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ORIGINAL ARTICLE

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The impairment of hepatocytes and sinusoidal endothelial cells during cold preservation in rat fatty liver induced by alcohol and the beneficial effect of hepatocyte growth factor

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Abstract A fatty liver resulting from alcohol intake is often unattractive for grafting. In this study, we investigated the impairment of hepatocytes and sinusoidal endothelial cells (SECs) during cold preservation of alcohol-induced fatty liver and examined the efficacy of human recombinant hepatocyte growth factor (hrHGF). Rats were fed an alcohol diet. We performed histological examinations of the hepatocytes and observed the ultrastructural alteration of the SECs. Additionally, we measured hepatic transaminase and peroxidative lipids for hepatocellular injury and the hyaluronic acid uptake rate (HUR) to determine SEC injury. We added hrHGF to University of Wisconsin (UW) solution to assess the protective effect of the agent. Numerous fatty deposits were observed in ethanol-induced fatty livers. These grew with the duration of cold storage. Hepatic transaminases of the effluents increased during cold preservation in the livers of alcohol-treated rats. Additionally, peroxidative lipids in the effluents increased during cold preservation in the livers of alcohol-treated rats, whereas they were undetectable in non-alcohol-treated rat livers. The sinusoidal endothelium had severely deteriorated in the livers of alcohol-treated rats. Further, the HUR decreased with ethanol treatment and/or cold preservation. The addition of hrHGF suppressed the increase of hepatic transaminase in the effluent of cold-preserved alcohol-treated livers. Peroxidative lipids in the same effluents were undetectable. In fatty livers, both hepatocytes and SECs received severe damage during cold preservation. Furthermore, we demonstrated that hepatocellular injury was significantly inhibited by hrHGF.

Keywords Alcohol-induced fatty liver · Cold preservation · Hepatic injury · Human recombinant hepatocyte growth factor

Introduction

Liver transplantation is widely accepted as a treatment for adult and pediatric patients with end-stage liver diseases [21]. However, thousands of patients are waiting for donor livers and several hundred die each year without receiving an available organ [44]. Therefore, the shortage of donor livers is a serious problem. Moreover, the transplantation of a donor liver does not necessarily lead to successful restoration of liver function. With regard to primary nonfunction (PNF) in particular, it usually results in graft failure, and 5–15% of patients require a re-transplantation [19]. It is well known that fatty liver is one of the main risk factors for PNF leading to emergency re-transplantation [32, 33, 39]. For this reason, fatty-liver grafts are often discarded, except for

mildly fatty livers [1, 38]. It is, therefore, important that the mechanism through which fatty livers cause PNF after liver transplantation be resolved, so that they can be used as available grafts.

In a previous study, we investigated the alteration of hepatocytes and sinusoidal endothelial cells (SECs) during cold preservation in the context of a choline-deficient diet (CDD) that induced fatty liver. We demonstrated that injury in both hepatocytes and SECs in a cold-preserved fatty-liver graft developed more rapidly and severely than in a normal liver graft [35]. However, there are several differences in the mechanism that produces fatty liver and induces hepatic injury, between a CDD and alcohol. Accordingly, it is unknown whether the experimental findings in CDD-induced fatty liver are comparable with those in alcohol-induced fatty liver. Moreover, in clinical practice most fatty-liver cases are attributed to alcohol excess, and alcohol-induced steatosis in the liver is strongly related to PNF [5, 36, 38]. Thus, in this study, we analyzed hepatic injury, morphologically and biochemically, in both hepatocytes and SECs in cold-preserved liver grafts with ethanol-induced fatty-liver models.

Furthermore, we previously presented the effect of human recombinant hepatocyte growth factor (hrHGF), with both the addition of preservative solution and administration through the portal vein before removal, on the reduction of damage induced by cold preservation of alcohol-induced fatty-liver tissues [35]. However, the in vivo administration of hrHGF prior to removal is difficult in clinical application. Therefore, in the present study we also assessed the effect of hrHGF on the suppression of hepatic damage, with the addition of cold-preservative solution alone.

Materials and methods

Animals

Male Wistar rats (SLC, Shizuoka, Japan) weighing 200–250 g were used in all experiments. The animals received humane care in compliance with the institution's guidelines. All rats were housed at room temperature in chip-bedding cages under 12-h light/dark cycles.

Induction of fatty liver

We induced fatty changes in the livers by feeding the rats an alcohol diet (Oriental Yeast, Tokyo, Japan) for 12 weeks according to the methods described by Lieber and Decarli [20]. In short, both non-alcohol and alcohol groups received equivalent amounts of corn oil. Thirty-six percent of the calories was provided by ethanol, in the alcohol groups, and isocaloric maltose–dextrin was given to the non-alcohol groups.

