Chronic iron overload stimulates hepatocyte proliferation and cyclin D1 expression in rodent liver

KYLE E. BROWN, M. MELEAH MATHAHS, KIMBERLY A. BROADHURST, and JAMIE WEYDERT

Hepatomegaly is commonly observed in hepatic iron overload due to human hemochromatosis and in animal models of iron loading, but the mechanisms underlying liver enlargement in these conditions have received scant attention. In this study, male rats were treated with iron dextran or dextran alone for 6 months. Chronic iron loading resulted in a >50-fold increase in hepatic iron concentration. Both liver weights and liver/body weight ratios were increased ~2-fold in the iron-loaded rats ($P < 0.001$ for both). Hepatocyte nuclei expressing proliferating cell nuclear antigen (PCNA), a marker of S phase, were significantly increased in the iron-loaded livers, suggesting enhanced proliferation. To assess the mechanisms by which iron promotes proliferation, the expression of tumor necrosis factor-α (TNF-α), interleukin (IL)-6, hepatocyte growth factor (HGF), and transforming growth factor-α (TGF-α) were assessed by reverse transcription-polymerase chain reaction (RT-PCR). Of these growth-associated factors, only TNF-α messenger RNA (mRNA) was significantly increased by iron loading (about 3-fold; $P = 0.005$). Because cyclin D1 is required for entry of hepatocytes into the cell cycle after partial hepatectomy or treatment with direct mitogens, levels of immunoreactive cyclin D1 were examined and found to be significantly increased in the iron-loaded livers. The increase in cyclin D1 protein in the iron-loaded livers was paralleled by an increase in the abundance of its transcript as measured by real-time PCR. Taken together, these results suggest that iron is a direct mitogen in the liver and raise the possibility that chronic stimulation of hepatocyte proliferation may play a role in the pathophysiology of iron overload states. (Translational Research 2006;148:55–62)

Abbreviations: GADPH = glyceraldehyde 3-phosphate dehydrogenase; GSK-3β = glycogen synthase kinase-3β; HCC = hepatocellular carcinoma; HGF = hepatocyte growth factor; IL = interleukin; mRNA = messenger RNA; PCNA = proliferating cell nuclear antigen; RT-PCR = reverse transcription-polymerase chain reaction; SD = standard deviation; TGF = transforming growth factor; TNF = tumor necrosis factor

As terminally differentiated cells, hepatocytes are unique in their ability to proliferate in response to various stimuli while exhibiting a low level of cell division under normal conditions. Hepatocyte proliferation is a common feature of animal models of experimental liver injury[1–4] and of chronic human liver disease.[5–10] In chronic hepatitis C, for example, hepatocyte proliferation is reported to correlate with fibrosis progression.[6,7] With advanced cirrhosis, however, a reduction in hepatocyte proliferation has been observed.[5–9] Given that normal somatic cells undergo a finite number of cell divisions before entering

From the Iowa City Veterans Administration Medical Center, Iowa City, Ia; and the Division of Gastroenterology-Hepatology, Program in Free Radical and Radiation Biology, and the Department of Pathology, University of Iowa Roy J. and Lucille A. Carver College of Medicine, Iowa City, Ia.

Supported in part by a Merit Review grant from the Veterans Administration (K.E.B.).

Submitted for publication August 7, 2005; revision submitted January 12, 2006; accepted for publication March 23, 2006.

Reprint requests: Kyle E. Brown, Division of Gastroenterology-Hepatology, University of Iowa Roy J. and Lucille A. Carver College of Medicine, 4553 JCP, 200 Hawkins Drive, Iowa City, IA 52242; e-mail: kyle-brown@uiowa.edu.

1931-5244/$ – see front matter

doi:10.1016/j.trsl.2006.03.002

a state of replicative senescence, the identification of senescent hepatocytes in livers with advanced fibrosis[10] supports the concept that progressive liver disease is accompanied by chronic stimulation of parenchymal cell division. These observations raise the possibility that although persistent hepatocyte proliferative activity has the beneficial effect of maintaining functional liver mass in the context of liver injury, it ultimately participates in the pathophysiology of chronic liver disease.

