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Hepatic pseudocapillarization in aged mice

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Abstract

Age-related changes in the hepatic sinusoid of the rat, human and baboons called pseudocapillarization have been discovered and are important because they are considered to be implicated in the pathogenesis of some age-related diseases. In this study, we investigated whether similar changes occur in the livers of old mice. Livers of young (3–4 months) and old (20–24 months) mice were perfusion-fixed and studied using electron microscopy and immunohistochemistry. The thickness of the sinusoidal endothelium was increased in old mice (154 ± 4 versus 244 ± 8 nm, $P < 0.001$). There was a reduction in fenestrations within the endothelium (porosity decreased from 4.1 ± 0.3 to $2.2 \pm 0.2\%$, $P < 0.001$). There was perisinusoidal staining with Sirius red in old mice, however, expression of laminin and von Willebrands factor was similar in young and old mice. Novel perisinusoidal fat-engorged stellate cells were found extensively in the old mice. This study confirmed that pseudocapillarization is a widespread aging change in the liver, now documented in several species including the mouse. Mice are an appropriate animal model for studying aging and the hepatic sinusoid.

Keywords: Liver; Aging; Pseudocapillarization; Mouse; Liver sinusoidal endothelial cell; Fenestration

1. Introduction

In the past, the liver has been considered to be relatively unaffected by the aging process and age-related diseases. Functional changes, particularly related to impaired drug metabolism (which for some drugs may be reduced by up to 40–50% in old age), were attributed mainly to age-related reduction in blood flow and liver mass (Cotreau et al., 2005; Le Couteur and McLean, 1998). Because such mechanisms cannot fully explain the age-related impairment of hepatic drug metabolism (Le Couteur and McLean, 1998), we examined the effects of old age on the structures lying between the blood and the hepatocyte that could impose a barrier to substrate transfer: the liver sinusoidal endothelium and extravascular space of Disse.

Liver sinusoidal endothelial cells occupy a strategic position between the hepatic sinusoid and hepatocytes. Liver sinusoidal endothelial cells are highly specialized endothelial cells lining the wall of the hepatic sinusoid, which separate the sinusoidal blood derived primarily from the portal vein, from hepatocytes (Fraser et al., 1995). Liver sinusoidal endothelial cells are perforated with fenestrations. Fenestrations are pores approximately 100 nm in diameter grouped together in clusters known as liver sieve plates (Wisse et al., 1985). Fenestrations in liver sinusoidal endothelial cells are true discontinuities in the endothelium, lacking either a diaphragm or underlying basal lamina (Fraser et al., 1995).

We found that aging is associated with marked changes in liver sinusoidal endothelial cells and space of Disse in the rat (Le Couteur et al., 2001), human (McLean et al., 2003), and baboon (Cogger et al., 2003). We termed this 'pseudocapillarization' because the aging sinusoidal endothelium had become more like capillaries seen in other vascular beds and in order to differentiate these changes from those seen in cirrhosis called 'capillarization'

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(Le Couteur et al., 2001). Overall there was a 40–80% increase in liver sinusoidal endothelial cell thickness and a 60–80% reduction in porosity (the percentage of the endothelial surface perforated with fenestrations). This was associated with basal lamina deposition in 25–40% of old livers. Major age-related changes in the liver were confined to the perisinusoidal regions—there was some deposition of lipofuscin and multinucleate cells in the hepatic parenchyma, however, no other ultrastructural changes were observed in hepatocytes.

Here, we investigated whether pseudocapillarization occurs with old age in mice. Confirmation of the presence of pseudocapillarization in mice would establish pseudocapillarization as a widespread aging change and confirm that old mice are an appropriate model for the study of aging and the hepatic sinusoid.

2. Methods

2.1. Animals and preparation of liver samples

Young and old B10.BR (H-2^k) mice (age 3–4 and 20–24 months) were purchased from the Animal Resources Centre, Perth, WA, Australia and maintained in the Centenary Institute Animal Facility (University of Sydney, Australia) under full SPF conditions and with adlibitum feeding. The study had the approval of the Sydney South West Area Health Service Animal Welfare Committee. Mice at 20–24 months are senescent (Liang et al., 2003).

Animals were sacrificed with CO_2 and livers immediately perfusion-fixed via a 23 G needle inserted into the portal vein. For light microscopy, liver tissue was fixed with 4% buffered paraformaldehyde. For electron microscopy, liver tissue was fixed with 1% glutaraldehyde/4% paraformaldehyde in PBS (0.1 M sucrose).

