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"Varicoid change" of bile canaliculi in rat liver at an early phase of ischaemia-reperfusion injury

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Abstract To elucidate early changes and the mechanism of ischaemia-reperfusion liver injury, we investigated three-dimensional microstructural changes of cellular actin filaments in rat livers using confocal laser scanning microscopy. The liver tissues of a reperfusion group were examined 12 h after removal of a vascular clamp. Fixed tissues were stained with fluorescein-labelled phalloidin to obtain stereoscopic images of the actin filaments and these were compared with histological findings. The images of bile canaliculi showed that multiple abnormal minute diverticula arose from the canalicular membranes and fused with one another, resulting in irregular dilation of the bile canaliculi. These changes were observed after 15 min of ischaemia and reperfusion in which no significant necrosis was seen. The frequency and degree of these changes were strictly dependent on the periods of ischaemia (15–60 min). We called these bile canaciluar lesions "varicoid changes". The liver of an ischaemia group taken after persistent clamping without reperfusion did not show these changes. Our findings suggest that the varicoid change in the bile canaliculi is probably due to alterations in the actin polymerization-depolymerization cycle and is a pathognomonic change of ischaemia-reperfusion liver injury.

Key words Ischaemia-reperfusion injury
Actin filament · Bile canaliculus
Confocal laser scanning microscopy
3-D microstructure

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Introduction

Liver transplantation surgery has been established as a common therapy for irreversible liver diseases. Early liver graft failure is, however, one of the most important complications [17]. In this graft failure, liver injury induced by ischaemia-reperfusion is believed to play a critical role. The ischaemia-reperfusion injury may result from several processes in the transplantation including donor organ harvesting, cold ischaemic preservation of the graft, warm ischaemia before anastomosis of the hepatic artery and the portal vein, and reperfusion after restoration of blood flow in recipients.

It is well known that temporary occlusion followed by restoration of blood flow to rats liver causes focal or submassive necrosis, depending on the duration of ischaemia [2, 12, 32]. McKeown et al. [22] reported that liver parenchymal cell damage was dominant in warm-ischaemic preservation, and that sinusoidal lining cell abnormalities were the main changes in cold-ischaemic preservation. The changes could be used to predict the viability of the liver graft. Lemasters et al. [20] reported that blebs developed on the cell surface of perfused rat livers with hypoxia-reoxygenation injury. Such blebs were observed in cultured rat hepatocytes treated with cyanide and iodoacetate, agents used to cause chemical hypoxia, resulting in cell death [21]. Recently, several investigators have indicated that injury in the reperfusion phase played a more important role in inducing the graft failure in liver transplantation than injury in ischaemia [5, 15, 35]. No adequate marker for the evaluation of damage in liver cells in ischaemia-reperfusion injury exists.

Actin filaments are abundant at the periphery of the hepatocytic cytoplasm, especially just beneath the cytoplasmic membranes of bile canaliculi. The bile canalicular actin filaments are known to have a specialized arrangement [16] and play an important role in contraction of the bile canaliculi [28, 29]. Dysfunction of the hepatocytic cytoskeleton has been found to be involved in some pathological states of the liver such as cholestasis [1, 37].

Because actin polymerization-depolymerization cycle is an energy-consumptive process [3, 14, 19, 30, 33], deficiencies of high-energy molecules such as adenosine triphosphate (ATP) under ischaemic conditions could unbalance the conversion of monomeric G-actin to polymeric F-actin. On clamping the pedicle of the kidney, proximal tubular cells show disruption of the intracellular actin filaments [18, 24]. The liver might also be expected to exhibit aberrant organization of the actin filaments under ischaemic conditions, but microstructural data on actin filaments in hepatocytes subjected to in vivo ischaemia-reperfusion injury have not been reported. This study was designed to investigate three-dimensional (3-D) changes of the hepatocytic actin filaments around the bile canaliculi in rat livers in an early phase of ischaemia-reperfusion injury, using confocal laser scanning microscopy (CLSM) characterized by its high resolution and depth-discriminating ability [23, 34].

Confocal and reconstructed stereoscopic images showed that reperfusion following ischaemia caused multiple abnormal minute diverticula in the bile canalicular membranes and irregular dilation of the bile canaliculi. We called such characteristic alterations of the bile canaliculi "varicoid changes" because of their morphological resemblance to varices of the lower extremity. The varicoid changes occurred earlier and their distribution was more uniform and extensive than frank histological necrosis. The results indicate that this change is an early microstructural sign of hepatocyte detriment induced by ischaemia-reperfusion injury and that it may be a sensitive marker for evaluating the viability of livers.

