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Hepatic progenitor cell proliferation and liver injury in alpha-1-antitrypsin deficiency

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Abstract

Homozygous, ZZ, alpha-1-antitrypsin (a1AT) deficiency is a common genetic liver disease, which causes liver injury and hepatocellular carcinoma (HCC) in children and adults. The a1AT mutant Z gene encodes a mutant protein which accumulates within hepatocytes leading to hepatocellular death, and a hepatic regenerative response. However, the mechanisms linking hepatocellular injury to these responses are poorly understood. In this study, we examined liver injury and response in human liver and in transgenic mice and found involvement of hepatic progenitor cells. Liver biopsy specimens of low grade, early stage human ZZ liver exhibiting minimal inflammation and minimal fibrosis (grade 1 and stage 1) were examined for hepatic progenitor cell (HPC) proliferation using immunoreactivity for cytokeratin-7 (CK7). The results showed increased CK7 positive HPC proliferation in human ZZ liver above normal liver, but five-fold less HPC proliferation than in grade and stage-matched disease control specimens of hepatitis C infected liver. Livers from PiZ mice, a model transgenic for the human a1AT mutant Z gene which recapitulates features of the human injury, also showed HPC proliferation. Human ZZ liver and PiZ mice are both known to develop dysplasia in the liver and HCC. HCC in PiZ mice was also characterized by HPC proliferation. Progressive hepatic fibrosis with age in the PiZ mice is demonstrated for the first time in these studies. In conclusion, the chronic injury in both ZZ human and PiZ mouse liver is associated with hepatic fibrosis, and a unique magnitude of HPC proliferation associated with the hepatic proliferative response.

Keywords

Alpha-1-antitrypsin; cytokeratin-7; hepatic progenitor cell; hepatocellular carcinoma; oval cell

Alpha-1-antitrypsin (a1AT) deficiency is caused by homozygosity for the a1AT mutant Z gene, and occurs in 1 in 2,000 births in many North American and European populations [1]. The Z mutation in the a1AT gene confers an abnormal conformation on the resultant nascent polypeptide[2]. This abnormal, mutant protein accumulates within the endoplasmic reticulum (ER) of hepatocytes rather than the appropriate, highly efficient secretion of the wild type protein. Homozygous, ZZ individuals have a known increased risk of chronic liver disease and hepatocellular carcinoma (HCC) resulting from this intracellular accumulation of the a1AT mutant Z protein[3,4]. Studies of the mutant Z protein molecule have shown that it has the

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tendency to form unique protein polymers within the hepatocellular ER. In some, but not all hepatocytes the accumulations of a1AT mutant Z protein become large enough to be visualized by light microscopy as the intracytoplasmic “globules” classically described in this disease [5]. Published data has shown that the liver injury in humans with a1AT deficiency is directly related to the hepatic accumulation of the a1AT mutant Z protein, yet many questions about the disease processes remain unanswered [6-9].

Our laboratory, and others, have reported a series of studies that have begun to examine the mechanisms of liver cell injury in a1AT deficiency[6-13]. The data suggest that accumulation of the a1AT mutant Z protein in the ER of hepatocytes activates autophagy, causes mitochondrial injury and mitochondrial autophagy, and is associated with caspase activation and apoptosis. A “low grade” hepatocellular regenerative response, presumably compensatory, has also been identified, although the mechanisms linking injury, regeneration, and the development of HCC are poorly understood[6]. It is well known that this, and other, metabolic diseases can be a risk factor for HCC, but possible links to pre-existing dysplasia, adenoma formation, the proliferative response, and other mechanisms are unknown [14,15]. As a result of our previous observations in the transgenic mouse system, we proposed that at least part of the mechanism of the regeneration in this disease characterized by chronic liver injury could involve hepatic progenitor cells, and that knowledge of the characteristics of this response and the cells involved could further advance the understanding of the liver injury, dysplasia, and HCC formation in this disease. Furthermore, we took advantage of the unique opportunity to examine human liver biopsy specimens from low grade and stage livers, rather than the severely fibrotic, cirrhotic, or excessively inflamed samples most commonly available for analysis, to make comparisons to a well described animal model liver.

Materials and Methods

Histologic methods for tissue preparation and examination were carried out using standard techniques, as previously described [6,16]. Cytokeratin-7 (CK-7) immunohistochemical staining was carried out as previously described, as were the p21 and MIB-1 studies [17-19]. Liver biopsies from four adult ZZ a1AT deficient human livers and four adult hepatitis C infected human livers were examined by a single, blinded pathologist (EB). A mean of 15 portal tracts were examined and the total number of CK-7 positive cells were counted. Electron microscopy was performed using standard techniques, as previously described[16]. Liver analysis involving two thirds partial hepatectomy was performed as previously described[6]. Further examinations, as described, were performed on 6 neonatal human ZZ samples. Data analysis was performed using SPSS, as noted (Chicago, IL).