2.2. Light microscopy and immunohistochemistry

Liver specimens were embedded in paraffin blocks. Sections from each animal were stained with Hematoxylin and Eosin. In addition, Masson trichrome, Wilder's reticulin and Sirius red stains, which identify extracellular matrix and fibrosis, were performed. All tissue was examined by a specialist hepatopathologist (RF) to exclude underlying disease such as infection, tumour, fibrosis or cirrhosis.

Immunohistochemistry was used to detect the expression of laminin and von Willebrands factor (vWF) as described (Cogger et al., 2003; Le Couteur et al., 2001). Laminin identifies basal lamina. Von Willebrands factor is an endothelial antigen that is not expressed in normal hepatic sinusoidal endothelium but is upregulated in cirrhosis. Sections were pre-treated with proteinase K after deparaffinization. To prevent endogenous peroxidase activity and biotin binding, sections were pre-treated with 0.3% H_2O_2 in PBS and avidin–biotin blocking solutions. Sections were then incubated with the primary antibodies specific for vWf (1:800 rabbit, Sigma, St Louis, MO) and laminin (1:30 rabbit, Sigma, St Louis, MO). Secondary biotinylated antibodies (anti-rabbit 1:800, Sigma, St Louis, MO) were incubated for 45 min. Sections were incubated with peroxidase-conjugated streptavidin (30 min, 1:50, Sigma, St Louis, MO) and peroxidase activity was revealed using 3,3′-diaminobenzidine. All slides were graded according to intensity of staining (0, +, ++, +++) and differentiation between periportal and pericentral staining was undertaken.

2.3. Electron microscopy

Transmission electron microscopy was performed as described previously (Cogger et al., 2003; Le Couteur et al., 2001). Fixed liver tissue was processed and embedded in Spurr's resin. Blocks were sampled at random for light microscopic assessment. Three blocks per liver were finally studied, selected randomly from those satisfying requirements for quality of fixation and tissue integrity. Ultra-thin (70–90 nm) sections were taken from each block and 10 Zone 2 regions at random were chosen and photographed for ultrastructural measurement from each liver using a Zeiss 902 Transmission Microscope (magnification $\times$2000). Transmission electron micrographic measurements (100 per liver) of the thickness of the sinusoidal endothelial cells were made using Zeiss KS Image Analysis program.

Scanning electron microscopy was performed as described previously (Cogger et al., 2003; Le Couteur et al., 2001). Perfusion-fixed tissue was osmicated (1% OsO_4/0.1 mol/L sodium cacodylate buffer), dehydrated in an ethanol gradient to 100% and incubated for 10 min in hexamethyl-disilazane (Sigma, St Louis, MO). Tissue was then mounted on stubs, splutter-coated with gold and examined using a Cambridge S360 Scanning Microscope. Ten images (magnification $\times$15,000) were taken from each animal for analysis of fenestrations diameter and endothelial porosity using the Zeiss KS Image Analysis program.

2.4. Statistics

The results are expressed as mean $\pm$ SEM and comparison of the two age groups was performed using the Student's *t*-test. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Light microscopy

On Hematoxylin and Eosin staining, lipofuscin deposits and multinucleate hepatocytes were noted in the older animals.

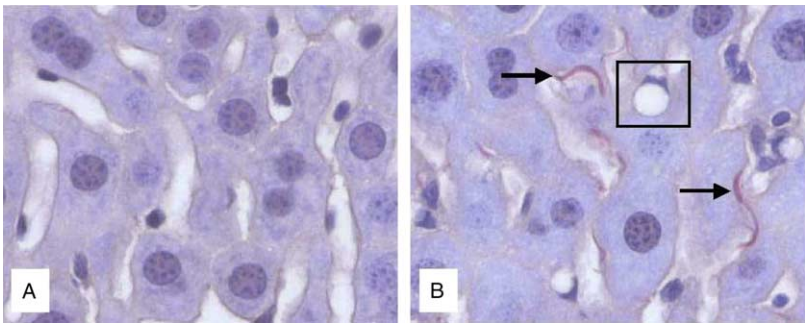

Fig. 1. Liver from young (3 months, A) and old (24 months, B) mice. The tissue has been stained with Sirius Red. In the old livers, there is linear perisinusoidal staining (→) and large signet shaped perisinusoidal cells (□).

Sirius red staining showed patches of linear perisinusoidal staining in all old mice but not the young mice (Fig. 1). Reticulin staining was similar in young and old livers. There were no major age-related changes in von Willebrands factor, laminin or Trichrome Masson stains although some perisinusoidal staining was noted in young and old livers.

Large, round cells were noted only in the livers of older animals (Fig. 1). The nucleus of these cells was peripherally located giving the appearance of a signet ring. They were present in livers of all old mice and in high frequency.

