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ORIGINAL ARTICLE

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Sequential changes in intercellular junctions between hepatocytes during the course of acute liver injury and restoration after thioacetamide treatment

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Abstract Sequential changes of gap junctions (GJs), tight junctions (TJs) and desmosomes (DSs) between hepatocytes during restorative proliferation were studied in rats after a single intraperitoneal administration of 200 mg/kg thioacetamide (TAA). Antibody against connexin 32 was used to demonstrate GJs; simultaneously the changes in TJs and DSs were studied using antibodies against 7H6 protein and desmoplakins. Propidium iodide and bromodeoxyuridine were used to recognize necrotic and proliferative cells. GJs were evenly distributed in early necrotic hepatocytes at 16 h after TAA treatment, then disappeared from necrotic and surrounding cells at 24 h. At 48 h, GJs had disappeared completely from hepatocytes in whole liver lobules, while many hepatocytes were heavily labelled with BrdU. At 72 h, GJs reappeared, firstly in perinecrotic areas. At 96 h after treatment, when the injured areas had disappeared and restorative proliferation ceased, GJs were distributed evenly throughout the lobules. Immunohistochemical observation of GJs in centrilobular, perinecrotic and periportal areas after TAA-induced hepatic necrosis was confirmed by counting the number of connexin-32-positive spots in the respective areas. TJs and DSs disappeared from necrotic cells at 24 h, but then increased between 24 and 48 h in perinecrotic areas, though the increased intensity of these junctions was more evident at 48 h. At 72 h, localization of TJs and DSs returned to normal. These results suggest that during the course of acute hepatic injury, GJs (cell-cell communication) behave differently from other intercellular junctions.

Key words Gap junctions · Tight junctions
Desmosomes · Acute liver injury · Thioacetamid

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Introduction

Epithelial cells are connected with each other cells by intercellular junctional complexes, consisting of gap junctions (GJs), desmosomes (DSs), tight junctions (TJs) and adherence junctions. These junctions may play different roles in the maintenance and regulation of tissues [2, 4]. GJs mediate intercellular communication between cells by allowing inorganic ions and other small water-soluble molecules to pass from the cytoplasm of one cell to the adjacent cells [15]. It has been shown that GJs change dramatically in regenerating liver following partical hepatectomy as well as in the course of hepatocarcinogenesis in rats [14, 19]. After injury to individual cells in tissues, it has been speculated that there is cell-cell communication via GJs between the damaged and intact cells [16], but the changes in GJs during acute liver injury and restorative proliferation have not been studied. Changes in the junctional complex during cholestasis induced by bile duct ligation, and chronic administration of thioacetamide (TAA) were investigated by mean of electron microscopy in ultrathin sections or freeze-fracture replicas [6, 7, 9, 20], but these studies are not quantitative, due to strict limitation of areas to be investigated, and therefore detailed comparison between the GJs in injured and regenerating cells was technically difficult.

We examined sequential changes in GJs in the liver of the rat after TAA treatment. Cells were studied semiquantitatively in the course of injury and during restorative proliferation using immunohistochemistry.

Materials and methods

Male Fischer 344 rats (Charles River Japan, Kanagawa, Japan) weighing about 200 g were fed a laboratory chew (MF; Oriental Yeast Col,, Tokyo, Japan) and given water ad libitum. Animals were maintained at 22±1° C and subjected to a standard 12 h light-dark schedule (9 a.m.–9 p.m.).

Twenty-one rats were injected intraperitoneally with TAA (Sigma, St. Louis, Mo., USA) dissolved in 0.9% NaCl at a dose of 200 mg/kg body weight [3]. Three rats were sacrificed 0, 16, 24,

48, 72, 96, and 168 h after the injection. All animals were intraperitoneally administered bromodeoxyuridine (BrdU; 100 mg/kg body weight, Dako, Copenhagen, Denmark) 1 h before sacrifice. To identify dead cells, the liver was perfused through the portal vein with Dulbecco's modified Eagle's medium (Kyokuto, Tokyo, Japan) containing 10 mg/ml propidium iodide (PI, Sigma) for 10 min, 16 and 24 h after TAA injection.

