EVIDENCE OF OXIDATIVE INJURY DURING AGING OF THE LIVER IN A MOUSE MODEL.

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ABSTRACT

The aim of the present study was to determine whether oxidative stress contributes to aging of the liver in a mouse model. Liver was obtained from young (3-5 months old) and aged (18-24 months old) mice. No age-induced gross changes in liver morphology were detected by light microscopy. Apoptosis was measured using the fragment end labeling of DNA for the immunohistochemical identification of the apoptotic nuclei. The total apoptotic cells represented 1 % of the total cells in livers of young mice and 8% in those of aged mice. Among the total apoptotic cells in livers of aged animals, 15% were hepatocytes, 40% sinusoidal endothelial cells, and 45% bile duct cells. Hepatic lipid peroxidation, expressed as malonaldehyde levels, protein oxidation, measured by protein carbonyl content, and DNA oxidation, measured as 8-hydroxy-2'-deoxyguanosine (oxo(8)dG), were significantly increased in the livers of aged animals as compared to younger mice. The apoptotic cells presented elevated levels of oxidized DNA, detected by immunohistochemistry using an antibody directed against oxo(8)dG in serial sections. These results suggest that livers of aged animals presents evidence of increased oxidative injury and apoptosis. Because the apoptotic cells in the aged livers are mostly bile duct cells and sinusoidal endothelial cells, the cells most sensitive to oxidative stress injury, it can be hypothesized that reactive oxygen species-induced apoptosis in these cells contributes to the aging of the liver.

INTRODUCTION

While no major age-specific liver diseases exist, the prognosis of many liver diseases is worse in individuals aged over 70 years. The elderly are extremely prone to develop drug induced and ischemic hepatitis, both of which are associated with a high mortality [1]. The high rate of adverse drug reactions, which is tripled among the geriatric population [2], provides clinical relevance for investigations on the effect of age on the liver. The observed changes in the clearance of drugs that undergo hepatic metabolism were originally attributed to changes in enzyme activity and more recently to altered hepatic size and a decrease in hepatic blood flow [3]. However, it has been recognized that none of these mechanisms fully explain the age-related changes in hepatic drug clearance observed in the elderly.

The liver is composed of parenchymal cells (hepatocytes) and non-parenchymal cells consisting of sinusoidal endothelial cells, bile duct cells and resident macrophages. Each of these cell populations has unique and specific functional characteristics, has a different half-life as well as responses to various insults. The interaction between hepatocytes and non-parenchymal liver cells is essential to maintain a wide array of physiologic and pathophysiologic responses of the organ [4]. It is therefore reasonable to hypothesize that each cell type within the organ may have a peculiar susceptibility to aging or may be differentially subjected to changes in surrounding microenvironment.

Oxidative stress is involved in the aging process. The free radical theory of aging is widely accepted and is the basis of several hypotheses explaining how the production of reactive oxygen species (ROS) promotes aging and death of cells in all of the organs and systems of the body. A central role for lipid peroxidation, protein and DNA oxidation in age-related changes has been documented [5]. Oxidative injury is known to be involved in the promotion of apoptosis, a process which is recognized as an important feature of aging [6].

The goal of the present study were to determine whether oxidative stress contributes to aging of the liver in a mouse model. In particular, the study addresses whether: 1) oxidative injury in the liver is increased as

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result of aging; 2) liver cell apoptosis is present in aged mouse livers and, if this is the case, which cell population is most affected; and finally 3) is oxidative injury to DNA present in apoptotic cells in the liver.

MATERIALS AND METHODS

Animals

Female BALB/c mice (Harlan labs, Indianapolis, IN through the National Institute for Aging contract) aged 3 to 5 months (young) and 18 to 24 months (aged) were studied. They all had free access to food and water and were maintained on a 12-hour light/dark cycle. i.p. for at least 3 days after shipment. The present study was performed in accordance with the Loyola University Institutional Animal Care and Use guidelines.

