Histol Histopathol (1997) 12: 937-943

Histology and Histopathology

Prolonged oval cell proliferation with Ito cell activation and extracellular matrix accumulation in galactosamine-induced acute hepatitis in mini rats

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Summary. Histopathological and immunohistochemical studies were carried out on D-galactosamine (GalNAc)-induced acute hepatitis in rats of the JCl: Wistar TgN (ARGHGEN) 1 Nts strain (Mini rats), in which expression of the growth hormone gene is suppressed by an antisense transgene. Hepatitis characterized by hepatocellular acidophilic necrosis with inflammatory cell infiltration was most prominent at 2 days after GalNAc (1000mg/kg)-injection, when proliferation of Ito cells and deposition of fibronectin and laminin were found along the sinusoidal linings. At 72 hours after GalNAc-injection, Ito cell proliferation with deposition of laminin and fibronectin became more prominent, and marked proliferation of small epithelial cells was observed in the periportal area. At 7 days after GalNAc-injection, quite a number of α-smooth muscle actin-positive Ito cells, surrounded by abundant fibronectin, laminin and type IV collagen, were still observed in close juxtaposition to rapidly proliferating small epithelial cells. The small epithelial cells were found to be positive for both α-fetoprotein and cytokeratin 7 and were therefore considered to be so-called oval cells. The results suggest that there may be some relation between oval cell proliferation, Ito cell activation and extracellular matrix accumulation in GalNAc-induced acute hepatitis in Mini rats.

Key words: Extracellular matrix, Galactosamine, Ito cell, Mini rat, Oval cell

Introduction

A single intraperitoneal injection of D-galactosamine hydrochloride (GalNAc) induces acute hepatitis in rats which morphologically resembles drug-induced hepatitis in man (Keppler et al., 1968; Decker and Keppler, 1972). GalNAc causes hepatocyte necrosis due to depletion of uridine nucleotides (Farber et al., 1973; Decker and Keppler, 1974), which in turn results in the inhibition of hepatocyte division. In GalNAc-induced hepatitis, proliferation of so-called oval cells is transiently observed instead of hepatocyte proliferation (Lemire et al., 1991; Dabeva and Shafritz, 1993). Oval cells are considered to be bipotential precursors for the two hepatic parenchymal cell lineages, i.e. bile duct epithelial cells and hepatocytes (Hayner et al., 1984; Germain et al., 1985, 1988; Evarts et al., 1987; Sirica, 1995). They are thought to be progeny of a hepatic stem cell compartment, and liver regeneration via this route occurs primarily under conditions in which the capacity of adult hepatocytes to replicate is suppressed (Thorgeirsson, 1993). However, the processes underlying generation and differentiation of the cells are not completely understood. Furthermore, how the Ito cells and extracellular matrix participate in liver regeneration and their relation to the proliferation of oval cells remain uncertain.

For the present study, we used rats of the Jcl: Wistar TgN (ARGHGEN) 1Nts strain (Mini rats), a newly developed Wistar rat-derived transgenic strain in which the expression of growth hormone (GH) gene is suppressed by the presence of an antisense transgene (Matsumoto et al., 1993, 1995). In our preliminary studies on GalNAc-induced acute hepatitis, proliferation of oval cells was only transient in rats of several strains including Wistars irrespective of the dose levels, whereas prominent proliferation of oval cells as well as increased amount of extracellular matrix components and activation of Ito cells was observed at even 7 days after injection in Mini rats at high dose levels. This suggests that the GalNAc-induced hepatitis in Mini rats is a good experimental system to investigate how extracellular matrix and Ito cells participate in the proliferation and differentiation of oval cells.

The purpose of this study was to clarify the process of proliferation of oval cells and to examine immunohistochemically the relationship between oval cells, Ito cells and extracellular matrix in GalNAc-induced acute hepatitis in Mini rats.

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Materials and methods

Forty-eight 6-week-old male rats of the Jcl: Wistar-TgN (ARGHGEN) 1Nts strain (Mini rats) (Matsumoto et al., 1993, 1995) (body weight 95-115g) were used. The animals were confirmed to be free from specific pathogens, were kept under controlled conditions (temperature, 23±2 °C; relative humidity, 55±5%) in an isolator caging system (Niki Shoji Co., Tokyo) and were fed pellets (MF, Oriental Yeast Co., Tokyo) and water *ad libitum*.