After perfusion for 10 min, 2- to 3-mm-thick liver slices were made. They were further fixed in buffered 20% formalin, embedded in paraffin, sectioned into 4-μm-thick slices, and stained with haematoxylin and eosin.

For immunohistochemistry liver slices, 2–3 mm thick, were frozen in liquid nitrogen, and frozen sections were made with a cryostat. The sections were fixed with acetone for 30 min at –20° C. After rinsing in phosphate-buffered saline (PBS), the sections were first incubated at room temperature (RT) for 1 h with either rabbit anti-J-peptide antiserum against connexin 32 (Cx32) [14], mouse 7H6 monoclonal antibody [22] recognizing a 155-kDa tight junction protein, mouse anti-desmoplakin I/II monoclonal antibody (Boehringer Mannheim, Mannheim, Germany). After washing in PBS, the sections were incubated with FITC-conjugated anti-rabbit IgG or anti-mouse IgG (Dako, Copenhagen, Denmark) at RT for 1 h. Some sections were used for double staining for Cx32 and BrdU by utilizing mouse anti-BrdU monoclonal antibody (Amersham, Buckinghamshire, UK). After immunostaining with Cx32 antibody, five randomly selected areas per rat liver were photographed at a magnification of 90. The number of Cx32-positive spots on hepatocyte plasma membranes was counted using an image analysis system (LA-555; Pias, Osaka, Japan) as previously reported [14].

Results

Immunohistochemical examinations of GJs, TJs and DSs in normal rat liver showed that these junctions were uniformly distributed throughout the liver lobules. Regarding patterns of immunolocalization in hepatocytes, Cx32 was observed as macular spots at the cell border, whereas 7H6 and desmoplakin I/II were observed along the bile canaliculi between adjacent hepatocytes (Fig. 1a, b and c, respectively).

Microscopic alterations in the livers were observed 16, 24, 48 and 72 h after TAA injection. At 16 h, centrilobular necrosis was observed (Fig. 2a). At 24 h, extensive necrosis was observed in the central vein area (CV) (Fig. 3a). At 48 h, necrosis still remained in the CV (Fig. 3b). At 72 h, necrotic areas were reduced in the CV and many mitoses of hepatocytes were observed evenly in entire liver lobules (Fig. 3c). By 96 h, these changes disappeared and hepatic tissues mostly recovered to normal conditions.

At 16 h after TAA injection, centrilobular necrosis was clearly demonstrated by PI incorporation [17]. Cx32-positive spots were distributed at the cell border of hepatocytes located in the necrotic area (Fig. 2b). In the PI-positive hepatocytes, Cx32-positive spots were still observed (Fig. 2c). At 24 h, in the necrotic lesions, the number of Cx32-positive spots decreased (Fig. 4a, b). Similarly, there were fewer GJs in hepatocytes located in the areas surrounding the necrotic lesions. In hepatocytes located in the periportal areas, however, the number of GJs was in the normal range. At 48 h, approximately 30% of the hepatocyte nuclei were positive for BrdU. BrdU-positive cells were seen throughout the normal-looking areas, including the areas surrounding the necrotic lesions (Fig. 4c, d). Cx32-positive spots were virtually absent in the entire lobule at 48 h after TAA treatment. At 72 h, Cx32-positive spots reappeared in the areas surrounding the necrotic lesions, while the number of GJs in necrotic lesions and the periportal areas still decreased (Fig. 4e, f). By 96 h, the number and distribution of GJs returned to normal. Figure 5 summarizes changes in the number of GJs in the hepatocytes of three areas; central areas, perinecrotic areas and periportal areas.