The mice were sacrificed by cervical dislocation and the livers were immediately dissected free. One portion of the organ was fixed in buffered formalin for morphological evaluation and immunohistochemical assays while several additional liver samples were immediately frozen in liquid nitrogen and stored at -80°C until being used.

Histopathology

Paraffin sections of each rat liver were stained with hematoxylin-eosin. The morphology of young and aged mouse liver was evaluated by two observers who received the specimens individually and in a blinded manner. All differences in interpretation between these two observers were resolved by consensus rereading of the material.

Lipid peroxidation

Hepatic lipid peroxidation was measured by malonaldehyde (MDA) content of tissue homogenates as previously described [7,8]. Liver samples were homogenized using a Teflon pestle diluted in Tris-HCl to 10% (w/v). A solution of 10 mM 1,1,3,3-tetramethoxypropane was used as the standard for MDA. The protein content for each liver sample was measured by the Coomassie G250 reagent assay (Pierce Chemical Company, Rockford, IL). The results were expressed as nmol MDA/mg of total protein.

Protein oxidation

Protein oxidation was measured as protein carbonyl content (PCC). PCC levels were determined in liver homogenates by their reaction with 2,4-dinitrophenylhydrazine, as previously described [9]. The results were expressed as nmol PCC/mg of total protein.

DNA oxidation

DNA oxidation was measured as 8-hydroxy-2'-deoxyguanosine (oxo(8)dG) levels in tissue homogenates. DNA was extracted from frozen liver samples using a commercially available genomic DNA purification kit (Promega, Madison, WI). A competitive enzyme-linked immunosorbent assay was used for the quantitative measurement of oxo(8)dG (OXIS,

Portland, OR). The results were expressed as pmol/ μg DNA.

To determine which cell population in the liver was more susceptible to oxidative stress and to investigate whether there was a relationship between apoptosis and DNA oxidation, the immunohistochemical localization of oxidized DNA was performed. The oxo(8)dG was purchased from Oxis (Portland, OR) and the immunohistochemical staining performed on formalin fixed liver tissue. For each sample, 10 microscopic fields ($\times 25$) were evaluated for positive stained liver cells.

Apoptosis

Apoptosis was measured by immunohistochemical identification of apoptotic nuclei in paraffin embedded sections of liver by fragment end labeling of DNA (FragEL, Oncogene, Boston, MA). Endogenous peroxidase activity was inactivated by immersion in methanol containing 0.3% hydrogen peroxide. A positive and a negative control, obtained by incubating the tissue with DNase and by staining without deoxynucleotide substrate, respectively, were produced for each liver studied. The sections were incubated in a solution containing 3,3'-diaminobenzidine 4-HCl and counterstained with methyl green. For each sample, 10 random microscopic fields ($\times 25$) were evaluated for positive stained liver cells. About 5,000 cells were counted for each liver. Data are expressed as the percent ($\text{mean} \pm \text{SEM}$) of cells positive relative to the total number of cells counted.

Statistical analysis

The results are expressed as $\text{mean} \pm \text{standard error of the mean}$. The number of individual animals tested in each assay ranged from 5 to 10. The Student t-test was used to assess statistical significance. A p value <0.05 was considered to be significant.

RESULTS

The morphological analysis of the liver collected from young and aged mice did not reveal any pathological alterations such as necrosis, steatosis or inflammatory infiltration in any of the animals used in the study. Therefore, the occurrence of any histologic age-related process within the liver of the mice studied was ruled out specifically. At the level of light microscopy, the livers obtained from aged mice did not differ from these of younger animals. No pathological changes were seen in either young or aged mouse liver.

The influence of age on oxidative injury in the liver was assessed by measuring tissue levels of lipid peroxidation, protein oxidation and DNA oxidation. Lipid peroxidation was measured as MDA content in the liver samples obtained from young and aged mice. Aging of the liver was associated with a significant increase in hepatic MDA levels, which were 0.51 ± 0.01 nmol/mg protein in aged mouse liver as compared to 0.21 ± 0.02 nmol/mg protein in young livers ($p=0.02$) (Figure 1, panel A).