D-galactosamine hydrochloride (GalNAc) (Sigma Chemical Co., MO, USA) was purchased from a commercial company.

Thirty rats were intraperitoneally (ip) administered with 1000mg/kg b.w. of GalNAc. The injection volume was adjusted to 10ml/kg b.w. using 0.9% saline. The remaining 18 rats received the same volume of 0.9% saline and served as controls. Five rats of the GalNAc-group and 3 controls were killed by exanguination under ether anesthesia at 12, 24, 48, and 72 hours (HAI) and 5 and 7 days after the injection (DAI). Slices of liver were frozen in dry ice-hexane for immunohistochemistry, and small pieces were fixed in 1% glutaraldehyde for electron microscopy. The remaining liver was fixed in 10% neutral-buffered formalin for routine processing and histology. Paraffin sections (4 μm) were stained with hematoxylin and eosin (HE).

Cryosections were fixed in acetone at -20 °C for 10 min before performing immunohistochemical staining for all parameters other than α-fetoprotein. In the latter case, cryosections were fixed in 4% paraformaldehyde at 4 °C for 10 min before staining. Antibodies against fibronectin, laminin, type I collagen, type IV collagen (L.S.L. Co., Japan), desmin (Medac, Germany), vimentin, cytokeratin 7, α-smooth muscle actin (DAKO, Japan), and α-fetoprotein (Nordic Immuno-logical Laboratories, CA, USA) were applied as the primary antibodies for immunohistochemical staining, using the avidin-biotin-peroxidase complex (ABC) method and Vector ABC kits (Vector Lab. Inc., U.S.A). Counter-staining was with methyl green.

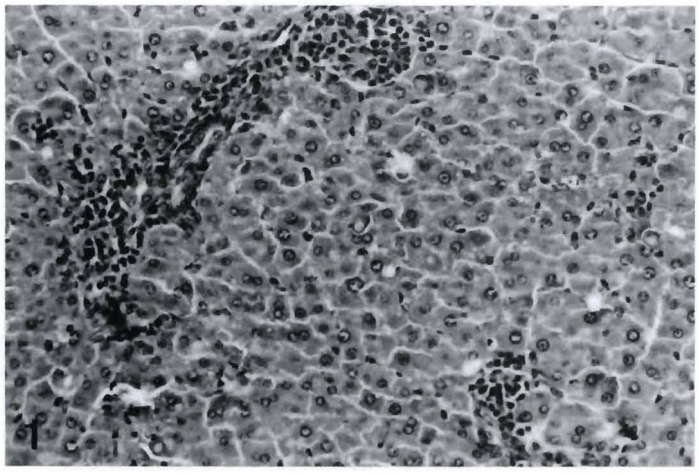
For electron microscopy, after fixation in 1% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) at 4 °C for 3hr, the liver samples were post-fixed in 1% osmium tetroxide in the same buffer, and embedded in epoxy resin (Epok 812, Oken Co., Tokyo). Ultrathin sections were double contrasted with uranyl acetate and lead citrate and observed under a JEOL 1200EX electron microscope (JEOL Co. Ltd., Tokyo).

Results

In the liver of rats in the GalNAc-group, small foci composed of necrotic hepatocytes and inflammatory cells were sporadically seen in the hepatic lobule at 12 HAI (Fig. 1). Subsequently, acute hepatitis progressed, and, at 48 HAI, prominent hepatocyte degeneration developed and inflammatory cells were found surrounding many small foci of necrotic hepatocytes (Fig. 2). The number of Ito cells (Fig.3a) and the amounts of laminin (Fig. 3b) and fibronectin (Fig. 3c) increased along the sinusoidal lining.

At 72 HAI, Ito cell proliferation with extracellular matrix deposition became more prominent, and marked proliferation of small epithelial cells was observed in the periportal areas (Fig. 4). The small epithelial cells were elongated or polygonal in shape with scant, weakly basophilic cytoplasm and oval, light-staining nuclei. Near these small epithelial cells, large hepatocytes with enlarged nuclei were noted. Inflammatory infiltration began to decrease and almost diminished at 5 DAI.