TJs and DSs showed similar alterations in respone to TAA-induced liver injury. At 16 h, they were observed in

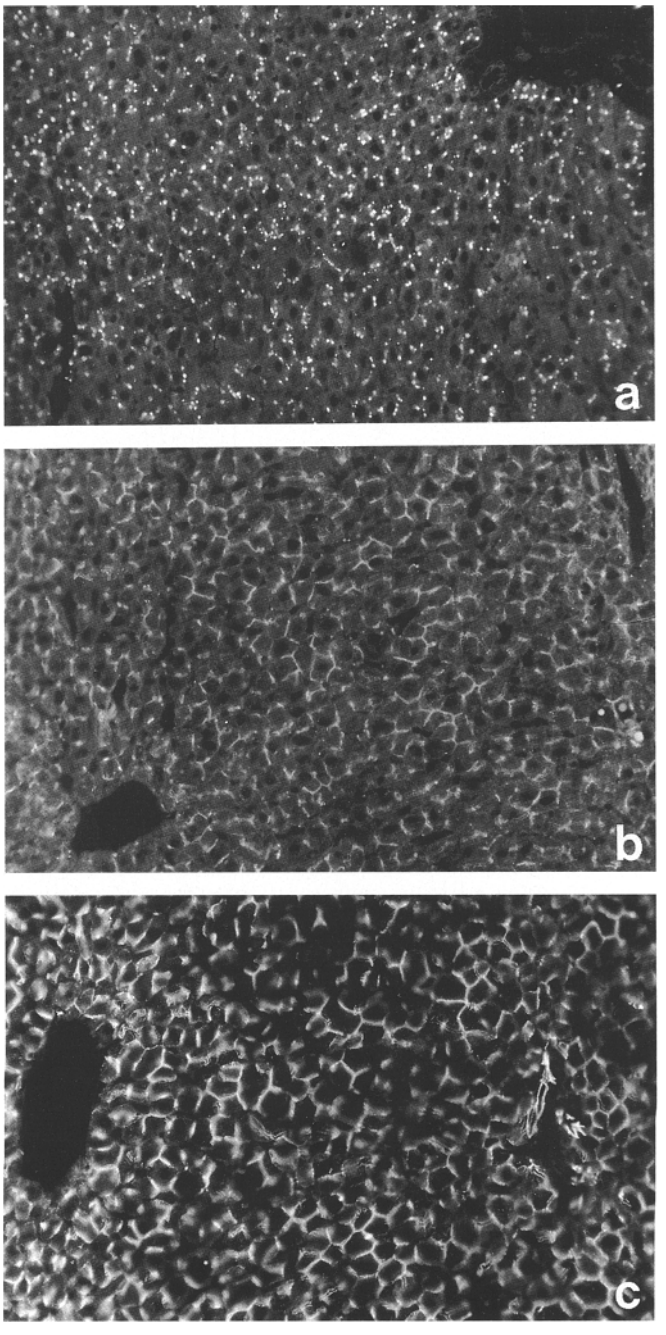


Fig. 1 Immunofluorescent staining of connexin 32 (Cx32) (**a**), 7H6 (**b**) and desmoplakin I/II (**c**) of normal livers. ×90