All animal studies and all studies using human tissues were approved by the appropriate animal studies committee and institutional review boards. PiZ mice, a model transgenic for the human a1AT mutant Z gene, were maintained on a C57Bl/6J background as previously described[6, 20]. Age-matched C57Bl/6J mice (Jackson Laboratories) were used as wild type controls. Mice were maintained on 12-h dark/light cycles and on *ad libitum* mouse chow and water in an approved barrier facility.

Results

Hepatic progenitor cell (HPC) response is present in early stage injury in human liver

There were two reasons we chose to study HPC response in early stage human livers. The first was our observation of cells that by light microscopy resembled HPC in the PiZ mouse; as this had not been previously described, this was pursued with further evaluation, as discussed below. The second was the mounting evidence of the role of the ductular reaction in portal fibrosis in human chronic liver disease[19,21]. While previous studies have shown evidence

of hepatocellular proliferation in human ZZ liver and in mouse models (PiZ mouse), a model transgenic for the human $\alpha 1$ AT mutant Z gene[6,15], no evidence has thus far been presented regarding the possible involvement of a progenitor cell population in this proliferative response. We chose to analyze the earliest stage of injury, and therefore we searched our tissue repository for liver biopsy specimens read as having low levels of injury. This was because analysis of possible progenitor cell activity might be confounded by extensive inflammation or cirrhosis. We identified four specimens of human ZZ liver with early injury (grade 1 inflammation and stage 1 fibrosis), which came from young adult patients, and analyzed them for the presence of hepatic progenitor cells identified by cytokeratin-7 (CK-7) immunohistochemical staining, using previously established techniques (Figure 1a)[17,18]. These ZZ samples were compared to normal, control specimens and to four specimens of hepatitis C virus infected liver matched for adult specimens with the same low grade and stage. The total number of CK-7 positive cells were then counted, and the mean per portal tract are shown in figure 1b. The result highlights two findings. First, a unique magnitude of HPC response in the human ZZ pre-fibrotic liver significantly greater than that in normals was seen. However, the HPC response in the ZZ liver was less than in similar stage injury in hepatitis C ($p < 0.02$ by ANOVA).

We next proposed that other features of injury, or a response to injury, might be detectable in these early stage specimens. Therefore, we examined these same specimens for a ductular reaction, as evidenced by periportal progenitor cells and stromal proliferation. We found such a response to be absent or minimal in the ZZ livers, but consistently increased in the HCV infected livers (figure 1c)[18, 19]. Evaluation of hepatocytes for evidence of replicative arrest by p21 stain, and/or increased entry into the proliferation cycle by Ki-67 (MIB-1) stain both showed no significant difference between $\alpha 1$ AT and HCV livers (Fig 1d.)[19].

The PiZ mouse model recapitulates the progenitor cell response of the human liver

Next, we repeated this analysis in liver specimens from PiZ mice, a well characterized model on the C57Bl6 background, transgenic for the human $\alpha 1$ AT mutant Z gene, by using murine CK-7 immunohistochemical staining for oval cells, the correlate of the human hepatic progenitor cell[22,23]. The PiZ mouse recapitulates many aspects of the liver injury found in ZZ humans[5-8,16,20]. 3 month old, male PiZ samples were compared to age and sex matched wild type (WT), C57Bl6 mice. CK-7 immunohistochemical staining easily identified biliary epithelium in the portal tracts and putative oval cells in the hepatic lobules (figure 2a). Samples of liver were also examined by transmission electron microscopy to confirm the ultrastructural characteristics of oval cells (figure 2b). Globules of $\alpha 1$ AT mutant Z protein within dilated ER is also seen along with many abnormal mitochondria, as previously described[5,8]. Putative oval cells were identified in 50% of twelve electron microscopy grids examined, from a total of six different PiZ mice, but were seen in only 8% of twelve grids from WT mice ($p < 0.02$). Quantification of the immunohistochemical CK-7 studies in young adult, non-fibrotic PiZ mouse liver showed a significant increase in oval cells compared to age-matched, non-fibrotic WT mouse livers (figure 2c). These data indicate the involvement of a progenitor cell population in the chronic liver injury related to the intracellular accumulation of $\alpha 1$ AT mutant Z protein. There has been the suggestion in other work that chronic activation of such a pathway might play a role in fibrosis or in hepatocellular death[18,19].