3.2. Transmission electron microscopy

In all specimens, the morphology of the hepatocytes was well preserved with no evidence of autolytic changes or fixation artifacts. In particular, there were no signs of organelle swelling and the structure of the mitochondria and microvilli was intact.

On transmission electron microscopy sinusoidal endothelium appeared thicker in the old animals and the number of endothelial fenestrations appeared to be diminished (Fig. 2). The age-related effect on the thickness of the sinusoidal endothelium was quantified (Table 1). There was a significant increase in thickness of about 60% from 154 ± 4 nm ($n = 3$ young mice) to 245 ± 8 nm ($n = 3$ old mice, $P < 0.001$). Basal lamina was seen in 25 of 31 microphotographs taken from the livers of old mice but only five of 31 microphotographs taken from the livers of the young mice. There was no marked increase in the amount of collagen seen in the space of Disse with old age.

The large round cells with peripheral nuclei that were observed on light microscopy in old animals were found to lie within the space of Disse and protrude into the lumen of the sinusoid. These cells contained a single large osmium-dense lipid droplet, surrounded by a thin layer of cytoplasm (Fig. 3).

3.3. Scanning electron microscopy

Scanning electron microscopy revealed defenestration and loss of sieve plate formation in the sinusoidal endothelium of the older animals (Fig. 2). These changes

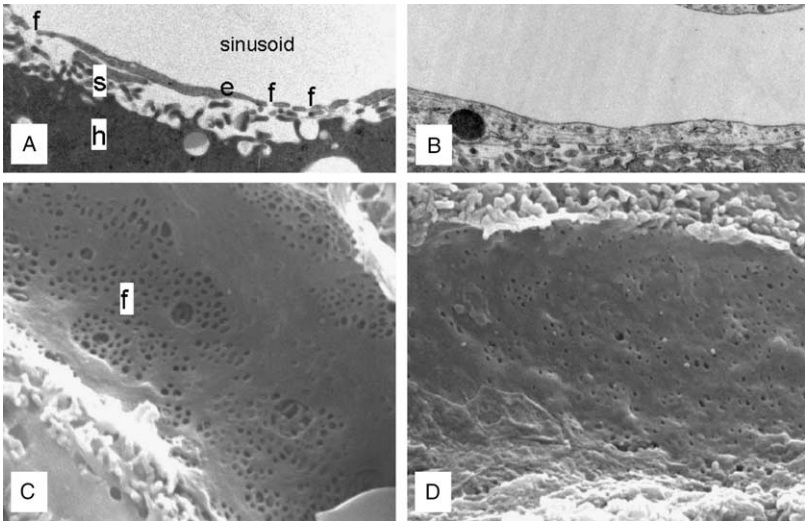

Fig. 2. Electron microscopy of the hepatic sinusoid. Transmission electron microscopy (original magnification × 12,000) shows many fenestrations in the thin endothelium of liver from a young mouse (A) [h, hepatocytes; s, space of Disse; e, endothelium; f, fenestration]. In the old mouse (B) the endothelium is much thicker and the fenestrations are reduced. Scanning electron microscopy (original magnification × 15,000) shows numerous fenestrations (f) clustered into liver sieve plates in liver of young mouse (C). In the old mouse (D) the fenestrations are markedly reduced.

Table 1
Pooled results of the effects of old age on the thickness and porosity of the hepatic sinusoidal endothelium in mice from this study compared with published results in rats (Le Couteur et al., 2001), baboons (Cogger et al., 2003), and humans (McLean et al., 2003)

	Young	Old	Fractional change
Porosity determined by scanning electron microscopy (% of endothelial area perforated by fenestrations)			
Mouse	4.1±2.2	2.2±3.5	0.53
Rat	4.1±2.3	2.5±1.2	0.61
Baboon	4.2±0.5	2.4±0.4	0.57
Human		Not done	
Endothelial thickness determined by transmission electron microscopy (nm)			
Mouse	154±4	245±8	1.59
Rat	230±50	320±80	1.39
Baboon	130±8	186±9	1.43
Human	165±17	289±9	1.75
Porosity determined by transmission electron microscopy (fenestrations per 10 μm of endothelium)			
Mouse		Not done	
Rat	2.7±1.1	0.9±0.8	0.33
Baboon	9.4±0.9	5.5±0.7	0.58
Human	7.7±0.7	1.5±0.4	0.19
Diameter of fenestrations (nm)			
Mouse	74±4	58±12	0.78
Rat	73±1	60±1	0.82
Baboon	58±1	70±2	1.20
Human		Not done	