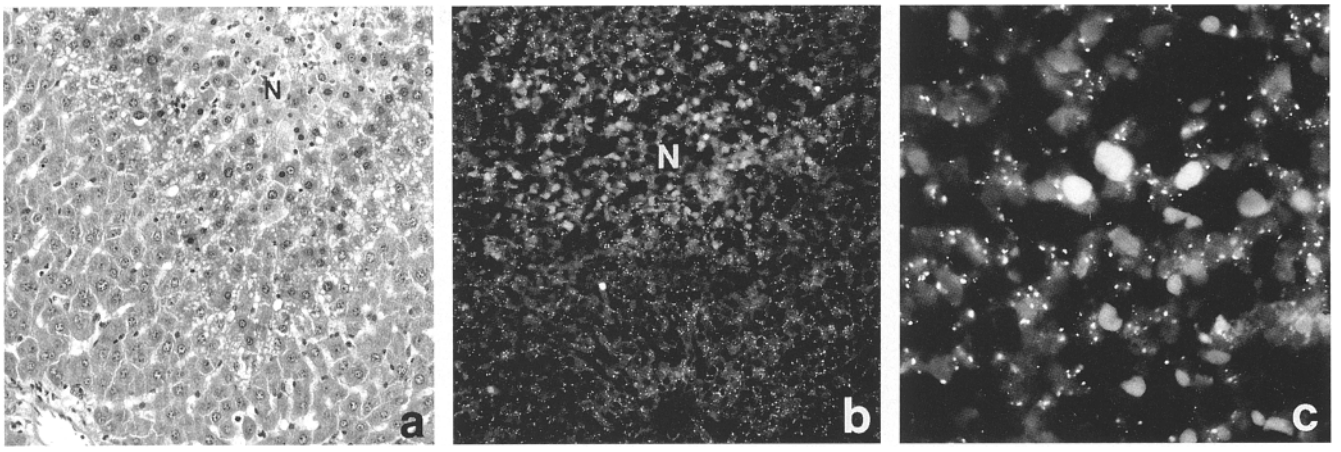
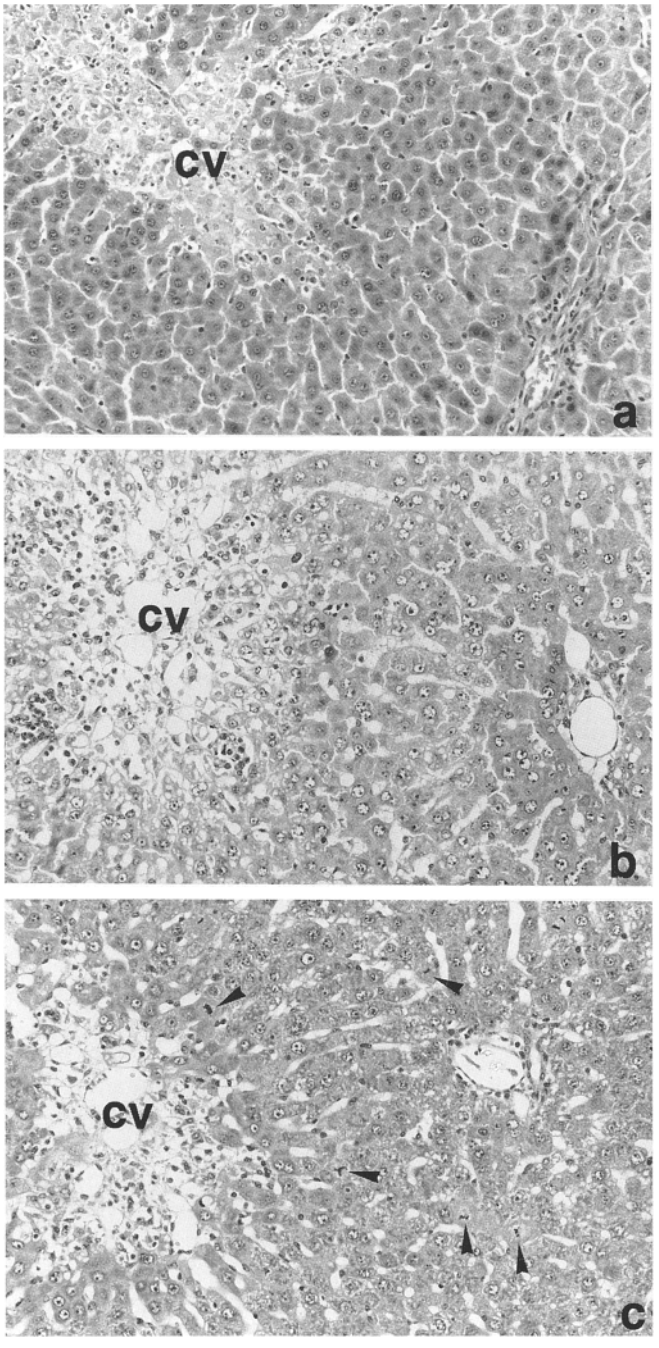