Surgical procedure for removal and storage of the livers

We removed the livers as described previously [26]. Briefly, while the rats were under ether anesthesia in the supine position, we made an upper and middle midline incision in the abdomen. The portal vein and suprahepatic vena cava were cannulated with JMS cut-down tubes (i.d. 0.9 and 1.2 mm, JMS, Japan) and then tied securely with 3-0 silk ligatures. The inferior vena cava was ligated with 3-0 silk at the upper level of the right renal vein before the liver was perfused via the portal vein with 30 ml of warm (37 °C) phosphate-buffered saline solution to wash out blood. Subsequently, the liver was carefully removed and immediately flushed with 10 ml of cold (4 °C) University of Wisconsin (UW) solution and then stored in UW solution at 4 °C until required for use.

Animal groups for experiments

The animals were divided into the following eight groups of different combinations of duration of cold preservation and mixture of alcohol to diet: (a) non-preserved, non-alcohol group, (b) non-preserved, alcohol group, (c) 4-h-preserved, non-alcohol group, (d) 4-h-preserved, alcohol group, (e) 24-h-preserved, non-alcohol group, (f) 24-h-preserved, alcohol group, (g) hepatocyte growth factor (HGF) group, prepared by administration of 1 μg/ml of HGF for "group d", and (h) HGF group, prepared by administration of 1 μg/ml of HGF for "group f".

Assessment of fatty changes in the liver

The removed livers in all groups were cut and immersed in 10% formalin and subjected to Sudan-III staining. We used light microscopy to examine the degree of fatty change.

Observation by scanning and transmission electron microscope

For the determination of ultrastructural changes in SECs and sinusoidal architecture, the livers of all groups were fixed for observation by scanning electron microscopy as described previously [35]. They were perfused with 2% glutaraldehyde in 0.1 M cacodylate or phosphate buffer and 0.1 M sucrose through the portal vein at 10-mmHg pressure for approximately 20 min. The specimens were cut to 1×1×5 mm for immersion fixation.

The specimens were immersed in 2% glutaraldehyde in 0.1 M cacodylate buffer and 0.1 M sucrose for 2 h, then post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer and 0.1 M sucrose for 5 h. Subsequently, they were dehydrated with staged ethanol and frozen in *t*-butyl alcohol at −20 °C until required for observation. After they had been dried in a vacuum, we examined the cut surface to study the sinusoidal architecture and SECs (SEM: Hitachi S-450X EDX).

Biochemical evaluation of injury in hepatocytes

The livers in all but the non-preserved group were perfused with warm (37 °C) Krebs–Henseleit bicarbonate-buffered (KHBB) solution. The first effluents of 0.8 ml were collected for measurement of the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH), for the evaluation of hepatocellular damage. Moreover, to investigate the mechanism of injury in the hepatocytes, we measured the lipid peroxide levels in the same samples. The assay was performed according to the methods described by Yagi [41]. The results were expressed as nanomoles of malondialdehyde per milliliter.

Hyaluronic acid uptake rate for the determination of SEC injury

Hyaluronic acid (HA) is removed from circulation mainly by the SECs in the liver [9, 10]. Therefore, the HA level in circulation is

thought to reflect SEC function [6, 26, 35]. We examined the hyaluronic acid uptake rate (HUR) to study the damage to the SECs. The livers in all groups were perfused in a non-recirculating system at a flow rate of 1 ml/min per gram of liver weight, kept constant by a digital flow controller (Digi-staltic, Cole Palmer Instruments) with warm (37 °C) KHBB solution saturated with 95% oxygen and 5% carbon dioxide at pH 7.4 for 5 min, to wash out the UW solution. Subsequently, the livers were perfused with KHBB solution containing 300 ng/ml of HA (Wako, Jyunyaku, Japan). After 20 min, we collected 1 ml of influent and effluent to measure the HA concentration, using sandwich enzyme binding assay (Chugai Paramecia, Tokyo, Japan). The HUR was calculated from the difference between the HA concentration of the influent and effluent samples.

Administration of hrHGF

hrHGF was prepared as previously described [42]. It was dissolved in 10 mmol of phosphate buffer, pH 7.4, containing 0.7 mol/l NaCl and 0.05% bovine serum albumin (Sigma Chemical, St. Louis, Mo., USA), with the final concentration adjusted to 0.5 mg/ml. To study the effect of hrHGF on the suppression of hepatic damage, we added 10 or 100 μg of the agent to 100 ml of cold UW solution to preserve the removed livers for 4 or 24 h, with the concentration adjusted to 0.1 or 1 μg/ml.

Statistics

All values are expressed as mean ± SEM. Statistical analyses were performed with Student's *t*-test. *P* values below 0.05 were considered significant.

Results

Assessment of fatty changes in the livers without cold preservation

No fatty changes were observed in the control livers. Numerous fatty droplets were present in the hepatocytes of the alcohol-treated groups, i.e., fat droplets existed more abundantly in zone 3 than in zones 1 and 2. The fatty change induced by ethanol exhibited a heterogeneous distribution.