Materials and methods

Materials and operations

Fifty male Wistar rats weighing between 200 and 250 g were categorized into three groups and underwent laparotomy under intraperitoneal anaesthesia with sodium pentobarbital (30 mg/kg body weight). Except for the hepato-duodenal ligament, peritoneal membranous attachments to the liver were divided. The rats were maintained at room temperature throughout the duration of experiments, and the livers were handled as infrequently as possible to prevent intrahepatic vasospasm and thrombus formation.

In the reperfusion group (n=30) both the hepatic artery and the portal vein were temporarily clamped with a haemostat at the left main branches to the left lateral and medial lobes as described by Baker [2]. Blood flow into the two left lobes, encompassing approximately 70% of the whole liver, was interrupted; portal blood flowed out via non-clamped lobes without causing intestinal congestion. The bile duct was carefully excluded from clamping to avoid obstructive cholestasis induced by disturbances of bile flow. Reperfusion was induced by gently removing the haemostat after timed ischaemia for 15, 30, or 60 min, and then the abdominal wall was immediately closed. The abdomen was reopened 12 h after declamping and the left lateral lobe was harvested, since our preliminary study using haematoxylin-eosin (H&E) staining had confirmed that the extension of necrotic areas was complete within 12 h reperfusion. The reperfusion group was thus divided into three subgroups based on duration of ischaemia: the 15-min subgroup (n=10), the 30-min subgroup (n=10), and the 60-min subgroup (n=10).

To study the effect of ischaemia alone, left main branches of the hepatic artery and the portal vein were persistently clamped for 12 h without reperfusion in 10 animals. The left lateral lobe was then harvested.

In 10 control animals the left lateral lobe was harvested 12 h after a sham operation including exposure of the hepatic artery and the portal vein, and removal of perihepatic membranous attachments.

Actin fluorescence and H&E stain

A block of 5 mm^3 taken from the central region of each harvested lobe was sliced into thick sections of 200 μm by a rotor-slicer (Dosaka EM, Kyoto, Japan), then fixed with 3.5% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 15 min at room temperature. After washing in PBS, the specimen was stained with fluorescein isothiocyanate (FITC)-phalloidin (Molecular Probes Inc., Eugene, Ore., USA; 1:2 dilution in PBS) for 30 min at room temperature to visualize hepatocytic actin filaments in situ. After washing in PBS, the specimen was embedded on a glass slide with 90% glycerin containing 1% p-phenylenediamine (Sigma, St. Louis, Mo., USA). Four small blocks were simultaneously taken at random from each harvested lobe, then stained with H&E.

Confocal laser scanning microscopy

CLSM (LSM-GB; Olympus Optical Co., Tokyo, Japan) [23, 34] was employed in 3-D observation of hepatocytic actin filaments. Briefly, an argon laser beam (λ=488 nm) focused by an objective lens (SPlan-Apo ×60, NA=1.40 oil; Olympus) was used to excite the specimens. Fluorescent emissions passing through a dichroic mirror (λ=500 nm) were sent to a photomultiplier through a 30-μm pinhole. Each confocal image was generated by 16 times integration of a single frame scan and 2 or 4 time-zooming. The area 5 μm below the surface of each specimen was omitted from imaging to avoid superficial tissue damage. Forty sequential confocal images of intracellular actin filaments were taken at 0.5-μm intervals. These confocal images, so-called microscopic tomograms, were stored in the 512×480×8 bit frame memory of an image processor (Nexus 6400; Nexus Inc., Tokyo, Japan), then reconstructed into stereoscopic images [34].

Results

At relaparotomy, the rats in all three groups were alive and appeared active with neither jaundice nor congestion in the liver and the digestive organs.

Control group

In controls confocal imaging showed abundant hepatocytic actin filaments represented by intense fluorescence just beneath the plasma membranes, especially adjacent to bile canaliculi (Fig. 1A). The canalicular and lateral membrane domains were represented by tubular and thin linear fluorescence, respectively. Thick strips of fluorescence were also observed along the sinusoids in a fluffy pattern. Stereoscopic imaging reconstructed by sequential confocal images exhibited the 3-D microstructures of rat livers including the bile canaliculi, the hepatocellular plates, and the sinusoids (Fig. 1B). Branching of the bile canaliculi extended like ivy along the planes between two or more adjacent hepatocytes. Another stereoscopic image showed that a bile canaliculus appeared smooth