Fig. 2a–c Rat livers 16 h after treatment with thioacetamide (TAA). **a** Centrilobular necrosis is observed. H&E, ×50. **b** Double immunofluorescent staining of Cx32 and propidium iodide (PI). Cx32-positive spots are observed in PI-positive cells. ×50. **c** Higher magnification of **a**. ×180



hepatocytes located in both the damaged and normal areas. At 24 h, the number of these junctions was decreased in the necrotic lesions, whereas at 24 and 48 h, their numbers and immunofluorescence intensities were increased in the areas surrounding the necrotic lesions (Figs. 6a, b, 7a, b). In these areas, dilated bile canaliculi lined with TJs and DSs were often observed (Figs. 6b, 7b). By 96 h, the distribution and number of these junctions had returned to normal.

Discussion

We have demonstrated that the changes in expression of Cx32 were strikingly different from markers for TJs and DSs during the course of acute hepatic injury and regeneration after a single dose of TAA. In the early phases of injury after TAA treatment (before 16 h), the number and localization of GJs did not change even in the dead hepatocytes identified by PI. In dead cells, the existence of immunohistochemically demonstrable GJ may not indicate functionally intact GJs per se, because a rapid and dramatic increase in the cytoplasmic Ca concentration is thought to close the gap junctional channels [11, 13, 16]. In the later phase of injury (around 24 h), reduction in the number of GJs occurred preferentially in the areas adjacent to the necrotic lesions. Since no BrdU labelling was observed in these cells, it was suggested that such reduction of intercellular communication is not associated with cell proliferation but rather the adaptation of surrounding hepatocytes against harmful substances flooding in from necrotic hepatocytes via GJs. In the late

Fig. 3 Rat livers 24 (**a**), 48 (**b**) and 72 (**c**) h after treatment with TAA. **a** Necrosis is observed in the central vein area (*CV*). **b** Necrosis still exists in the CV. **c** Necrotic areas reduced in the CV and many mitoses (*arrowheads*) of hepatocytes are evenly observed in entire liver lobules. H&E, ×90