Morphological changes in the sinusoidal architecture

Because the fatty change was heterogeneous in the alcohol-exposed model we discriminated zone 3 from other areas, to examine the morphological changes in the sinusoidal architecture in relation to the degree of fat accumulation. In zones 1 and 2 of the alcohol-treated groups, the architecture seemed to be moderately irregular with 24-h cold preservation (Fig. 1d, 1e, and Fig.1f). In contrast, they were moderately disarranged, and the spaces were narrowed, in zone 3 of the alcohol-exposed liver, even without cold preservation (Fig. 1g). The architecture was disrupted even more in zone 3 of group d with 4-h cold preservation (Fig. 1h). Finally, after 24-h cold preservation, the radiated structure had collapsed and the space was narrower in zone 3 of group f than in all zones of the other groups (Fig. 1i), whereas in the non-alcohol groups (controls) sinusoidal arrangements appeared to be linear in all areas, even with 24-h cold preservation (Fig. 1a, 1b, and 1c).

Morphological changes in the sinusoidal endothelium

In group a, the sinusoidal endothelium was well maintained in all areas, and many fenestrae were observed (Fig. 2a). The endothelium was slightly disrupted in group c, and detachment of the sinusoidal endothelium progressed, leading to the string-like appearance in group e (Fig. 2b and 2c). In zones 1 and 2 of the alcohol-exposed liver, the morphological alteration of the sinusoidal endothelium was nearly the same as that in non-alcohol groups (Fig. 2d, 2e, and 2f). However, there were still several gaps and a few blebs in zone 3, despite the liver not being preserved (Fig. 2g). Sinusoidal endothelial damage in the same area appeared severe, with enlarged gaps, leading to a string-like appearance with 4-h cold preservation (Fig. 2h). After 24-h cold preservation, the endothelium in zone 3 was mostly lost and exhibited sinusoidal denudation (Fig. 2i).

Morphological changes in the hepatocytes

Fatty changes were examined under Sudan-III staining. There were no stained areas in groups a, c, and e. In zone 3 of alcohol-exposed livers, the stained area increased with cold preservation, and many large fatty droplets were found in group f after 24 h of cold preservation of alcohol-treated livers (Fig. 3a, 3b, and 3c).

Biochemical evaluation of hepatocellular injury

The AST, ALT, and LDH levels of the first effluent were 16 ± 4.0, 17.5 ± 5.1, and 348 ± 86 IU/l, respectively, in 4-h cold-preserved non-alcohol livers (group c). They significantly increased in the alcohol-exposed livers (group d), with values of 89 ± 11.2, 96.3 ± 16.6, and 1523.8 ± 301.2 IU/l ($P < 0.05$), respectively. Subsequently, during 24-h cold preservation, the levels in control livers were 68.5 ± 3.5, 65.3 ± 5.8, and $1{,}035 \pm 218.2$ IU/l, respectively, and reached peak levels of 208 ± 23.2, 203.4 ± 14.7, and $3{,}339.2 \pm 134.3$ IU/l, with a significant increase in group f ($P < 0.005$). These findings showed a severe insult of hepatocytes with fatty droplets during 4 and 24 h of cold preservation (Fig. 4a).

