruloplasmin, and α-1 antitrypsin levels. Average alcohol consumption was less than 14 units per week in all subjects. The patients had a mean follow-up of 67 months (range 3–155 months).

Control tissue

Forty-three archived formalin-fixed paraffin-embedded liver needle biopsy specimens, obtained at the time of liver transplantation from age and sex matched donor livers (time zero biopsies), served as controls for telomere study using quantitative fluorescent *in situ* hybridization (Q-FISH). Donor data were reviewed and sections of adequate size were chosen to reflect normality according to the following criteria: no history of liver disease or senescence-related disease in the donor; a short medical illness preceding donor death; none or minimal reperfusion injury or steatosis at histological review; excellent graft function 1-year after transplantation.

Archived formalin-fixed paraffin-embedded liver biopsy specimens were obtained from 12 further patients, during the regenerative phase of acute ischaemic–reperfusion injury following liver transplantation, to serve as positive control tissue for expression of cell cycle phase markers. All of these patients made a rapid recovery after transplantation, with normal liver function at discharge from hospital. All the liver biopsy specimens showed evidence of regeneration on H&E-stained sections. Archived formalin-fixed paraffin-embedded liver needle biopsy specimens were also obtained from the background liver, from five patients with colorectal cancer metastases undergoing resection, to serve as negative control tissue for cell cycle phase markers. None of these patients had received chemotherapy prior to resection. Needle biopsies were distant from metastases, with normal histology on H&E-stained sections.

Telomere intensity and nuclear area

Nuclear area and telomere intensity (a surrogate marker of telomere length) of hepatocytes were assessed using Q-FISH, as previously described in detail [23]. Hepatocytes were identified using unconjugated mouse monoclonal anti-Hepar-1 (Dako, concentration 1:100). Cy3 fluorochrome-conjugated donkey anti-mouse secondary antibody was used to detect anti-Hepar-1-positive cells (Fig. 1A).

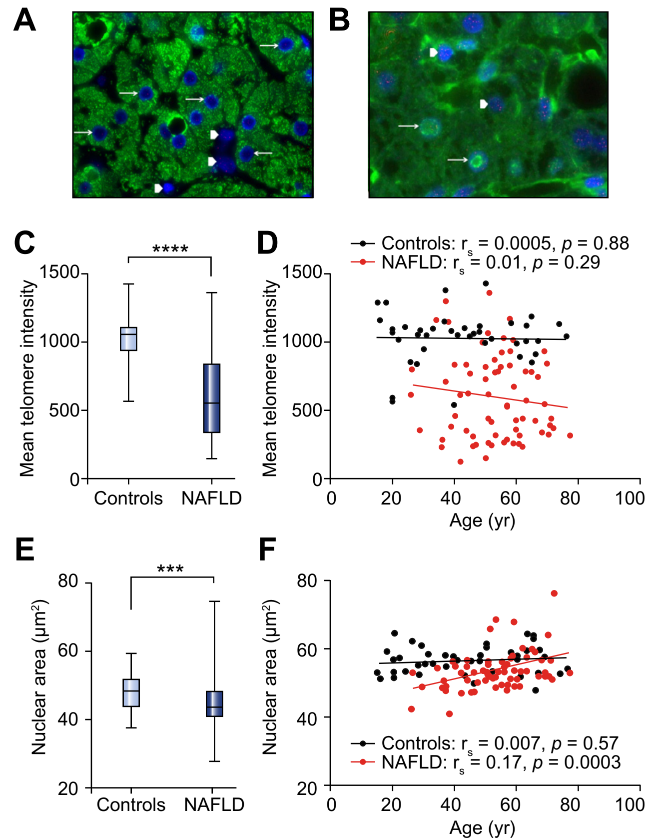

Fig. 1. Q-FISH analysis of hepatocytes. (A) Hepatocytes (arrow) are distinguished from other lineages (pentagon) by anti-Hepar-1 (green cytoplasm). Nuclei stain blue with DAPI; telomeres are identified by the pink intranuclear dots. (B) γ-H_2AX-positive hepatocytes (arrow) have green nuclei; adjacent γ-H_2AX-negative hepatocytes are shown (pentagon). (C) Hepatocyte telomere MFI was lower in NAFLD compared to controls (****p <0.0001) and (D) it did not correlate with age in NAFLD or controls. (E) Hepatocyte nuclear area was smaller in NAFLD and increased with age (***p = 0.0002), (F) but not in controls.

Rabbit polyclonal anti-γ-H_2AX (Abcam, concentration 1:25) was used to assess DDR in 30 patients across the spectrum of NAFLD. FITC fluorochrome-conjugated donkey anti-rabbit antibody identified γ-H_2AX-positive hepatocytes (Fig. 1B). These experiments were performed simultaneously with Q-FISH for hepatocyte telomere length.

Immunohistochemistry

A Bond™ machine (Leica Microsystems) was used to perform automated immunohistochemistry. Unconjugated mouse monoclonal anti-Mcm-2 was used as a marker of cell cycle entry (Novocastra, concentration 1:25, heat-induced citrate-based antigen retrieval, 30 min). Unconjugated mouse monoclonal anti-cyclin A (Novocastra; concentration 1:25; heat-induced citrate-based antigen retrieval, 10 min) and unconjugated mouse monoclonal anti-PH3 (Upstate Biotechnology; concentration 1:500; heat-induced EDTA-based antigen retrieval, 10 min) were used as markers of S phase and M phase, respectively. Unconjugated mouse monoclonal anti-p21 was used as a marker of cell cycle arrest (Dako; concentration 1:100, heat-induced EDTA-based antigen retrieval, 20 min). Unconjugated anti-CK19 (Dako; concentration 1:100, heat-induced EDTA-based antigen retrieval, 20 min) was used to identify progenitor cells in hepatic parenchyma.

Immunohistochemistry was assessed in a quantitative manner to ensure objectivity. Images obtained from DotSlide digital microscope were analysed using ScanR analysis software.

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