Two major pathways of hepatocyte proliferation have been described.[11] The first one is compensatory hyperplasia or regeneration, of which partial hepatectomy is the classic example. Similarly, hepatocyte loss resulting from necrogenic injury activates pathways that overlap considerably with those involved in the restoration of hepatic mass after partial hepatectomy. In these circumstances, there is prominent induction of immediate early genes such as c-fos and c-jun as well as pivotal roles for IL-6, TNF-α, and HGF in the proliferative response.[12,13] In contrast, diverse agents such as lead nitrate and peroxisome proliferators have been shown to act as direct mitogens.[11] These agents act through pathways that differ from those that drive regenerative hyperplasia and stimulate hepatocyte cell division in the absence of significant hepatocyte loss. Although it is reasonable to assume that hepatocyte proliferation occurring in the context of a chronic inflammatory disease such as viral hepatitis is "regenerative" in nature, ie, a response to attrition of parenchymal cells caused by the underlying disease, the possibility that some forms of progressive liver disease may involve hepatic hyperplasia warrants consideration.

One such condition is iron overload. Hepatomegaly is a frequent physical finding in hemochromatosis and is reported to regress after phlebotomy, suggesting that liver enlargement is directly related to the effects of excess iron.[14] Hepatomegaly has also been linked to the presence of hepatic fibrosis in patients with hemochromatosis.[15] Furthermore, liver enlargement is commonly observed in rodent models of iron overload. In view of these observations, it was hypothesized that iron might act as a mitogenic stimulus to hepatocytes. Accordingly, the aims of this study was to determine the effects of iron overload on hepatocyte proliferation in rodent liver and to explore potential mechanisms by which iron might stimulate proliferation.

MATERIALS AND METHODS

Animals: Male Sprague–Dawley rats (200–250 g) were purchased from Harlan Laboratories (Indianapolis, Ind). The rats were housed in individual polyethylene cages with stainless steel tops and were fed a standard rat diet (Dyets, Inc., Bethlehem, Pa) and allowed water *ad libitum*. After an acclimatization period of 1 week, i.p. injections of iron dextran or an equivalent quantity of dextran were administered on a biweekly basis. In each of the first 4 injections, 50 mg of iron was given. The dose was subsequently increased to 100 mg for the remainder of the experiment. A total of 900 mg of iron was administered over a 22-week period. The animals were cared for in accordance with criteria from the National Research Council, and the protocol was approved by the Animal Research Committee of the John Cochran Veterans Administration Medical Center.

Rats (n = 5 per group) were euthanized by exsanguination while anesthetized with pentobarbital sodium (65 mg/kg). At the time of death, the livers were quickly excised, weighed, and divided for analysis as described below.

Hepatic iron concentration: Nonheme iron concentrations in whole liver were determined by the method of Torrance and Bothwell as previously described.[16]

Histology and immunohistochemistry: Liver samples were fixed in 10% buffered formaldehyde solution and embedded in paraffin blocks. Sections were stained with Perls Prussian blue for nonheme ferric iron, hematoxylin and eosin for routine histology, and Masson trichrome for collagen using standard techniques. Immunostaining for PCNA was performed after heat-induced antigen retrieval for 10 min in citrate buffer (pH 6.0) using primary antibody (Oncogene Research Products, San Diego, Calif) applied at dilution of 1:500 for 1 h at room temperature. Apoptotic hepatocytes were detected by immunoreactivity with M30, an antibody that recognizes a neoepitope generated by caspase-mediated cleavage of cytokeratin 18.[17] Sections for M30 immunostaining were antigen-retrieved as above, and M30 antibody (Roche Applied Science, Indianapolis, Ind) was applied at a dilution of 1:10 and incubated at 4°C overnight. Immunoperoxidase staining was then performed as previously described.[18]

PCNA index: A total of 1000 hepatocytes on each slide were counted under light microscopy (60× objective, Olympus BX40; Olympus Corporation, Tokyo, Japan). Hepatocytes were scored as "positive" for PCNA staining if (1) staining was nuclear, (2) staining was characterized as strong (qualitatively 3 times the intensity of the weakest staining nucleus found on the slide), and (3) there were no morphologic changes of degeneration or apoptosis. The labeling index is reported as number of positive cells/100 nuclei.

DNA isolation: Liver samples were weighed, and DNA was isolated using a commercial kit (Dneasy, Qiagen, Valencia, Calif). Results are expressed as milligram/gram tissue.

RT-PCR: Total RNA was isolated from rat livers using the Trizol reagent (Invitrogen Life Technologies, Carlsbad, Calif) according to the manufacturer's instructions and quantitated spectrophotometrically. Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen Life Technologies) was used to synthesize cDNA as previously described.[18] The cDNA product was amplified by PCR using 800-nM oligonucleotide primers for rat TNF-α, IL-6, HGF, TGF-α, cyclin D1, and GAPDH. PCR-amplified DNA was electrophoresed on 2% agarose gels containing 1-μg/mL ethidium bromide,