At 7 DAI, proliferation of small epithelial cells was still prominent, and many large hepatocytes were also



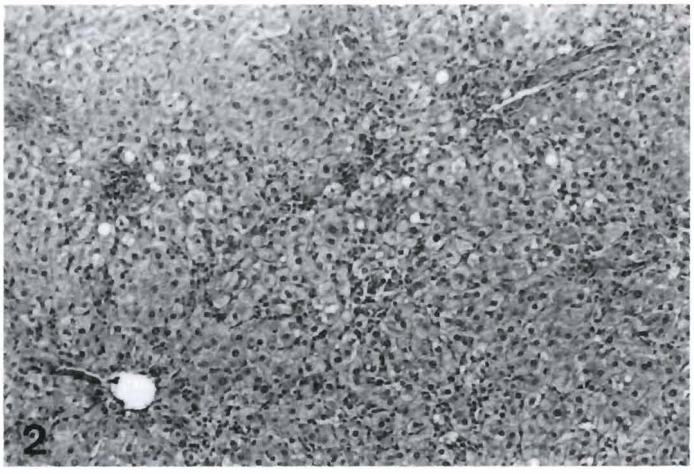


Fig. 1. Liver of a Mini rat at 12 hours after GalNAc-injection. Small foci of necrotic hepatocytes with inflammatory infiltration are sporadically seen. HE. x 50

Fig. 2. Liver of a Mini rat at 48 hours after GalNAc-injection. Many small foci of inflammatory cell infiltration are seen. HE. x 25

observed (Fig. 5). The small epithelial cells radiated in rows from the periportal area into the hepatic lobule or proliferated in clusters between the hepatocyte plates. Some made up duct-like structures. Surrounding these small epithelial cells, deposition of a prominent amount of laminin (Fig. 6a) and fibronectin (Fig. 6b) was found. In addition, an appreciable amount of type IV collagen (Fig. 6c) and a little amount of type I collagen were also detected. Intermingled with these extracellular matrix components, there were many desmin-positive Ito cells (Fig. 7a), most of which also stained strongly for α-smooth muscle actin (Fig. 7b). The small epithelial cells described above were always positive for cytokeratin 7 (Fig.8a), and most of them additionally expressed α-fetoprotein (Fig. 8b) at even 7 DAI. Their ultrastructural characteristics were intermediate between those of typical hepatocytes and bile duct cells. The cells had electron-lucent round to oval nuclei and scanty intracytoplasmic organella (Fig. 9a), the latter being somewhat more developed in the epithelial components forming duct-like structures than in those arranged in rows or clusters (Fig. 9b).