Hepatic PCC was taken as index of protein oxidation. PCC levels were 2.5 fold higher in the livers obtained from aged (3.5 ± 0.3 nmol/mg protein) as compared to younger animals (1.1 ± 0.2 nmol/mg protein, $p = 0.01$) (Figure 1, panel B). Aged mouse livers show significantly higher levels of DNA oxidation, measured as oxo(8)dG, than did the livers of younger animals. In particular, in aged mouse liver, oxo(8)dG levels were twice those of young mouse liver (Figure 1, panel C).

The percentage of total apoptotic cells in aged mice was 8.0 ± 1.0 vs 1.0 ± 0.5 in young livers. Hepatocytes were only marginally apoptotic in aged mice. In fact, only 5% of the total hepatocytes were apoptotic. Staining for fragmented DNA was localized in the hepatic sinusoids. The hepatic sinusoid contains 4 distinct cell types, each with its own characteristic morphology: Kupffer cells, perisinusoidal (fat-storing) cells, sinusoidal endothelial cells (SEC) and pit cells. Sinusoidal cells represent 30 to 35% of the total number of liver cells. Each cell type within the liver was recognized based on well-characterized morphological parameters [10]. The present study reports that 11% of SEC population, which forms the wall of the sinusoid separating the sinusoidal lumen and the Disse space, was apoptotic in aged mouse liver. They represented 40% of the total apoptotic cells. Sinusoidal endothelial cells account for 45% of the total sinusoidal cells, a percentage that is not age-dependent in rodents [11]. In the liver obtained from aged mice, 15% of the bile duct cells were apoptotic, accounting for 45% of the total apoptotic cells (Figure 2). To determine whether any relationship exists between oxidative injury, in particular to DNA, and apoptosis, an immunohistochemical localization of oxidized DNA, measured oxo(8)dG was performed in livers from young and aged mice. The cells identified as showing apoptosis were the same cells that, in serial sections, stained positive for DNA oxidation.

DISCUSSION

Epidemiological data show that certain diseases including biliary tract diseases, toxic or ischemic hepatitis exhibit an age-related increase in overall incidence [1,2]. This observation raises the question whether the elderly are predisposed to certain liver disease because they are not able to effectively respond to a toxic stimulus due to an impairment of hepatocyte function or cell-cell communication. Senescent changes in the liver, when present, include accumulation of lipofuscin pigment and increased hepatocyte size and ploidy levels. The mitochondria of older rodents have an impaired performance as compared to those of younger animals [12-14]. Mitochondria are the most important source of reactive oxygen species (ROS) within the cells, under physiologic or pathologic conditions [15]. An electron leak from the respiratory chain of mitochondria has been reported as the membranes loose fluidity and changes their composition.