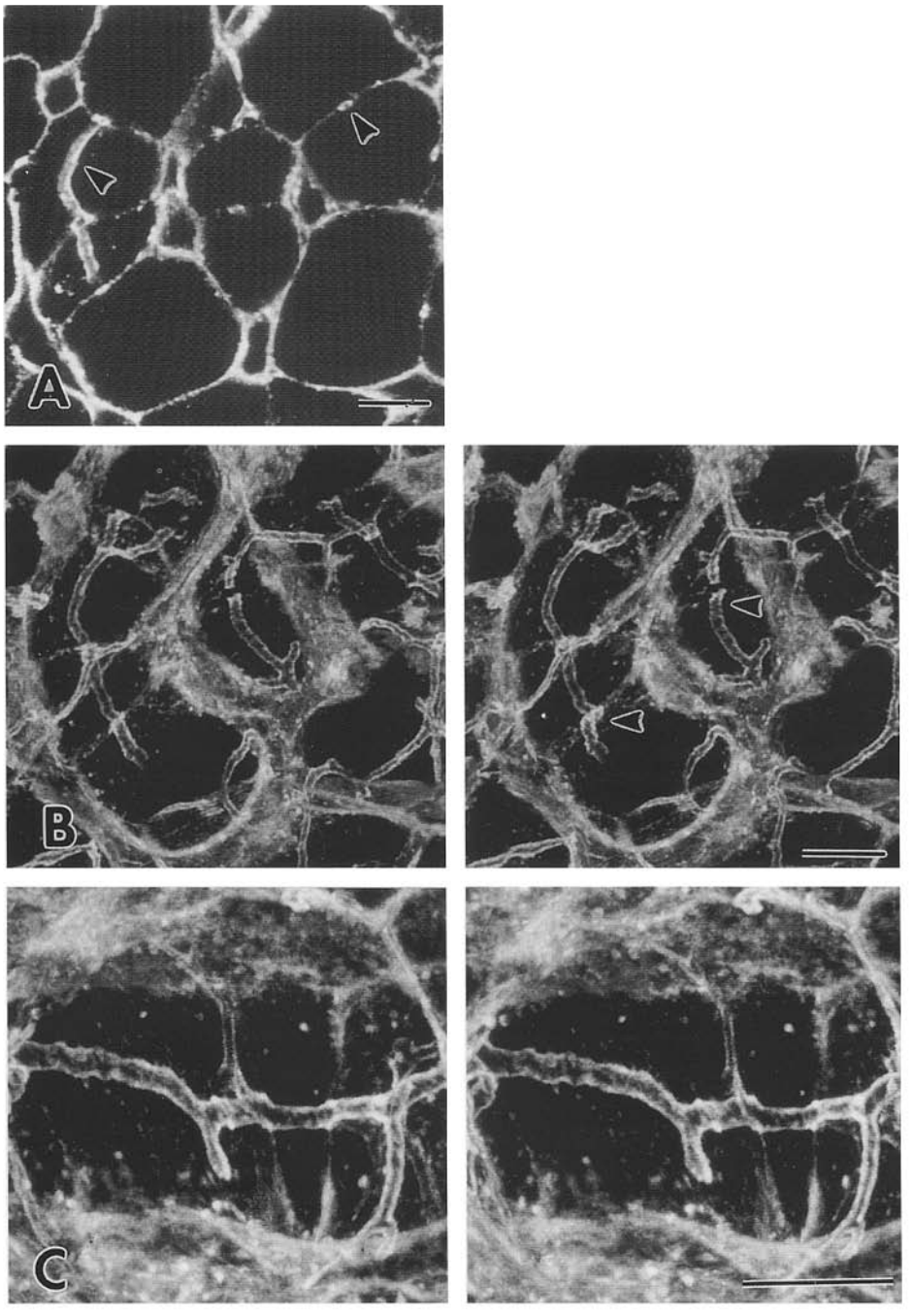

Fig. 1 Confocal (**A**) and stereoscopic (**B**, **C**) fluorescence microscopy images of actin filaments in control rat hepatocytes. **A** Optically sectioned bile canaliculi are represented as tubular fluorescence (*arrowheads*). Thin linear fluorescence beneath the hepatocytic plasma membranes and thick strips of fluorescence along the sinusoids are also shown. **B** An image reconstructed by sequential images including **A**. Bile canaliculi (*arrowheads*) branch out in an ivy-like configuration between two or more adjacent hepatocytes. **C** Another image showing a smooth and slender bile canaliculus with a uniform diameter of less than 2 μm. Objective lens: ×60 (**A**, **C**); zoom ratio: ×2 (**A**, **B**), ×4 (**C**); reconstruction with 40 sequential confocal images taken at 0.5-μm intervals (**B**, **C**). *Bars* 10 μm

and slender with a uniform diameter of less than 2 μm (Fig. 1C).