Discussion

In the present study, prominent and prolonged

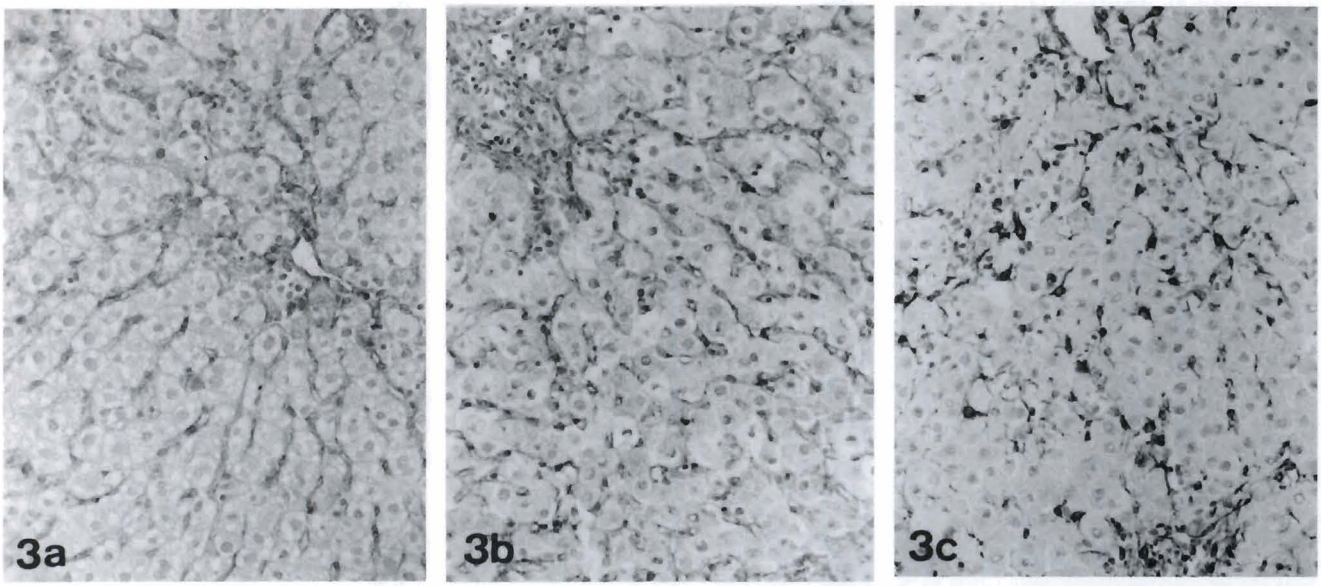


Fig. 3. Liver of a Mini rat at 48 hours after GalNAc-injection. The amounts of laminin **(a)** and fibronectin **(b)** and the number of desmin-positive Ito cells **(c)** increase along the sinusoidal lining. Immunostaining. x 50

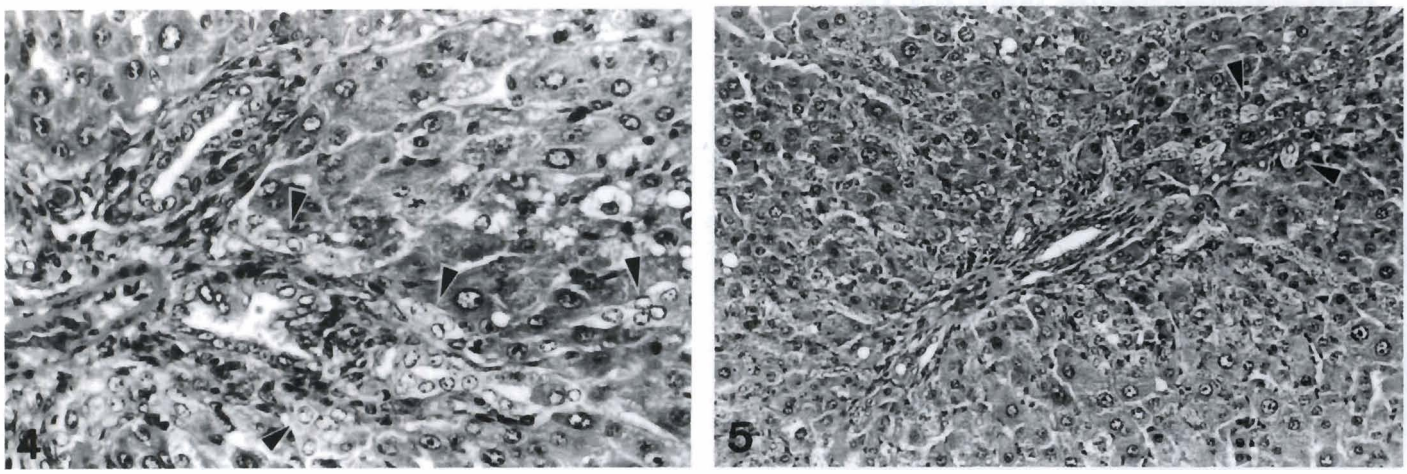


Fig. 4. Liver of a Mini rat at 72 hours after GalNAc-injection. Proliferation of small epithelial cells is seen in the periportal area (arrowheads). HE, x 160

Fig. 5. Liver of a Mini rat at 7 days after GalNAc-injection. Prominent proliferation of small epithelial cells is seen in all the hepatic lobules. Some of the cells make up duct-like structures (arrowheads). HE. x 50

proliferation of small epithelial cells was observed in the livers of Mini rats injected ip with 1000mg/kg b.w. of GalNAc, at even 7 DAI. The positive immunoreactivities for both bile duct epithelial cell (cytokeratin 7) and fetal hepatocyte (α-fetoprotein) markers, and the electronlucent, round to oval nuclei and scanty intracytoplasmic organella were consistent with an oval cell nature (Grisham and Thorgeirsson, 1997). As mentioned earlier, it is considered that oval cells proliferate when the regeneration of hepatocytes is suppressed. Therefore, studies of oval cells have been performed mainly in experimental models in which partial hepatectomy and chemical injury are combined (Alison and Hully, 1991; Golding et al., 1995) or a mitoinhibitory hepatocarcinogen is applied (Lenzi et al., 1992). Earlier investigations of GalNAc-induced hepatitis revealed a transient proliferation of oval cells (Lemire et al., 1991; Daveba and Shafritz, 1993) with suppression of hepatocyte regeneration due to depletion of uridine nucleotides (Farber et al., 1973; Decker and Keppler, 1974).