The oxidation of enzymatic proteins can block the metabolic processes essential for cell functioning and repair. The peroxidation of cell membrane lipids and oxidative damage of nucleic acids can induce cell death [16]. Apoptosis is presumed to represent the final common pathway of cell injury mediated by oxidants, being induced by ROS either directly or indirectly through an alteration of the intracellular redox state and the expression of pro-inflammatory cytokines [17-20]. Because of the critical role of mitochondria in cell physiology, mitochondrial damage has been intensively investigated and it is thought to represent a major trigger for apoptosis. The permeability transition and the production of ROS are factors required for the orchestration of apoptosis. The oxidation of proteins to include enzymes critical for cell function, the peroxidation of membrane lipids and DNA injury are all important parameters to investigate the relationship between aging, oxidative damage and apoptosis [21, 22]. The present study reports that aging of the liver is associated with increased lipid peroxidation [23,24], protein [5] and DNA oxidation. [25]. This finding is consistent with other reports of increased oxidative stress during aging. A recent report shows an increased oxidative stress in the brain and peripheral organs in accelerated senescence-prone mouse model. It has therefore been proposed that oxidative injury may be the basis of the senescence-related degeneration and the functional impairments seen in such mice [26]. A low fatty acid unsaturation in longevous animals would be consistent with the free radical theory of aging, since membrane lipids increase their sensitivity to ROS attack as a function of their degree of unsaturation. To evaluate the relationship between fatty acid composition and oxidative stress during aging, liver fatty acids and proteins from several species were studied. The peroxidizability index of fatty acids and the sensitivity to *in vitro* lipid peroxidation were negatively correlated with the maximum life span. It has been shown that liver proteins present markers of oxidative modification of their structure [27]. A low degree of fatty acid unsaturation may have been selected in longevous mammals to protect lipids and proteins against ROS while maintaining an appropriate environment for membrane function. Nevertheless, in our experience, the percentage of cells undergoing apoptosis, a process closely related to oxidative damage to intracellular macromolecules and membranes, is greater in aged than young mouse liver. Importantly, the cells that present apoptosis are those more sensitive to oxidative injury. Those cells are not the hepatocytes, which represent the majority of the cells in the liver, but DNA oxidation has been shown to be present in bile duct cells and SEC. Therefore, the impact of aging and oxidative injury in the liver may vary between cell types. SEC form a monolayer that provides a semipermeable protective mechanical barrier between the blood and the hepatocytes. By virtue of their unique location, SEC play a central role in the regulation of the interactions between blood cells, agents of intestinal origin and toxic agents [11]. Although age-induced

Figure 1. A. Hepatic levels of malonaldehyde (MDA) in young (white bar) and aged (black bar) mice. B. Protein oxidation measured as protein carbonyl content (PCC) in the liver of young and aged mice. C. Hepatic levels of 8-hydroxy-2'-deoxyguanosine (Oxo(8)hdG), an index of DNA oxidation, in young and aged mice. The results are expressed as mean $\pm$ SEM. N=5 mice per group. * $p < 0.02$, vs young mouse liver as determined by Student t-test.

Figure 2. Apoptosis was measured by immunohistochemical identification of apoptotic nuclei by fragment end labeling of DNA. A. Liver obtained from a young mouse. B. Aged mouse liver (magnification x 200).

morphologic and ultrastructural changes observed in SEC are minimal, an age-dependent functional impairment of endothelial cells has been proposed recently. SEC are sensitive to oxidative injury following hypoxia-reoxygenation [28-33]. The finding of an age-associated increase in oxidative stress induced apoptosis in this cell population provides new information on the aging process in the liver. Hypoxia-reoxygenation may contribute to aging of the liver by affecting the cells most

sensitive to alterations in oxygen supply and oxidative injury upon reoxygenation. An alteration in the function of SEC contributes to the decreased resistance of aged liver to injury.

Bile duct cells also demonstrate an age-dependent increase in apoptosis and oxidative injury. Intrahepatic biliary epithelial cells account for approximately 5% of the hepatic cell population and form a complex network of interconnecting conduits comprising the intrahepatic

biliary ductal system. The intrahepatic bile duct epithelium determines the characteristics of the primary hepatocellular bile from which it reabsorbs fluids, amino acids, glucose and bile acids, while secreting water, electrolytes and immunoglobulin A [34]. The intrahepatic biliary epithelium is a primary target in a broad group of hepatic disorders that represent an important cause of morbidity and mortality, particularly among the elderly. Bile duct cells are known to be extremely sensitive to oxidative stress [35,36]. These results suggest that aging of the liver is characterized by increased oxidative injury and increased programmed cell death involving almost exclusively SEC and bile duct cells. Oxidative injury may play a role in the aging of the liver by causing apoptosis of SEC and bile duct cells.

LIST OF ABBREVIATIONS

ROS: reactive oxygen species
MDA: malonaldehyde
PCC: protein carbonyl content
Oxo(8)hdG: 8-hydroxy-2'-deoxyguanosine
SEC: sinusoidal endothelial cells

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