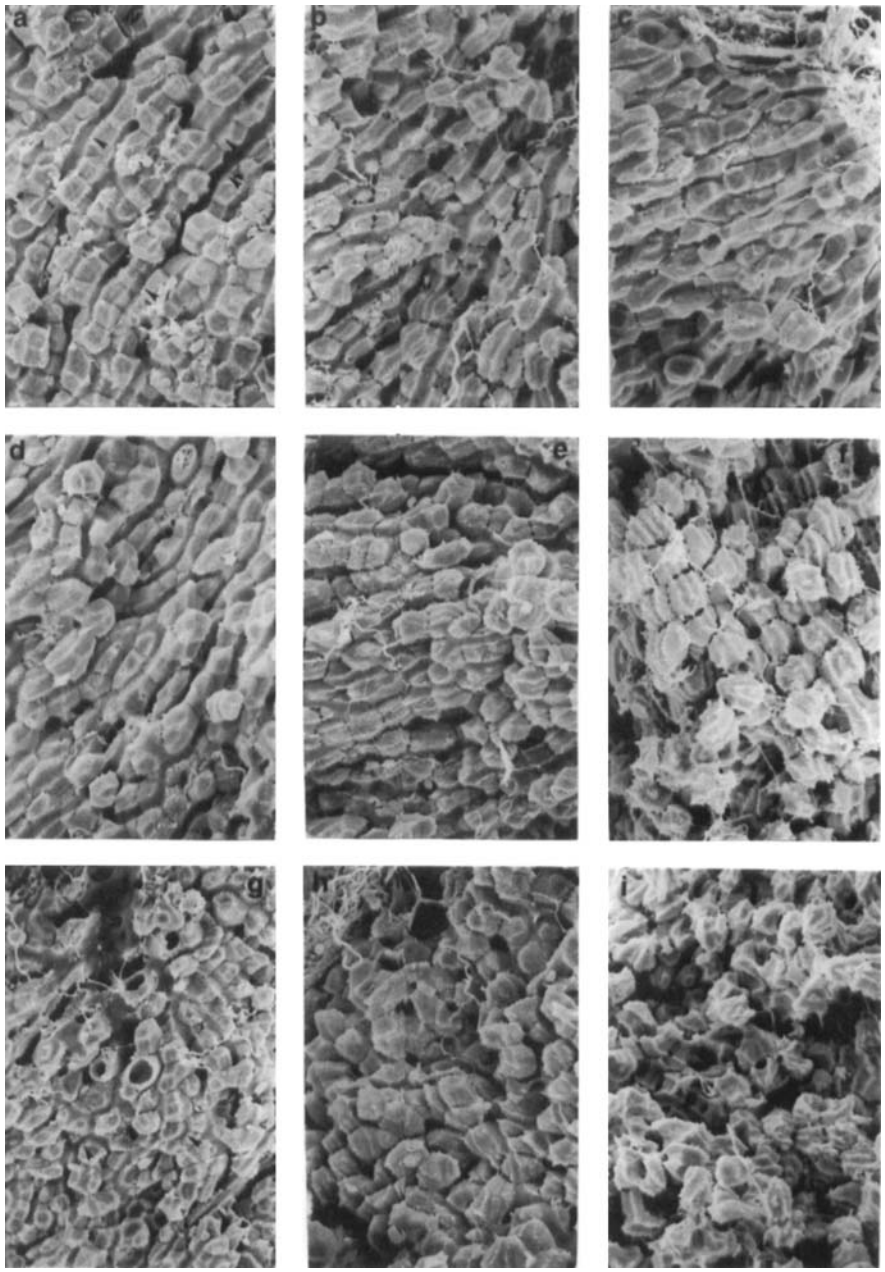


Fig. 1a–i Scanning electron microscopy images of sinusoidal architecture. **a–c** Normal livers at zone 2. **a** Without cold preservation. The sinusoidal arrangements (*arrow heads*) are linear and clear. **b** After 4 h of cold preservation. The sinusoidal arrangements appear to be slightly irregular. **c** After 24 h of cold preservation. The sinusoidal arrangements are irregular but linear. **d–i** Alcohol-exposed livers. **d** At zone 2, without cold preservation. The sinusoidal arrangements are almost linear and clear. **e** At zone 2, after 4 h of cold preservation. The sinusoidal arrangements are slightly irregular. **f** At zone 2, after 24 h of cold preservation. The sinusoidal arrangements are irregular and the spaces are narrow. **g** At zone 3, without cold preservation. Note that the sinusoidal arrangements are moderately disarranged. **h** At zone 3, after 4 h of cold preservation. The sinusoidal arrangements are irregular and the spaces are narrow. **i** At zone 3, after 24 h of cold preservation. The radiated structure of the sinusoid is collapsed and the spaces are not detectable (approximate original magnification: ×500)

Biochemical evaluation of lipid peroxidation in hepatocytes

We examined the concentrations of peroxidative lipids in the first effluent as a marker of intrahepatocellular lipid peroxidated during cold preservation. They were undetectable in the cold-preserved non-alcohol and non-preserved alcohol groups because the values below 0.5 μmol/ml could not be determined. The values in the alcohol groups were 0.96 ± 0.14 μmol/ml with 4-h cold preservation and increased to 2.02 ± 0.19 μmol/ml with 24-h cold preservation (Fig. 4b).

Determination of SEC injury with HUR

The HUR value of non-alcohol liver without cold preservation was 4.6 ± 0.78 and declined as the cold preservation time was prolonged; the values in 4-h and 24-h cold preservation were 3.5 ± 0.62 and 1.4 ± 0.24 μg/g per h, respectively. However, in alcohol-exposed livers, they showed low levels even without cold preservation and decreased more in the 4-h and 24-h preserved liver groups: 2.9 ± 0.22, 1.7 ± 0.56, and 0.6 ± 0.14 μg/g per h for groups b, d, and f, respectively (Fig. 5), but the