As noted above, the Mini rat is a newly developed transgenic rat in which the expression of GH gene is suppressed by introduction of an antisense transgene (Matsumoto et al., 1993, 1995). In this context, studies using hypophysectomized rats are of interest. The results suggest that GH augments the regeneration of liver after partial hepatectomy (Untermann and Phillips, 1896; Hemingway and Cater, 1958; Rabes and Brandle, 1969; Uthne and Uthne, 1972), and Ekberg et al. (1992) have

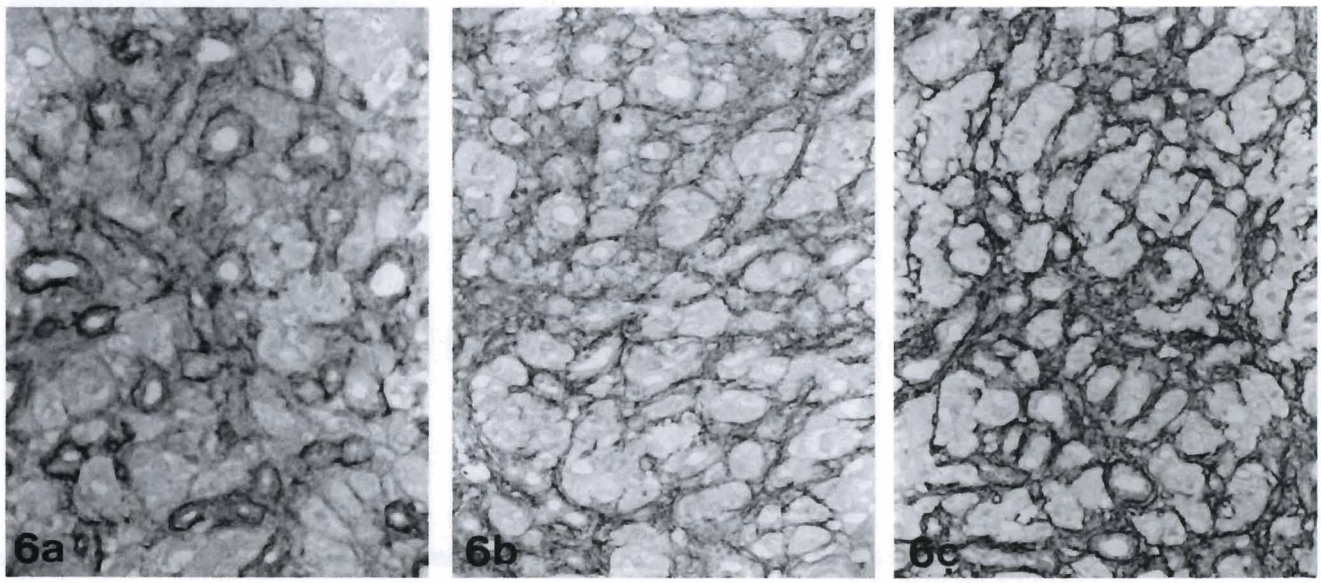


Fig. 6. Liver of a Mini rat at 7 days after GalNAc-injection. Prominent deposition of laminin **(a)**, fibronectin **(b)** and type IV collagen **(c)** is seen around the proliferating small epithelial cells. Immunostaining. x 50