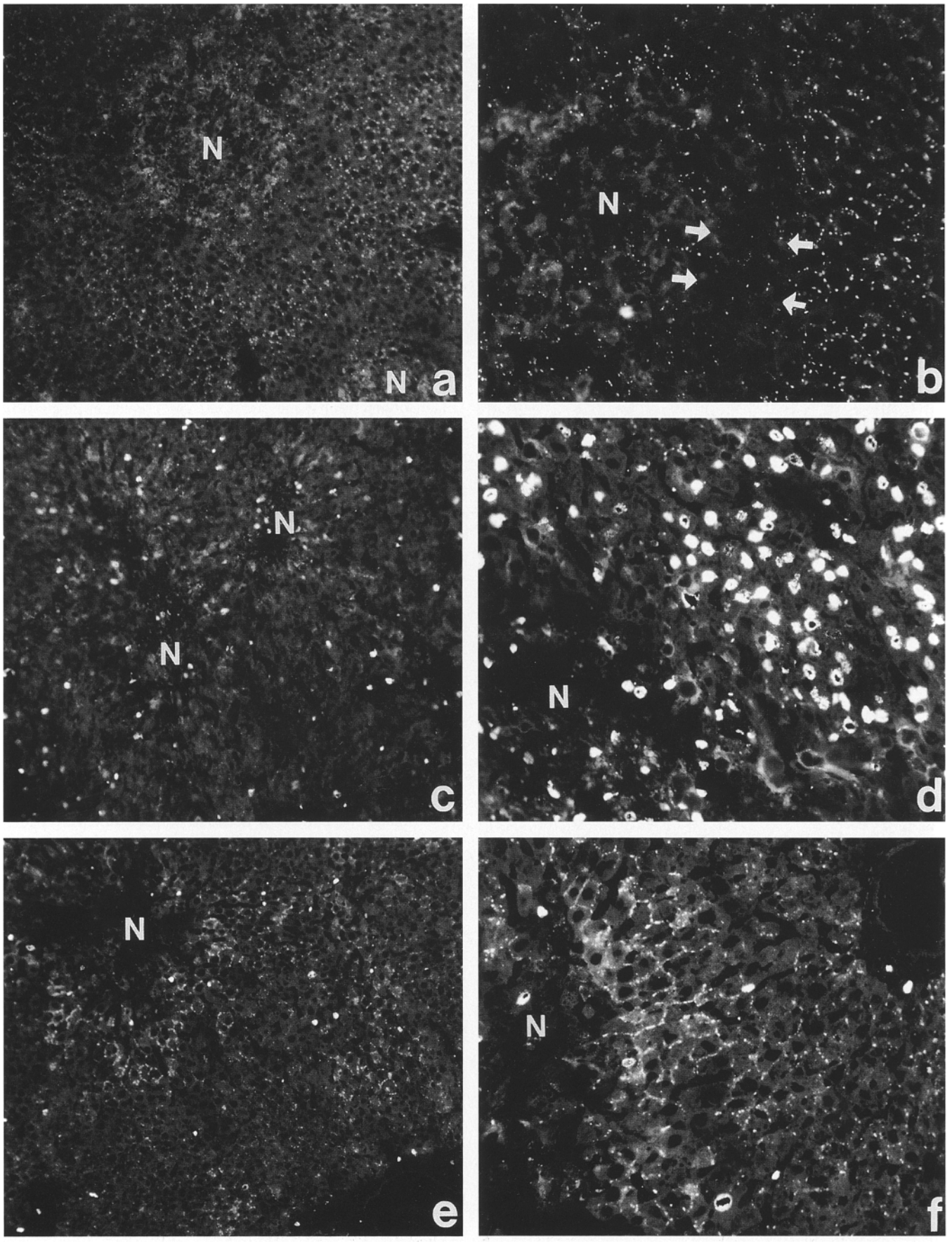


Fig. 4 Double immunofluorescent staining of Cx32 and bromodeoxyuridine (BrdU) of livers 24 (**a**, **b**), 48 (**c**, **d**) and 72 (**e**, **f**) h after treatment with TAA. **a** The number of Cx32-positive spots zonally decreases in necrotic lesions and the areas surrounding necrosis. ×50. **b** Higher magnification of **a**. Cx32-positive spots decrease zonally in the areas surrounding necrosis (*arrow*). ×90. **c** Cx32-positive spots mostly disappear and many BrdU-positive cells are found in the same area. ×50. **d** Higher magnification of **c**. ×90. **e** Cx32-positive spots reappeared in the area surrounding necrosis. ×50. **f** Higher magnification of **d**. ×90. *N* Necrotic lesions

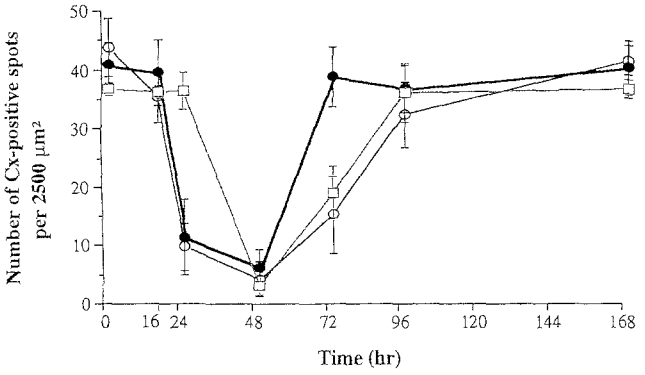


Fig. 5 Changes in the number of Cx32-positive spots in the areas after treatment with TAA. ○ central area, ● perinecrotic area, □ periportal area