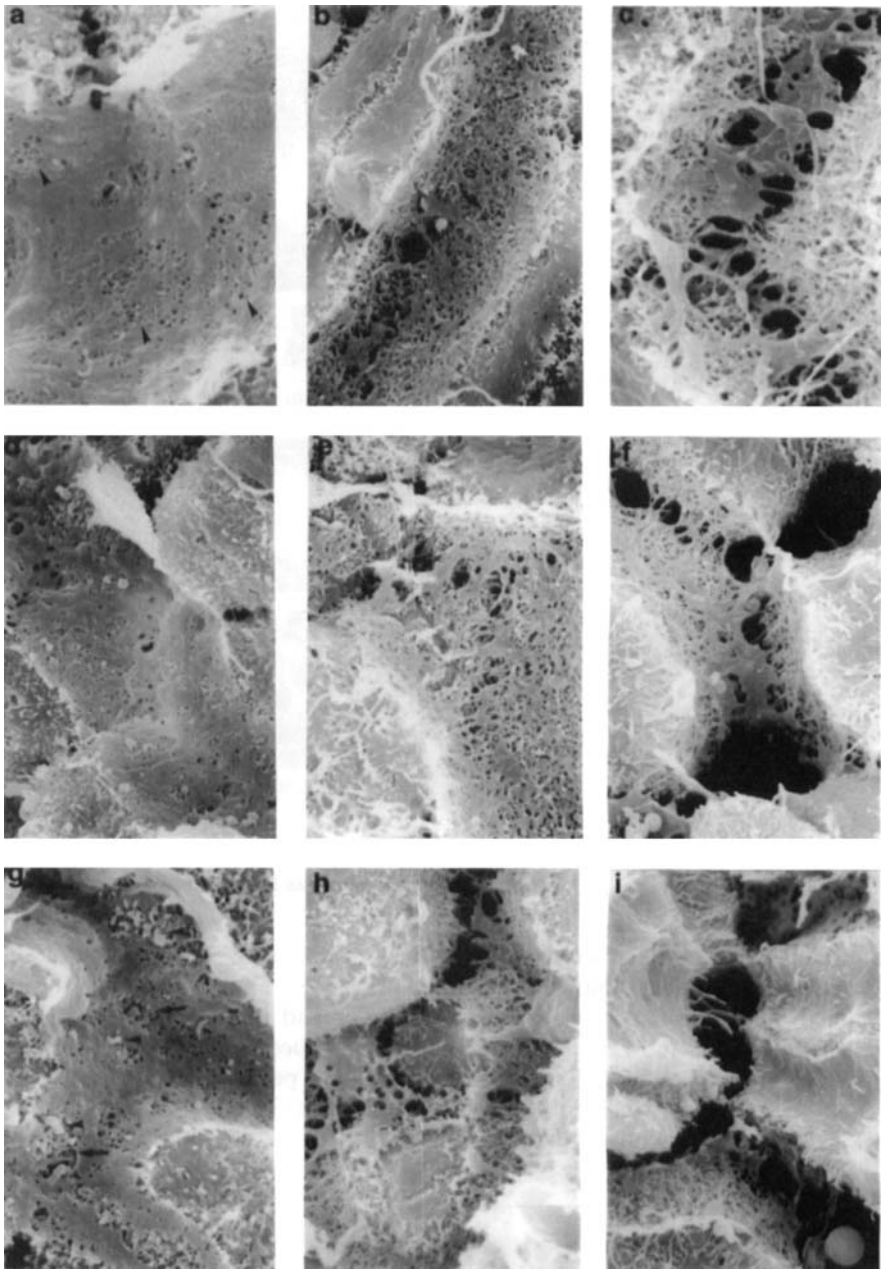


Fig. 2a–i Scanning electron microscopy images of the sinusoidal endothelium. **a–c** Normal livers at zone 2. **a** Without cold preservation. Many fenestrae (*arrow heads*) are clearly observed. **b** After 4 h of cold preservation. The endothelium is slightly disrupted with a few gaps and blebs. **c** After 24 h of cold preservation. A string-like appearance is observed. **d–i** Alcohol-exposed livers. **d** At zone 2, without cold preservation. The sinusoidal endothelium is mostly well maintained. **e** At zone 2, after 4 h of cold preservation. The endothelium is slightly disrupted with a few gaps and blebs. **f** At zone 2, after 24 h of cold preservation. A string-like appearance is observed. **g** At zone 3, without cold preservation. Several large gaps and blebs are shown. **h** At zone 3, after 4 h of cold preservation. The detachment of the sinusoidal endothelium progressed resulting in a string-like appearance. **i** At zone 3, after 24 h of cold preservation. The denudation of the sinusoidal endothelium is severe (approximate original magnification: ×5,000)

differences between non-alcohol and alcohol livers were not significant.

Effect of hrHGF on cold-preserved alcohol-exposed livers

Using the methods described above, we compared groups g and h, which received an addition of hrHGF in UW solution, with groups d and f, respectively, to examine the effect of the agent. With regard to hepatocellular injury during 4-h cold preservation, the values of AST, ALT, and LDH in group g were 53 ± 17.8, 59 ± 20.9, and $1{,}035 \pm 218.2$ IU/l, respectively, which were not significantly, but moderately, lower than those in the corresponding control group d (Fig. 6a). In the 24-h cold-preserved, alcohol-exposed livers, the transaminases in group h were 82 ± 18.8 for AST, 84 ± 24.5 for ALT, and $1{,}312 \pm 326.7$ for LDH, all of which were significantly lower than those of group f (all P values < 0.005) (Fig. 6a). The peroxidative lipid levels in the first effluent of both groups treated with hrHGF could not reach the detectable level (Fig. 6b).

