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# Hepatic nerve growth factor induced by iron overload triggers defenestration in liver sinusoidal endothelial cells

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ABSTRACT

The fenestrations of liver sinusoidal endothelial cells (LSECs) play important roles in the exchange of macromolecules, solutes, and fluid between blood and surrounding liver tissues in response to hepatotoxic drugs, toxins, and oxidative stress. As excess iron is a hepatotoxin, LSECs may be affected by excess iron. In this study, we found a novel link between LSEC defenestration and hepatic nerve growth factor (NGF) in iron-overloaded mice. By Western blotting, NGF was highly expressed, whereas VEGF and HGF were not, and hepatic NGF mRNA levels were increased according to digital PCR. Immunohistochemically, NGF staining was localized in hepatocytes, while TrkA, an NGF receptor, was localized in LSECs. Scanning electron microscopy revealed LSEC defenestration in mice overloaded with iron as well as mice treated with recombinant NGF. Treatment with conditioned medium from iron-overloaded primary hepatocytes reduced primary LSEC fenestrations, while treatment with an anti-NGF neutralizing antibody or TrkA inhibitor, K252a, reversed this effect. However, iron-loaded medium itself did not reduce fenestration. In conclusion, iron accumulation induces NGF expression in hepatocytes, which in turn leads to LSEC defenestration via TrkA. This novel link between iron and NGF may aid our understanding of the development of chronic liver disease.



## 1. Introduction

Liver sinusoidal endothelial cells (LSECs) are unique endothelial cells both morphologically and functionally. These cells line hepatic sinusoids and thus play important roles in regulating hepatic microcirculation. They also lack a basement membrane and are characterized by fenestrae, which occupy 6-8% of the endothelial surface [1–4] and act as dynamic filters that play an active role in regulating the exchange of macromolecules, solutes, and fluid between the blood and the surrounding tissues [5–7]. However, in disease states, the diameter and number of LSEC fenestrae undergo changes, such as loss of fenestrae (defenestration), in both animals and humans [8–17]. These changes can be induced by several factors, including drugs and toxins [18–22], and are believed to have adverse effects on liver function in general [4]. However, the precise mechanism by which these hepatotoxins induce defenestration remains to be elucidated. Iron, a vital requirement for normal cellular function, is also an important hepatotoxin when present in excess, which may affect endothelial cell function and induce defenestration.

It is well known that growth factors, such as hepatocyte growth factor (HGF), tumor necrosis factor (TNF), and interleukin-6 (IL-6), are involved in hepatic regeneration [24]. Furthermore, neurotrophins (NTs) may play a role in hepatic regeneration [25–27]. Nerve growth factor (NGF), a member of the NT family, is the most expressed NT in the adult mouse liver [28]. NGF is also proapoptotic in the liver [27] and is thought to protect the liver against oxidative stress and xenobiotic injury [29]. NGF was also shown to be highly expressed in hepatocytes and hepatoma cells in liver cirrhosis and hepatocellular carcinoma in both clinical [30, 31] and animal models [26,27], which suggests that NGF may contribute to the pathophysiology of liver disease. Thus, in the present study, we focused on the link between hepatic iron overload and NGF expression using mouse models of iron overload.

## 2. Materials and methods

### 2.1. Animals

Male C57Bl/6 mice (Clea Japan, Tokyo, Japan) were randomly assigned to three treatment groups: control, dietary iron (slight