Interestingly, in a previous study, we examined the proliferative response in the PiZ model following two thirds partial hepatectomy[6]. This is a well characterized, acute regenerative stimulus used to study hepatic responses to acute insults. The results in this previous study suggested that much of the recovery of the PiZ mouse livers to two thirds partial hepatectomy involved proliferation of mature hepatocytes and that a progenitor cell population was not *required* for recovery of hepatic mass and function following this acute insult[6]. However,

CK-7 analysis was not employed in these studies. We have now reexamined liver specimens from 72 hours post two thirds partial hepatectomy (4 PiZ and 4 WT) with the CK-7 immunohistochemistry using the same methods as above. The results revealed either no oval cell proliferation, or a low and inconsistent response in the PiZ livers exposed to partial hepatectomy, which was not different from the low or absent oval cell response observed in the WT control livers exposed to partial hepatectomy (CK-7 positive cells per portal tract; PiZ 1.02 ± 0.8 , WT 1.1, $p > 0.6$). These data confirm our conclusions from the previous study that recovery from two thirds partial hepatectomy does not require a progenitor cell population, but also suggests that the mechanism of hepatic response to acute injury and to a chronic injury of a1AT mutant Z protein accumulation are different.

The above examinations in the human and in the mouse samples had focused on evidence of progenitor cell proliferation under the control of the variables of inflammation, and without the presence of existing fibrotic injury. That is, we examined samples without variables, which themselves can alter the readouts we employed. Young adult ZZ human and young adult PiZ mouse samples were the only specimens identified which fit these criteria. We next attempted to repeat the CK-7, p21, and Ki-67 analysis of six, neonatal ZZ human and neonatal PiZ mouse liver samples. However, as is well described in the literature, these neonatal samples exhibited variable levels of extramedullary hematopoiesis, variable levels of lymphocytic inflammation, widespread giant cell transformation, and early peri-portal fibrosis. These findings impossibly confounded quantitative comparisons to the quiescent specimens. Therefore, we turned our attention to the controllable variable of injury over time.

Chronic injury in the PiZ model: fibrosis and HCC with oval cell proliferation

There have been a limited number of studies of age related injury in this model, and of susceptibility to fibrosis [10,24]. Thus, after examination of the early injury in the PiZ mice, we next examined a range of ages, including older mice (individuals at each month from one to twelve months of age and at 14, 16, 18, 20, and 24 months of age, see below) for evidence of oval cell proliferation to determine if the response changed over time. The results revealed continued oval cell proliferation which changed very little with advancing age (figure 3a). Advancing age in the PiZ mice is associated with hepatocellular dysplasia, and hepatic tumors grossly (Manuscript in press, *Hepatology Research*). HCC has also been reported in other mouse models of a1AT deficiency. Dysplasia and HCC are described in human ZZ liver and in some a1AT model systems, but not previously within the PiZ model we have used in these studies [14,15,25]. Oval cell proliferation, confirmed by CK7 immunohistochemistry, was present within many of the HCCs (figure 3b). Finally, since chronic liver disease with progressive fibrosis and cirrhosis are well described in human liver as a result of a1AT deficiency, we next examined the same PiZ mouse livers aged as above for fibrosis using Sirius red staining [10,24]. Sirius red highlights fibrosis in the liver as red on a neutral blue background. This was an especially interesting question because of the recently proposed link between oval cell proliferation and portal fibrosis [19,21]. Progressive fibrosis has not been reported in the PiZ model previously, and is generally uncommon in murine models of liver disease. Our results revealed a periportal fibrosis, detectable in the first few months of life, which progressed to bridging fibrosis in the second year (figure 4a). Elderly mice often exhibited early changes suggesting regenerative nodules (figure 4b). This is very similar to the progression of disease in ZZ humans who can, but uncommonly, exhibit fibrosis early in life, but who have been shown to develop significant fibrosis in old age in nearly all patients [26]. Scoring of the fibrosis, independently by two blinded examiners (EB, JT), confirmed a significant association of age with fibrosis in the PiZ mouse compared to WT controls (Ishak score applied to the murine livers range 0-6; WT mice age 14mo 0.4 ± 0.2 and PiZ mice age 14mo 3.4 ± 0.6 , $p < 0.01$). These data show that another aspect of human liver injury, namely

fibrosis, associated with accumulation of α 1AT mutant Z protein in the liver is recapitulated in the PiZ murine model, in addition to oval cell proliferation.