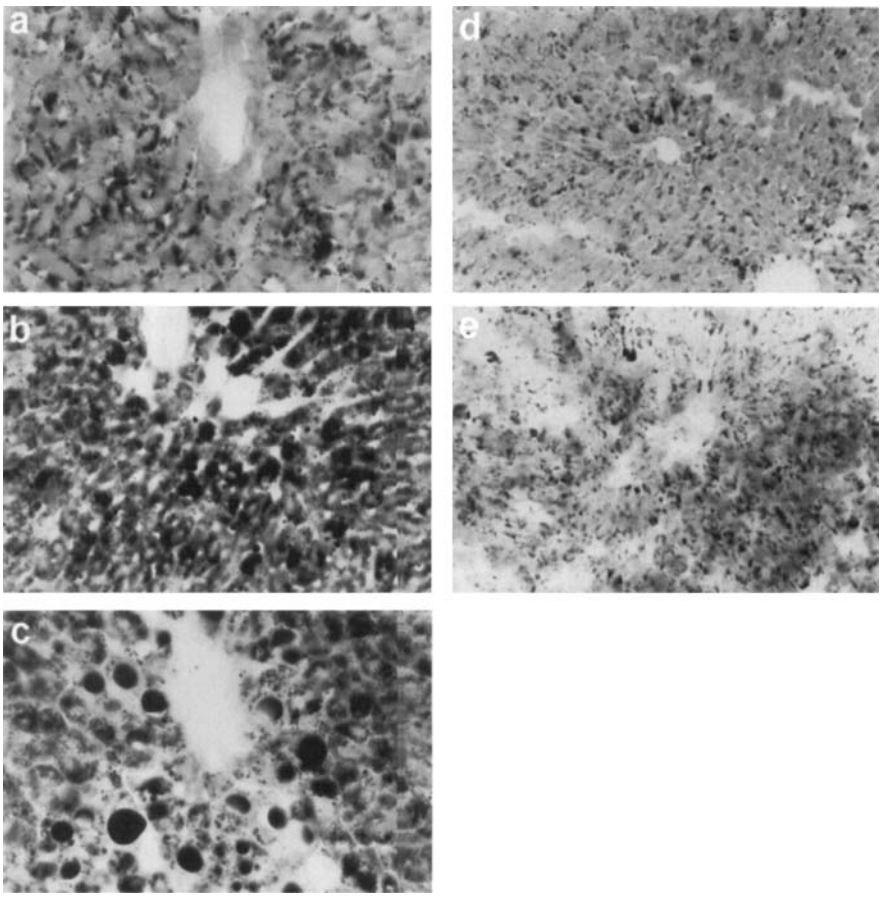


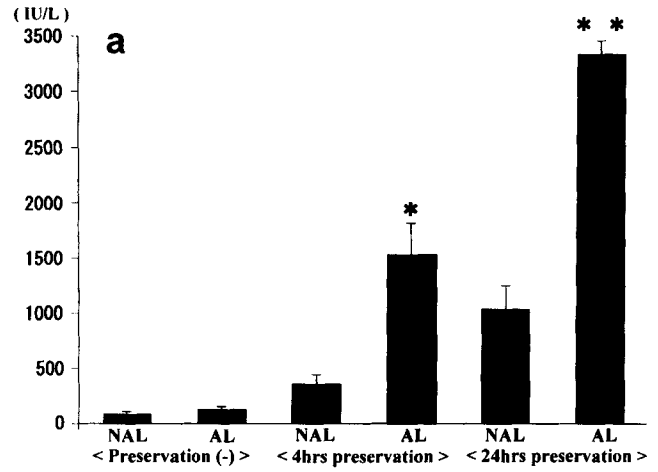
Fig. 3a–e Light microscope view of Sudan-III-stained species of the alcohol-exposed livers at zone 3. The stained area is shown in *black*. **a** No preservation. **b** After 4 h of preservation. The stained area appears to have increased. **c** After 24 h of cold preservation. Many large, round, stained droplets are observed. **d** After 4 h of cold preservation with hrHGF treatment. The stained area is reduced in comparison with that in group d, shown in Fig. 3b. **e** After 24 h of cold preservation with hrHGF treatment. Note that almost no large, round, stained droplets are observed (approximate original magnification: **a**, **b**, and **c**: ×400; **d** and **e**: ×200)

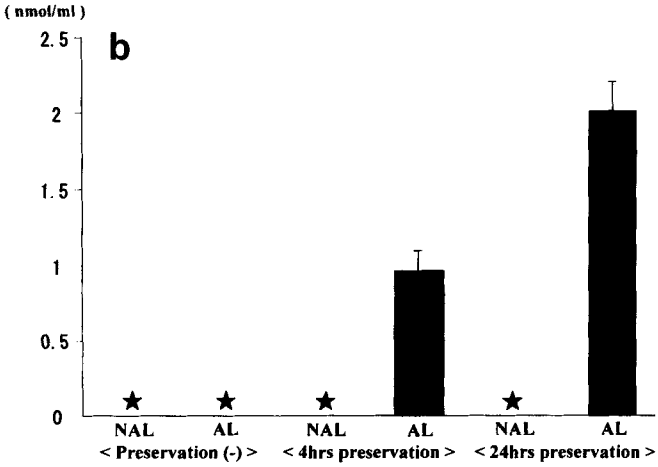
Sudan-III staining showed that fatty change in group g was milder than in group d (Fig. 3d). The stained area in group h was evidently smaller and less than that of group f (Fig. 3e). The HUR values of group g and h were 1.8 ± 0.61 and 0.8 ± 0.19 μg/g per h, respectively, which were not significantly different from the corresponding control groups d and f.