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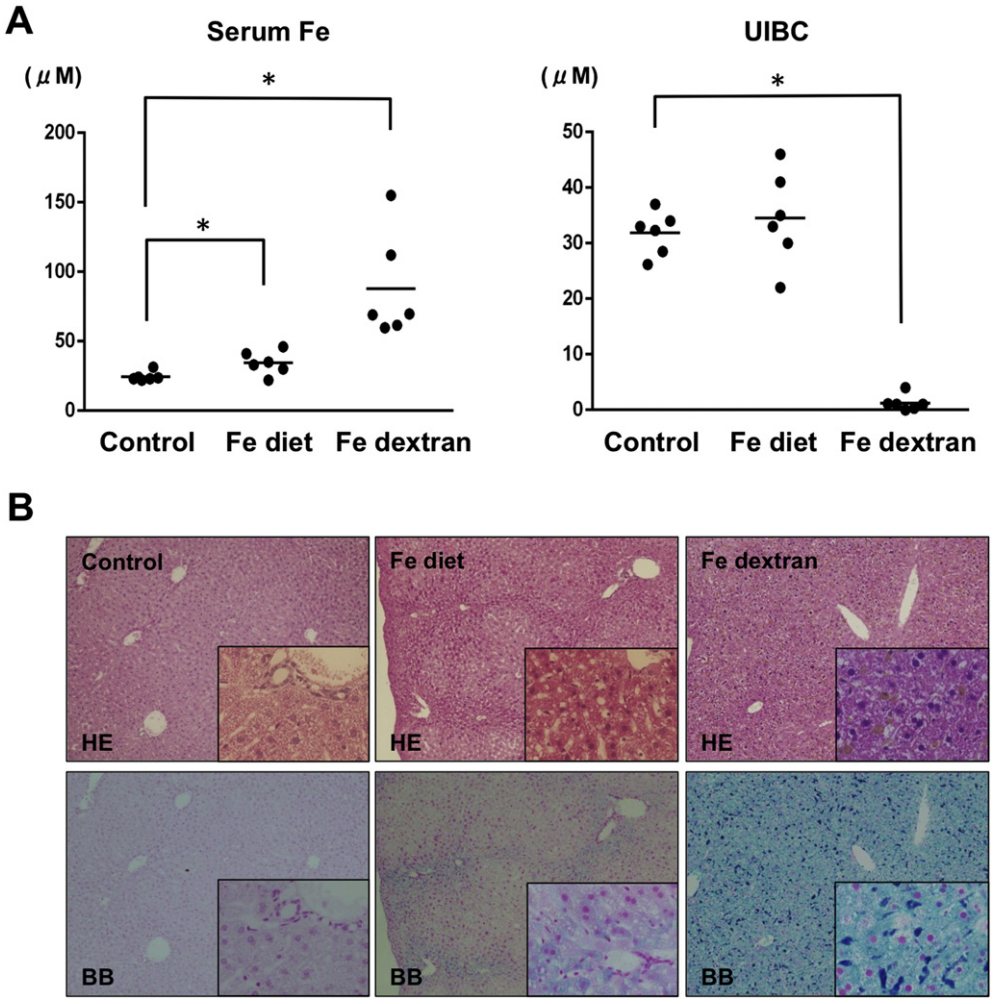


**Fig. 1.** Serum and histologic evidence of iron overload. (A) Serum iron was slightly increased in the iron diet group but significantly increased in the iron dextran group. However, UIBC was significantly reduced. (B) Slight iron accumulation was observed in the portal area of iron diet mice, with severe iron accumulation observed throughout the liver tissues of iron dextran mice (*$P < 0.05$). H&E staining shown in the upper row, Berlin Blue staining shown in the lower row.

iron overload), and iron dextran (severe iron overload). Each group comprised five mice. The control group was fed a regular mouse chow diet for 8 weeks, while the dietary iron overload group was fed a 2.5% (w/w) carbonyl iron diet for 8 weeks. The iron dextran group received intraperitoneal injections of iron dextran solution (10 mg iron/head/day) (Sigma-Aldrich, St. Louis, MO, USA) for 5 days. Another group of five mice received intraperitoneal injections of mouse recombinant NGF (1 μg/head/day) (Promega, Madison, WI, USA) for 3 days. The mice were sacrificed at the end of each treatment period, and serum and liver tissues were collected. The liver tissues were processed for formalin-fixed paraffin-embedded tissue blocks and then subjected to H&E and Berlin Blue staining. All animal experiments were approved by the animal experiments committee of the Asahikawa Medical University (Hokkaido, Japan) based on guidelines for the protection of animals.

### 2.2. Serum analysis

Serum iron and unsaturated iron binding capacity (UIBC) were measured with the automatic serum analyzer LABOSPECT 008 (Hitachi, Tokyo, Japan). Assay reagents were obtained from Shino-Test (Tokyo, Japan).

### 2.3. Western blotting

Liver tissues were lysed in RIPA buffer, separated in polyacrylamide gels and electro-transferred to nitrocellulose membranes. After blocking with 5% nonfat dry milk in PBST buffer (PBS containing 0.05% Tween-20), the membranes were probed with rabbit anti-NGF (Abcam, Cambridge, UK), rabbit anti-HGF (Abcam), mouse anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-TrkA (Novus Biologicals, Littleton, CO, USA), or mouse anti-Actin antibody (BD Bioscience, Franklin Lakes, NJ, USA). The membranes were then incubated with the respective HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (R&D Systems, Minneapolis, MN, USA). Antibody binding was visualized using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA).

### 2.4. Digital PCR analysis

Absolute copy numbers of mouse Ngf mRNA were analyzed using the digital PCR system with the TaqMan probe for mouse *Ngf* (Life Technologies, Carlsbad, CA, USA). RNA was extracted from the livers using the Purelink RNA mini kit (Life Technologies), and the RNA concentrations were measured by fluorometric quantification using