Reperfusion group

Focal necrosis was characteristic of the reperfusion group, as reported in previous studies [2, 12, 32]. Immediately after clamping, the two left hepatic lobes became pale and were well demarcated from non-clamped lobes. They returned to a completely normal colour after declamping. The clamped lobes in the 15-min reperfusion subgroup showed no macroscopic abnormalities 12 h after declamping, while those in the 30- and 60-min subgroups showed discrete whitish foci of necrosis.

Foci of hepatocellular necrosis with a neutrophil infiltration were detected microscopically in 24, 35, and 40 sections out of 40 in the 15-, 30-, and 60-min subgroups, respectively. There was a marked difference in the extent of the necrotic areas between the 15-min subgroup and both the 30- and 60-min subgroups; only a few small necrotic foci were present in superficial regions of the livers just below the capsules in the 15-min subgroup, while larger necrotic foci appeared in deep regions in the

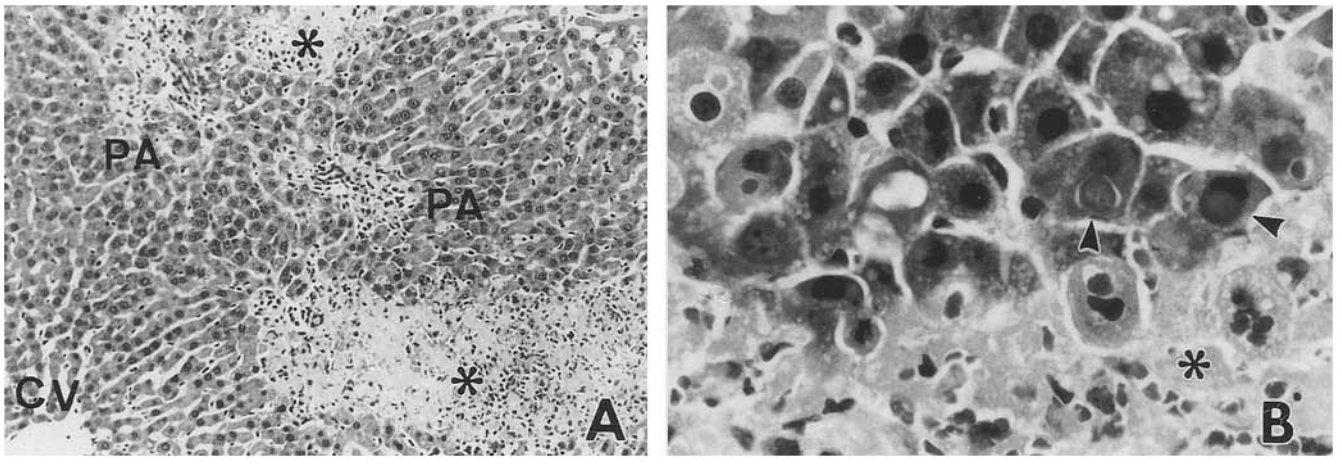

Fig. 2 H&E-stained liver tissues of the 30-(**A**) and 60-(**B**) min subgroups of the reperfusion group. **A** Foci of focal necrosis (*asterisks*) between portal areas (*PA*) and a central vein (*CV*), well demarcated from surviving hepatocyte zones. **B** A peripheral necrotic region (*asterisk*) and a surrounding bionecrotic zone. Most bionecrotic hepatocytes appear detached from one another, sometimes including large intracytoplasmic droplets (*arrowheads*). Objective lens: ×10 (**A**), ×40 (**B**)

30-min subgroup (Fig. 2A), or throughout the entire affected hepatic lobes in the 60 min subgroup. Necrotic foci were demarcated from the surviving hepatocytes by bionecrotic zones, in which most hepatocytes appeared detached from one another, sometimes including large intracytoplasmic droplets (Fig. 2B), as observed in partial hepatectomy [7, 25]. Neither microthrombi nor indicators of cholestasis such as intracanalicular bile plugs were seen in any section. H&E staining did not show contours of bile canaliculi in the livers subjected to ischaemia-reperfusion injury, nor in the control livers.