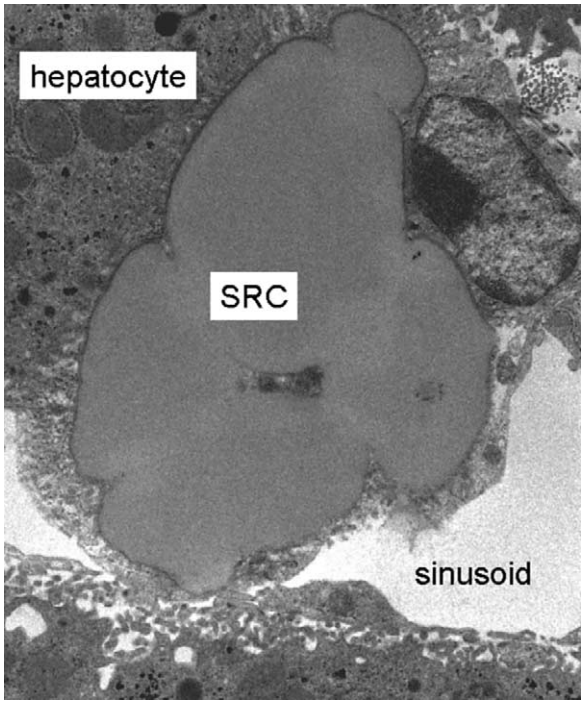

Fig. 3. Electron micrograph of a liver from an old mouse showing a signet ring-shaped cell. The cell appears to be filled with fat vesicles and protrudes into the sinusoidal lumen. These were only seen in the old mice and probably represent fat-engorged stellate cells.

were quantified (Table 1). The porosity of the endothelium (i.e. the percentage of the area of the sinusoidal endothelium perforated with fenestrations) was reduced by about 50% from 4.1±3.9% ($n=3$ young mice) to 2.2±3.5% ($n=3$ old mice, $P<0.001$).

Quantification with image analysis also revealed a reduction in the average diameter of the fenestrations from 74±4 nm in the young mice to 58±12 nm in the old mice ($P<0.01$).

4. Discussion

At the ultrastructural level, there were marked changes in the hepatic sinusoidal endothelium in the aged mice. These included an increase in the thickness of the endothelium, defenestration and patchy basal lamina formation. These findings are consistent with those reported in old age in rats (Le Couteur et al., 2001), humans (McLean et al., 2003), and non-human primates (Cogger et al., 2003) that have been termed 'age-related pseudocapillarization' (Table 1). Thus, we believe that old age is likely to be associated in all animals with a substantial increase in the thickness of the hepatic sinusoidal endothelium and perhaps more importantly, a substantial reduction in the porosity of the endothelium. Whether the reduction in porosity is secondary to a reduction in the numbers of fenestrations, or whether this is also associated with a decline in the diameter of the fenestrations is less clear. In mice, the diameter of the fenestrations was reduced by about 20%. Gap formation may explain why fenestral diameter was increased in old non-human primates.

At the light microscopic level there were no major abnormalities in the livers of the old mice. Sirius red staining was increased along the perisinusoidal margins in the old mice, however, other stains (Trichrome Masson, reticulin) and antibodies (laminin, von Willebrands factors) showed no major changes. This is different to changes seen in other species including humans where there were some age-related changes detected with these methodologies. However, overall the results indicate that ageing is associated primarily with ultrastructural changes in the endothelial cells rather than with the types of changes in the sub-endothelial matrix that are commonly seen in hepatic fibrosis and cirrhosis.

It is intriguing that the unusual large round cells with peripheral nuclei that we first observed in the old non-human primates (Cogger et al., 2003) were also found to occur in aged mice. In old baboons and old mice these cells appeared to be fat-engorged cells lining the sinusoid, possibly representing hepatic stellate cells on the basis of their location and fat engorgement. These cells are in concert with the observation by Vollmar et al. (2002) of age-related increases in stellate cell-associated areas of ultraviolet vitamin A-autofluorescence, which correlated with increasing tissue concentrations of vitamin A metabolites.

In the mice, we found that the cells protruded into the sinusoidal lumen, which may have hemodynamic implications. Although there are no major changes in intrahepatic hemodynamics in old rats (Le Couteur et al., 1992) it has been concluded that swollen hepatocytes and steatosis might contribute to portal hypertension in a rat model of alcohol-induced liver injury (Akamatsu et al., 1993).