Discussion

In our previous study, we found that impairment of both hepatocytes and SECs in cold-preserved fatty liver, which was induced by CDD, progressed more rapidly and severely than in the non-fatty liver [35]. The liver treated with CDD showed homogeneous fatty changes, whereas ethanol-treated livers were heterogeneous fatty livers because fat deposits accumulated in the centrilobular area, namely zone 3. In coincidence with the degree of fat accumulation, the sinusoidal disorder was more severe during cold preservation in zone 3 than in other zones. More importantly, the sinusoidal endothelium in zone 3 of the alcohol-exposed liver was already injured, even without cold preservation, and more susceptible than that in other areas to cold preservation. Sarphie et al. showed that the morphological alteration of SECs was a gap formation resulting from the fusion of fenestrae in centrilobular sections of the alcohol-treated liver [30]. Their finding is compatible with that in our observation. More interestingly, Haba et al. showed that SEC damage to cold storage with/without reperfusion with Euro-Collins solution was severe in the central vein area and slight in the portal vein area with normal liver models [14]. In addition, Fratte et al. reported that SECs were highly susceptible to cold preservation, even with UW solution [11]. We also recently demonstrated in a morphological study that Kupffer cells (KCs) were activated during cold preservation to induce SEC damage [27]. KCs are considered to be activated also by ethanol consumption, with involvement of endotoxin [8, 12, 37]. Thus, both an alcohol and cold-preserved effect may contribute to SEC injury, especially in zone 3 during cold storage.

The exact mechanism that causes increased fragility of hepatocytes in cold-preserved fatty liver is still unknown. A possible explanation is the involvement of lipid peroxidation. Chronic alcohol feeding, by which fatty liver is promoted, is considered to enhance lipid peroxidation in the liver [7, 28, 29]. Moreover, Dianzani reported that ethanol induced and provoked the





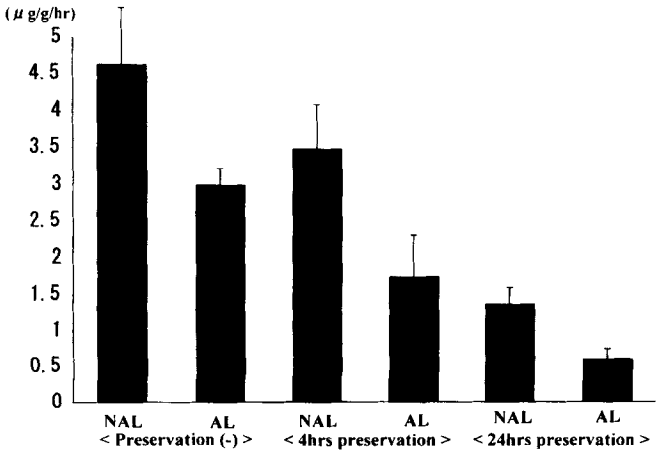


Fig. 5 HUR of livers reperfused for 20 min. *Each point* represents the mean ± SEM. All results represent four experiments ($n=4$) (*NAL* non-alcohol-exposed liver, *AL* alcohol-exposed liver)

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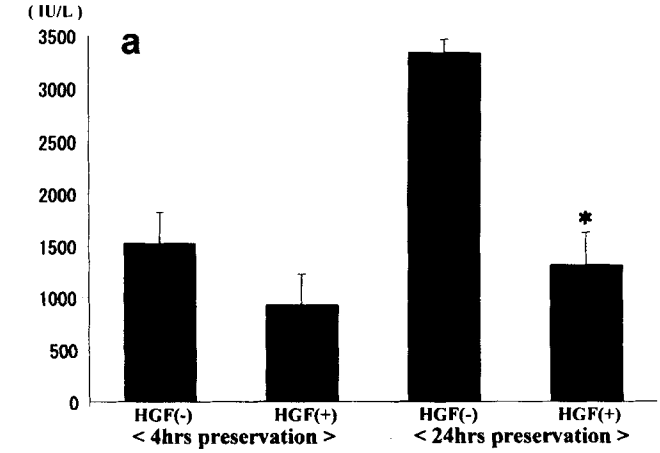
Fig. 4 a LDH release in the first effluent samples obtained from reperfused livers. *Each point* represents the mean ± SEM. All results represent five experiments ($n=5$). $*P<0.05$ vs 4-h-cold-preserved non-alcohol-exposed liver, $**P<0.005$ vs 24-h-cold-preserved non-alcohol-exposed liver. **b** Concentration of peroxidative lipid in the first effluent samples obtained from reperfused livers. *Each point* represents the mean ± SEM. *Black stars* represent undetectable levels. All results represent five experiments ($n=5$) (*NAL* non-alcohol-exposed liver, *AL* alcohol-exposed liver)