Discussion

In this study, we continued to pursue our interest in liver injury related to the intracellular accumulation of α 1AT mutant Z protein. We followed up on our recent reports of increased hepatocellular proliferation in ZZ liver, presumably a compensatory mechanism resulting from increased hepatocellular death, and examined more carefully the possible mechanisms and consequences of this proliferative response in the course of chronic injury. We hypothesized that hepatic progenitor cell proliferation might be associated with α 1AT mutant Z protein accumulation in human and in mouse model liver. We did, in fact, find a unique magnitude of HPC proliferation which was significantly above WT liver in both humans and in mouse model liver, but less than what was seen in human hepatitis C liver. Surprisingly, we saw the HPC proliferative response even in early stage injury, rather than in many previous reports from other systems in which HPC proliferation is known to be present in late injury and to be associated with a hepatocellular replicative block. These data fit the observation that most patients with hepatitis C and ZZ α 1AT deficiency have a chronic course of liver injury which progresses slowly over decades. In both diseases, only a small group of patients progresses rapidly, which could be a focus of future work to determine if the injury mechanisms in the small group with rapid progression is the same as in the chronic injury group. Interestingly, in this study and in other studies from our laboratory, a pre-cancerous, adenomatous phase associated with α 1AT mutant Z hepatic accumulation was not identified. These findings are provocative in light of previous work in this model which indicated that in PiZ mouse liver, proliferation overwhelmingly involved hepatocytes without globules of retained α 1AT mutant Z protein. This could suggest that the oval cell proliferation could be a more important part of the hepatic lesion in this disease than the simple proliferation of mature hepatocytes, because the intracellular globules form over weeks to months and new cells, developed out of progenitor cell population, might not have yet developed visible inclusions. Furthermore, these data suggest that the mechanism of the chronic hepatocellular proliferation in the PiZ model is likely different than the mechanism of response to the acute injury of a partial hepatectomy. The recovery from partial hepatectomy did not depend on oval cell proliferation.

Our findings are also surprising for the degree of hepatic fibrosis we have documented in the elderly PiZ mice. Most murine models of chronic liver disease do not exhibit this level of fibrosis, rather this finding is suggestive of the course of human ZZ liver disease, again confirming the utility of this model. We will now be able to use fibrosis, or the prevention of fibrosis, as a readout in future studies of potential therapies for ZZ liver disease. At present, there is no specific treatment, other than supportive care, although a variety of drug strategies are under active investigation. We will also propose evaluation of therapies for the non-specific development of fibrosis from chronic injury. The PiZ model may be a unique tool for these investigations as most murine studies of fibrosis and its prevention have employed more acute injury techniques, such as bile duct ligation or toxin administration. We will continue to investigate the links of fibrosis to progenitor cell proliferation in the model, as well as in human liver.

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Abbreviations

a1AT	Alpha-1-antitrypsin
CK-7	Cytokeratin-7
HPC	Hepatic progenitor cell
HCV	Hepatitis C Virus
HCC	Hepatocellular carcinoma
ZZ	Homozygous ZZ phenotype
WT	Wild type

Figure 1. Hepatic progenitor cells by CK-7 staining, and other features of early injury in human liver

Panel a, low magnification (left), and high magnification (right) of CK-7 positive cells in biliary epithelium (white arrows) and putative hepatic progenitor cells (black arrows) in the hepatic lobule of a representative sample of human ZZ liver. Panel b, mean CK-7 positive putative hepatic progenitor cells per portal tract from 4 samples of normal liver, 4 samples of ZZ liver, and 4 samples of HCV infected liver, as described ($p < 0.02$ by ANOVA of ZZ compared to HCV liver, bars $\pm$ S.D.). Panel c, CK-7 staining of peri-portal areas in human ZZ liver (left panel) and human HCV infected liver (right panel). Panel d, quantification of p21 staining (left panel) and MIB-1 staining (right panel) in the human liver samples described above (bars $\pm$ S.D., $p > 0.1$ for ZZ compared to HCV in both p21 and MIB-1 conditions)

Figure 2. Quantification of oval cells by CK-7 staining in PiZ mouse liver

Panel a, low magnification (left), and high magnification (right) of CK-7 positive cells in biliary epithelium (white arrows) and putative murine oval cells (black arrows) in the hepatic lobule of a representative sample of PiZ mouse liver. Panel b, transmission electron photomicrograph of PiZ mouse liver with oval cell (OC), adjacent to hepatocytes containing globules of a1AT mutant Z protein within dilated ER (G), a nucleus (N), and many cytoplasmic mitochondria. Bar = 500nm. Panel c, mean CK-7 positive putative murine oval cells per portal tract in WT and in PiZ mice.

Figure 3. Proliferation and HCC in the PiZ mouse model

Panel a, quantification of CK-7 positive, putative oval cells averaged in groups by age 2-6 months, 6-12 months, and 12-24 months (bars $\pm$ S. D., $p > 0.2$ between all groups). Panel b, CK-7 staining of HCC from PiZ mouse liver showing CK-7 positive cells in biliary epithelium (white arrows) and putative hepatic progenitor cells (black arrows).

Figure 4. Sirius Red staining of WT and PiZ mouse liver

Panel a, Photomicrographs of sirius red staining highlighting red fibrotic tissue of male WT mice (top row) and male PiZ mice (bottom) age 5, 14, and 24 months, as shown. Panel b, low power photomicrograph of Sirius red staining of 24 month old, male PiZ mouse liver.