Stereoscopic imaging of hepatocytic actin filaments from a specimen in the 15-min subgroup showed scattered but frequent deformities of bile canaliculi (Fig. 3A). Multiple minute diverticula of approximately 1 μm in diameter with high fluorescence intensity projected from the bile canalicular membranes to the surrounding ectoplasm. The outer surfaces of these minute diverticula attached to the canalicular membranes and their continuity was never interrupted. There was no isolated structure like a vesicle around the bile canaliculi in the cytoplasm. Minute diverticula appeared to fuse with one another, resulting in irregular dilation of the bile canaliculi. We called these characteristic changes of the bile canaliculi "varicoid changes". The varicoid changes were uniformly scattered throughout the affected liver tissue, even in deep regions far from the liver capsule where necrosis was not seen. The distribution of these varicoid changes was independent of three zones in the hepatic acinus.

Stereoscopic imaging of a specimen in the 30-min subgroups showed marked varicoid changes in a bile canaliculus resembling a cluster of grapes with a rough surface and irregular dilation up to approximately 7 μm in diameter (Fig. 3B). In contrast to the fluorescence intensity around the bile canaliculi, that along both the lateral and sinusoidal membranes was much weaker. The varicoid changes were detected throughout the livers in all specimens. In necrotic foci, arrangements of the actin filaments beneath the canalicular, lateral, and sinusoidal membranes were markedly disrupted and became speckled in appearance.

Stereoscopic imaging of the 60-min subgroup showed advanced varicoid changes in a bile canaliculus resembling a honeycomb structure with a cavernous surface and a huge diameter of approximately 14 μm (Fig. 3C). Actin filaments around bile canaliculi showed irregular but enhanced fluorescence. Observation by CLSM confirmed that spatial continuity of canalicular membranes in the varicoid changes was well preserved, and that the multiple abnormal minute diverticula attached to the canalicular membranes. Both the frequency and degree of the changes were clearly dependent on duration of ischaemia. They were uniformly and extensively scattered throughout the livers in all sections taken from the 15-, 30-, and 60-min subgroups of the reperfusion group, even in histologically intact regions far from the areas of necrosis.

Ischemia group

In livers from the ischaemic group each lobe clamped for 12 h became pale, soft, and friable. The tissues showed no obvious areas of necrosis, but uniform degenerative changes of hepatocytes including hyperchromatic nuclei and eosinophilic cytoplasm were observed (Fig. 4A). Although hepatocellular plates became a little thinner, the architecture of parenchymal cells was well maintained. Bile canaliculi were not detected in the H&E-stained sections.

Stereoscopic imaging of actin filaments demonstrated that each bile canaliculus was smooth and slender without any signs of the varicoid changes (Fig. 4B). Some bile canaliculi appeared regionally stretched and rigidified with sporadic discontinuities. In contrast to the clear fluorescence around the bile canaliculi, both the lateral