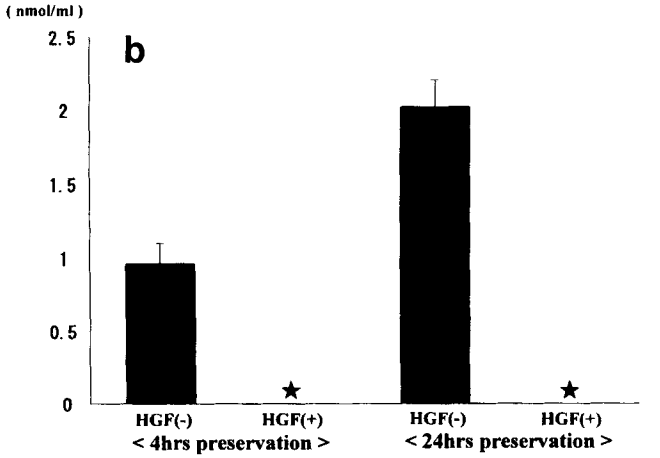


Fig. 6 a LDH release in first effluent samples obtained from reperfused alcohol-exposed livers with and without hrHGF treatment. *Each point* represents the mean ± SEM. All results represent five experiments ($n=5$). $*P<0.005$ vs 24-h-cold-preserved non-alcohol-exposed liver. **b** Peroxidative lipid levels in first effluent samples obtained from reperfused alcohol-exposed livers with and without hrHGF treatment. *Each point* represents the mean ± SEM. *Black stars* represent undetectable levels. All results represent five experiments ($n=5$)

formation of free-radical species and exhausted antioxidant substances in the liver [7]. More interestingly, cold preservation is also thought to stimulate free-radical formation through Kupffer cell activation [4, 40]. Indeed, we found that peroxidative lipids in the first

effluent could be detected only in cold-preserved alcohol-exposed livers and increased during extensive cold storage. It is certain that these values in the first effluent do not indicate the exact amount of peroxidative lipid in hepatocytes, but the cytoplasm membrane is thought to be freely permeable to some substances, such as sodium and chloride ions, adenosine, inosine, and hypoxanthine, during cold storage [2]. Therefore, the detection of peroxidative lipids in the first effluent is likely to express the approximate degree of up-regulated peroxidative lipid in hepatocytes. Thus, such an oxidative reaction through free radicals may be involved in intrahepatocellular injury of ethanol-induced fatty liver after cold preservation, including the expansion of fatty droplets.

We examined the effect of hrHGF in preventing hepatic injury during cold preservation. HGF derived from SECs, KCs, and stellate cells in the sinusoid is a potent growth factor and targets a variety of epithelial and endothelial cells as well as hepatocytes [3, 23, 24, 43]. Since HGF acts on hepatocytes as a strong mitogen and motogen to promote hepatocyte proliferation most potently, it is believed to express regenerative and therapeutic effects on various types of hepatic damage [13, 15, 16, 18, 22, 24]. Previously, we found a preventive effect of hrHGF on hepatic deterioration during cold storage in fatty liver induced by CDD [35]. The administration of hrHGF through the portal vein and in UW solution significantly ameliorated the impairment of both hepatocytes and SECs in that study. In the present study, the addition of the agent in UW solution improved hepatocellular injury during 4 and 24 h of cold preservation, as shown in the decreasing transaminase levels in the first effluent and histological examination. As to the beneficial action of hrHGF on alcohol-induced fatty liver, putative mechanisms can be raised. In a recent study, Tahara et al. demonstrated that HGF administration led to recovery from alcohol-induced fatty liver by enhancing apo B synthesis and subsequent very low density lipoprotein (VLDL) secretion [34]. Furthermore, we found that HGF prevented endotoxin-induced hepatocellular injury [17, 31]. Considering that free-radical formation should be greatly raised in endotoxic injury, hrHGF might contribute to the reduction of the toxic action of free-radical species. Indeed, we showed that peroxidative lipid levels in the first effluent of the groups treated with hrHGF remained at undetectable levels. These functions of hrHGF may act on diminishing hepatocellular damage of alcohol-induced fatty liver during cold preservation.

Contrary to previous reports that hrHGF pretreatment through the portal or systemic vein prevented SEC injury induced by cold preservation or endotoxin [31, 35], we could not obtain clear evidence indicating the beneficial effect on SECs as shown by the HUR. Although the reason for this discrepancy remains unclear, we suppose that these results derive from either a different pathogenesis of the fatty liver or a different method of hrHGF administration. Indeed, there has been some evidence supporting the effect of HGF on endothelial cell damage other than our studies [25]. Furthermore, we found in the preliminary study that the addition of UW solution alone ameliorated SEC injury in cold-preserved human livers (manuscript in preparation).

In conclusion, we presented evidence herein that the impairment of both hepatocytes and SECs developed more rapidly and severely during cold preservation in fatty liver from alcohol exposure than in non-fatty liver, and we suggest the partial involvement of oxidative reaction derived from radical formation. Moreover, we found that the simple addition of hrHGF to UW solution reduced hepatocellular damage of cold-preserved alcohol-exposed fatty liver. These results may provide some benefits for making fatty livers available as grafts, although future investigations are needed to improve the survival of such grafts after liver transplantation.

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