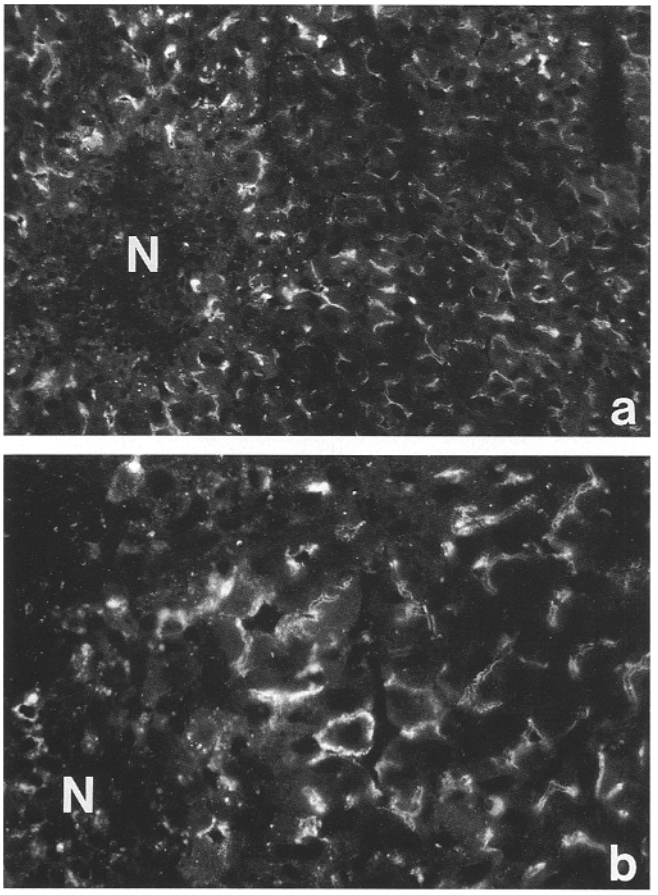


Fig. 6a, b Immunofluorescent staining of 7H6 of livers 48 h after treatment with TAA. **a** An increase in immunoreaction of 7H6 is observed in areas surrounding necrosis. ×90. **b** Higher magnification of **a**. × 180. *N* Necrotic lesions