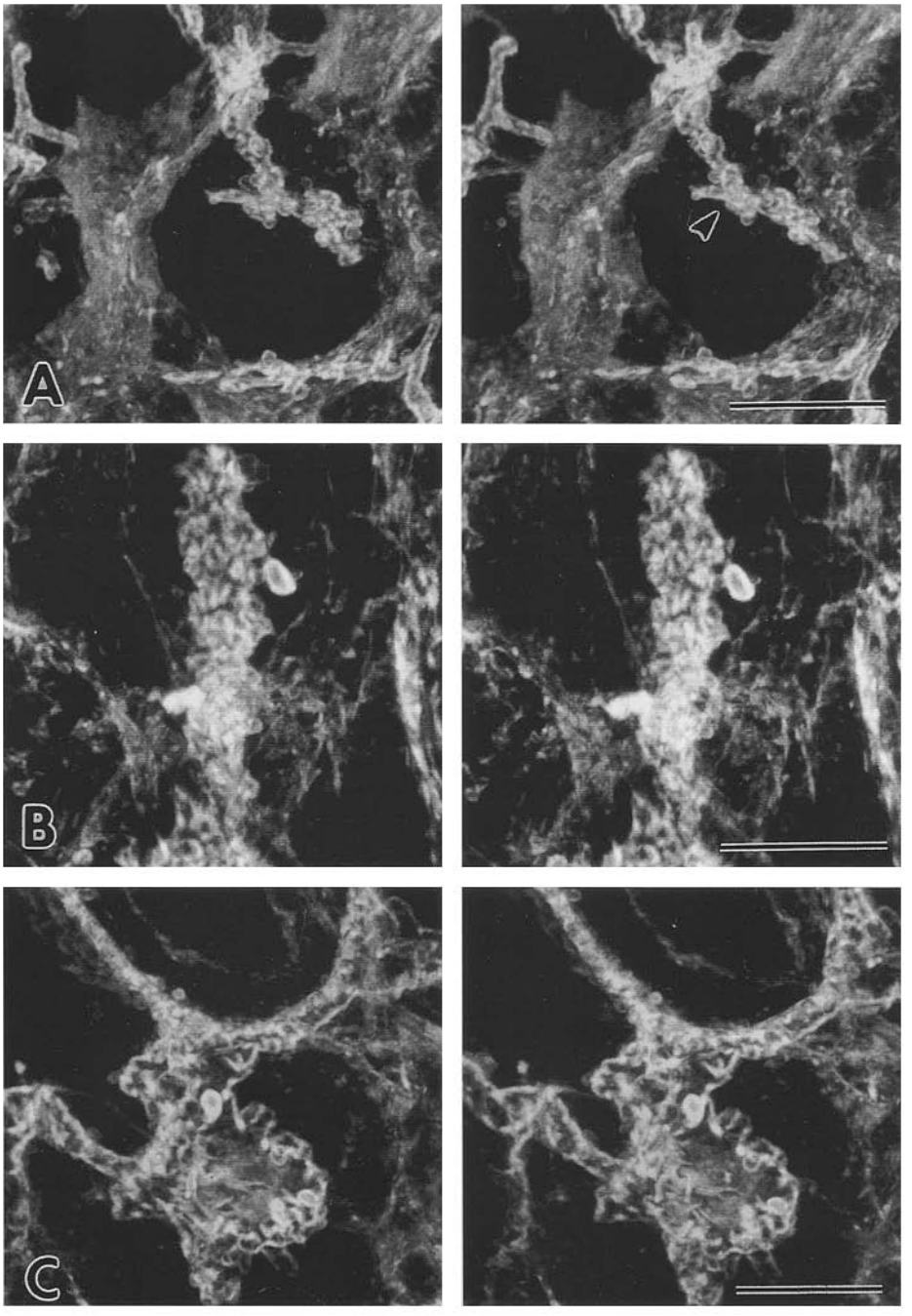

Fig. 3 Stereoscopic fluorescence images of actin filaments taken from the 15-(**A**), 30-(**B**), and 60-(**C**) min subgroups of the reperfusion group. **A** Bile canaliculi show mild but frequent varicoid changes (*arrowhead*): multiple intensely fluorescent minute diverticula arise from bile canalicular membranes to surrounding ectoplasm, fusing with one another and resulting in irregular dilation of bile canaliculi. **B** A bile canaliculus shows marked varicoid changes resembling a cluster of grapes with a rough surface and a large diameter of 7 μm. The lateral and sinusoidal membranes are decreased in fluorescence intensity. **C** A bile canaliculus shows advanced varicoid changes resembling a honeycomb structure with a cavernous surface and a huge diameter of 14 μm. Objective lens: ×60 (**A–C**); zoom ratio: ×4 (**A–C**); reconstruction with 40 (**A–C**) sequential confocal images taken at 0.5-μm intervals. *Bars* 10 μm

and sinusoidal membrane domains were markedly decreased in fluorescence intensity, showing a variegate pattern.

Discussion

In the present study, we detected characteristic changes of actin filaments around bile canaliculi; varicoid changes in all cases of rat livers subjected to ischaemia-reperfusion injury. These changes had unique microstructural features: an irregular increase in fluorescence intensity of actin filaments around bile canaliculi, multiple minute diverticula arising from bile canalicular membranes and extending to the surrounding ectoplasm, and marked irregular dilation of bile canaliculi.

It was certain that the varicoid changes did not result from artificial or direct physical factors involved in the experimental procedures. To prevent disturbances in extrahepatic bile flow due to increased intracanalicular hydrostatic pressure, we took special care to avoid inadvertently clamping the common bile duct. Persistent outflow of portal blood via the patent right main branches of the portal vein during clamp-induced ischaemia avoided re-