The possible functional implications of such age-related changes in hepatic endothelial structure are of considerable interest. The loss of fenestrations may contribute to the impaired hepatic clearance of lipoproteins that normally traverse through the fenestrations prior to binding to the LDL receptors on the hepatocyte membranes (Le Couteur et al., 2002). Age-related pseudocapillarization may also have implications for drug and oxygen transfer across the endothelial membrane (Le Couteur et al., 2005). This functional role of the liver sinusoidal endothelial cell in substrate transfer has been described using the term 'the liver sieve' (Fraser et al., 1995). It is possible to quantify these effects on the transfer of substrates using engineering principles related to membrane filtration. There are several, pressure-driven, membrane processes including microfiltration (particles 0.1–0.5 μm), ultrafiltration (10–100 nm), nanofiltration (1–10 nm), and reverse osmosis (<1 nm). Therefore, ultrafiltration is the best term to describe the passage across hepatic endothelial fenestrations, with diameters around 100 nm. The hydraulic pressure driving filtration is the sinusoidal pressure, which is very low and in the order of a few centimeters of water (Le Couteur et al., 1992), and in the axial direction consistent with tangential flow ultrafiltration. In ultrafiltration, the volume flux (J) is described by the Hagen–Poiseuille formula as

$$J = \frac{fR^2 \Delta P}{8\eta l}$$

where f is the porosity of the membrane, R diameter of the pores, ΔP the pressure gradient across the membrane, η viscosity, and l the thickness of the membrane (Cherkasov, 1990). In old age, it is unlikely that there is a substantial change in the sinusoidal pressure (Le Couteur et al., 1992) and from Table 1 it can be seen that with ageing, porosity is reduced by 40%, diameter decreased by 20% (excluding non-human primate result) and thickness increased by 50%. By application of the Hagen–Poiseuille law, it can be estimated that pseudocapillarization of the hepatic sinusoidal endothelium will be associated with a reduction in flux across the endothelial in the order of 75%.

The reduction in the diameter of fenestrations will also influence the size of particles that are able to transfer across the endothelium. Empirically, it has been found that ultrafiltration of particles through pores in synthetic membranes decreases once the radius of the particle exceeds 0.3 ± 0.05 of the radius of the pore ('critical diameter') (Cherkasov, 1990). More precisely, the membrane-sieving coefficient, χ, which is the ratio of the concentration of a macromolecule in the permeate to that in the bulk, can be calculated from the ratio of the size of the particle and the size of the pore, λ, by the classic sieving relationship (Cherkasov, 1990):

$$\chi = [2(1-\lambda)^2 - (1-\lambda)^4] \times \left[1 - \frac{2}{3}\lambda^2 - 0.163\lambda^3 + \ldots\right]$$

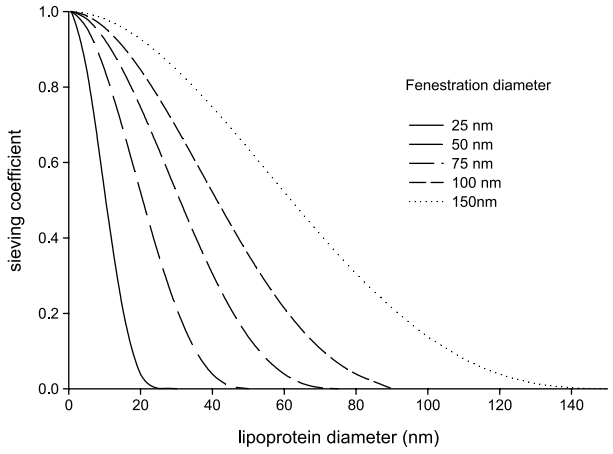

Fig. 4. The theoretical effects, according to the sieving equation, of fenestral diameter on the membrane sieving of (lipoprotein) particles with various diameters. The sieving coefficient is the ratio of the permeate to the bulk concentration of the particles.

It can be seen from Fig. 4 that alteration of the diameter of the fenestrations between 25 and 150 nm has a dramatic effect on the transfer of lipoproteins depending upon their diameter. This is likely to be of particular relevance to chylomicron remnants because their diameters range from 50 to 100 nm.

We have now confirmed the presence of major ultrastructural changes in the hepatic sinusoid with old age in several species including humans. This indicates that pseudocapillarization is a widespread aging change. It is now important to determine whether the changes can be ameliorated by caloric restriction. The observation that pseudocapillarization is found in the mouse model renders this area of aging research amenable to investigation using transgenic methodologies.

In conclusion, old age in mice is associated with ultrastructural changes in the hepatic sinusoidal endothelium that are similar to those documented in other species including man. These changes of pseudocapillarization include loss of endothelial fenestrations, thickening of the endothelium and some deposition of basal lamina and moreover indicate that the aged mouse is a suitable model for the study of such changes.

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