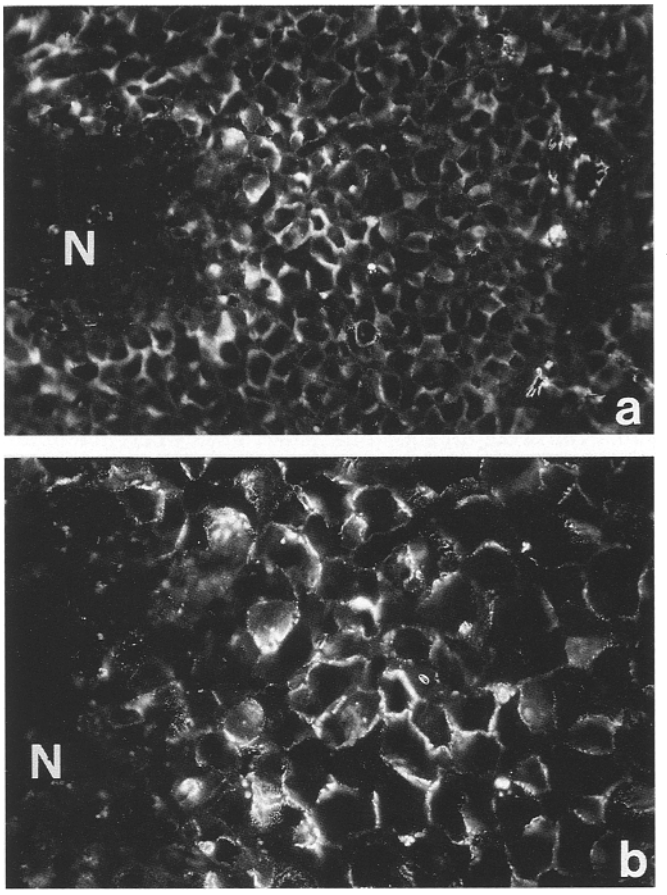


Fig. 7a, b Immunofluorescent staining of desmoplakin I/II of livers 48 h after treatment with TAA. **a** An increase in immunoreaction of desmoplakin I/II is observed in areas surrounding necrosis. ×90. **b** Higher magnification of **a**. ×180. *N* Necrotic lesions

phases of the recovery, GJs disappeared from virtually all hepatocytes located in the entire liver lobule. Such a reduction in the number of GJs corresponds well with the increase in the BrdU labelling, suggesting that this is due to cell proliferation as observed in the regenerating liver following partial hepatectomy [19]. Thus, the hepatocyte response against TAA-induced injury could be divided into three phases regarding changes in GJs: an acute functional response; a response by focal reduction in the number of GJs; and response cell proliferation. The first two phenomena are consistent with the idea that there are two types of mechanisms for the regulation of GJ against liver injury [8]: short-term regulation by closing channels [16], and long-term regulation by reducing synthesis and assembly of GJs [5, 18].

Small molecules, including second messengers such as cAMP [10], calcium ions and IP_3 [12], can be transferred between neighbouring hepatocytes via GJ channels [15]. This suggests that GJ communication plays an important role in the maintenance of liver functions which require each individual hepatocyte to work as part of an organized hepatocytic mass. In this sense, bile flow formation in the bile canaliculus needs organized and periodic contraction of bile canaliculi [21], presumably by cell-cell transduction of contraction signals through GJs [12]. In the course of liver regeneration following partial hepatectomy, the occurrence of slight cholestasis [19] and disappearance of GJs are observed [19]. Thus, it is conceivable that, in the areas surrounding necrosis at 24 h, a decrease of GJs causes weak cholestasis because we observed that the space of bile canaliculi is ultrastructurally expanded at the 48 h (data not shown).

TJs are now considered to function as the "gate" for a paracellular pathway and also as the "fence" for the protein and lipid composition of the plasma membrane [4]. TJs in hepatocytes are preferentially localized around the bile canaliculi, clearly suggesting that they function to prevent bile constituents from rushing into the circulation. This idea is supported by earlier electron microscopic observations using the freeze-fracture technique [7, 20] which demonstrated that the integrity of TJs decreased after bile duct ligation in parallel with the development of hyperbilirubinaemia. Immunohistochemical examination in this study showed that TJs stained with 7H6 antibody increase preferentially in perinecrotic areas 24 and 48 h after TAA treatment, and also that bile canaliculi were dilated in the same areas where the number of GJs markedly decrease. These findings suggest that cholestasis occurs in the perinecrotic areas due to hypofunction of GJs. Taking the function of TJs into consideration, the increased intensity of TJs in perinecrotic areas is considered to be an adaptive response of hepatocytes to fortify the "gate", function of the bile canaliculi to prevent bile juice from leaking out. This speculation is consistent with the observation that common bile duct ligation significantly induces the mRNA and protein of ZO-1α^+, one of tight junctional proteins [1]. In addition, GJs significantly decrease after common bile duct ligation (unpublished observation). However, the significance of increased DSs after hepatic injury remains unclear, though DSs in hepatocytes changed in a way similar to TJs during hepatic regeneration after TAA-induced injury, as well as after partial hepatectomy (unpublished observation).

Taken together we consider that upon the death of hepatocytes, adjoining hepatocytes respond rapidly by closing of GJ channels and then by a decrease in the number of GJs, which results in a disturbance in the organized contraction of the bile canaliculi. As a result of this, mild cholestasis occurs. TJs increase to fortify the "gate" function against bile in the areas surrounding the necrotic lesions.

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