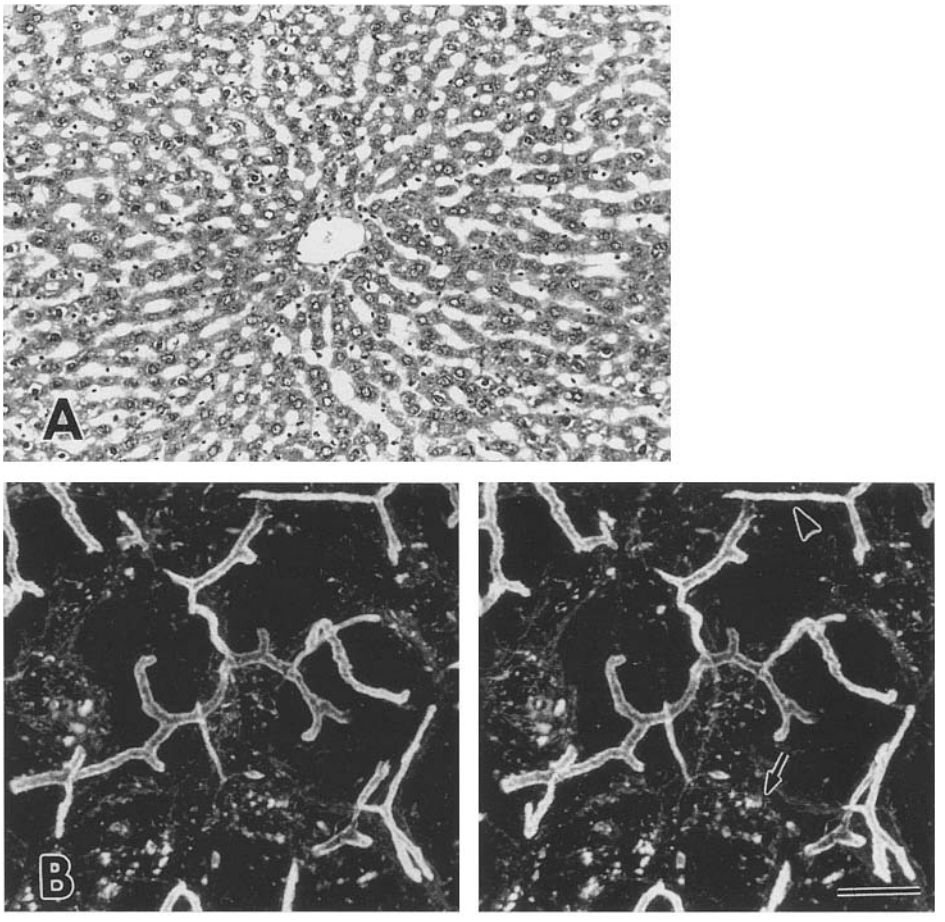

Fig. 4 H&E-stained liver tissue (**A**) and a stereoscopic fluorescence image of actin filaments (**B**) taken from the ischaemic group. **A** Hepatocytes show uniform degenerative changes including hyperchromatic nuclei and an eosinophilic cytoplasm. Although the hepatocellular plates become a little thinner, the architecture of parenchymal cells is well maintained. Objective lens: ×10. **B** Bile canaliculi are smooth and slender without any signs of varicoid changes, although some of them appear regionally stretched and rigidified (*arrowhead*) with sporadic discontinuities. Both the lateral and sinusoidal membranes are markedly decreased in fluorescence intensity, exhibiting a variegate pattern (*arrow*). Objective lens: ×60; zoom ratio: ×2; reconstruction with 40 sequential confocal images at 0.5-μm intervals. *Bar* 10 μm

flow of congested blood containing endotoxin into the ischaemic hepatic lobe during the reperfusion phase. In addition, imaging by CLSM allowed examination of thick liver sections of 200 μm, which required only simple preparations, and provided microscopic tomograms with very little artificial distortion [23, 34].

The varicoid change is an abnormal 3-D microstructural phenomenon in bile canaliculi embedded in the liver. Depth-discriminating ability is necessary to detect the phenomenon in a thick tissue section. Neither conventional light microscopy nor electron microscopy is adequate. It was extremely difficult to distinguish varicoid change from the sinusoidal lumen by conventional light microscopy (data not shown). Moreover, ultrastructural changes of the canalicular membrane and pericanalicular actin filaments were not available, since the changes were much less frequent than intact bile canaliculi.

Compared with data from present and previous histologic studies [2, 12, 32] varicoid changes of bile canaliculi possessed high sensitivity and specificity for ischaemia-reperfusion liver injury. It has been reported that the minimum interval of ischaemia required to induce definite necrosis in rat livers prior to reperfusion has been determined to be 30 min by H&E staining [2], 40 min by gallocyanin staining [12], and 60 min by triphenyltetrazolium chloride assay [32]. The present 3-D microstructural study by CLSM demonstrated uniform and extensive distribution of the varicoid changes throughout the livers in all specimens obtained after 15 min ischaemia followed by reperfusion. In this 15-min subgroup of the reperfusion group, only a few small necrotic foci were observed in superficial regions of the livers. The cause of the necrosis in the 15-min subgroup has been suspected to be the inevitable vasospasm induced by trauma during the handling of the livers, as reported previously [2]. In the cases with 30 and 60 min of ischaemic followed by reperfusion, marked varicoid changes were observed throughout the livers in all specimens, even in histologically intact regions far from necrotic foci. Both the frequency and degree of the changes were clearly dependent on the duration of ischaemia. These findings indicate that varicoid change in the bile canaliculi is an early microstructural sign of the hepatocytic damage leading to cell death, while histological necrosis becomes detectable only some time after cell death.

The varicoid change induced by ischaemia-reperfusion is morphologically similar to the bile canalicular changes in livers of the rats administered phalloidin daily for 3–7 days before sacrifice [8, 26, 40] or in living cultured hepatocytes poisoned by the addition phalloidin to the culture medium for 20–100 min [36]. Phalloidin, when administered to living liver cells, act as an agent that directly accelerates the polymerization of actin and inhibits its depolymerization [38], so that the actin fila-

ments lose the dynamism of polymerization. In the present study, the phalloidin was used only to stain the liver tissues after paraformaldehyde fixation. Points of similarity between the present ischaemia-reperfusion study by CLSM and the previous phalloidin-intoxication study [36] are the following: the density of actin filaments around bile canaliculi was irregularly increased; abnormal minute diverticula arose from bile canalicular membranes, resulting in irregular dilation of bile canaliculi; microstructural changes in the canalicular membrane domain appeared much earlier and were more pronounced than those in the lateral and sinusoidal domains; and the frequency and degree of bile canalicular changes were clearly dependent on the duration of ischaemia or doses of phalloidin. These striking similarities, therefore, suggest that the varicoid change is really caused by a disturbance of the actin polymerization-depolymerization cycle.

In addition, disorders of the actin polymerization-depolymerization cycle are inferred to be a critical cause of this change by the following evidence: actin polymerization is known to be regulated by the intracellular energy level [3, 14, 18, 19, 24, 30, 33] and the intracellular ATP concentration was rapid decreased in both ischaemic livers and hypoxic hepatocytes [9, 10]. Exhaustion of intracellular ATP during ischaemia should unbalance the conversion of G-actin to F-actin in the microfilaments, supporting the notion that disorders of actin organization around bile canaliculi play an important role in inducing the varicoid change.

The present study, however, raised a paradox; 12 h of ischaemic without reperfusion resulted in mild changes in the bile canaliculi and rather severe changes in the lateral and sinusoidal membranes, while brief ischaemia (15–60 min) followed by reperfusion caused the characteristic varicoid changes localized in the bile canaliculi within 4 h after initiation of the reperfusion (data not shown). The intracellular ATP level should be lower in the ischaemic phase than in the reperfusion phase. Stretched and rigid bile canaliculi seen in ischaemia are thought to be secondary changes induced by swelling of surrounding hepatocytes due to deficient supply of metabolic energy [11]. Mild hepatocellular swelling was really detected in biopsy samples of cold-preserved grafts in liver transplantation [17]. Hepatocytic damage in the ischaemic phase should nevertheless be important in formation of varicoid changes, because the frequency and degree of the changes correlated with the duration of ischaemia. These results strongly suggest that varicoid change is caused by disorders of the actin polymerization-depolymerization cycle around the bile canaliculi, and that both intracellular ATP depletion in the ischaemic phase and additional unidentified factors in the reperfusion phase are essential for the production of this change.

Reperfusion may accelerate is ischaemic damage of hepatocytes by mechanisms such as excessive influx of extracellular calcium ions [27], oxidative stress [13], or reflow of endotoxin-rich blood [31]. Cellular free calcium ion and calcium-binding proteins are known to produce significant effects on the organization of actin filaments [6] or on interactions between actin and actin-binding proteins [4]. Our previous study using cultured primary rat hepatocytes showed that characteristic deformities of bile canaliculi just like these varicoid changes were formed at low pH [39]. Further investigations of the kinetics of hepatocytic actin polymerization-depolymerization and the actin-binding proteins are necessary to understand the precise mechanism. Our observation suggests that varicoid change of the bile canaliculi is pathognomonic of an early phase of ischaemia-reperfusion liver injury. It has a high potential as a sensitive marker to evaluate damage to hepatocytes in predicting the viability of grafts in liver transplantation.

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