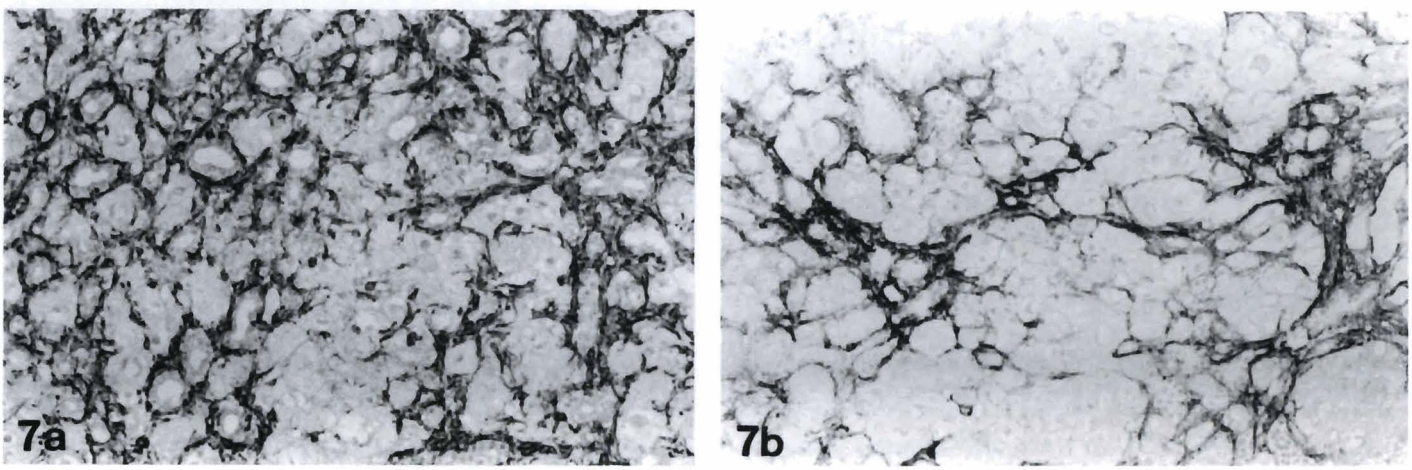


Fig. 7. Liver of a Mini rat at 7 days after GalNAc-injection. Proliferated Ito cells are always positive for desmin **(a)**, and most of them are also positive for α-smooth muscle actin **(b)**. Immunostaining. x 50

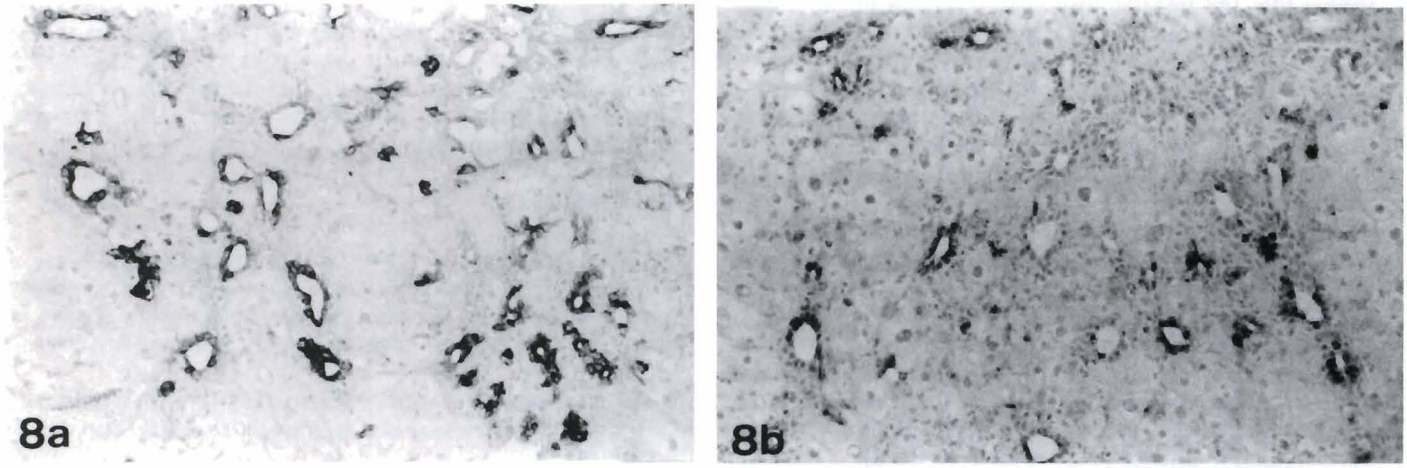


Fig. 8. Liver of a Mini rat at 7 days after GalNAc-injection. Small epithelial cells are positive for cytokeratin 7 **(a)**, and most of them are also positive for α-fetoprotein **(b)**. Immunostaining, x 50

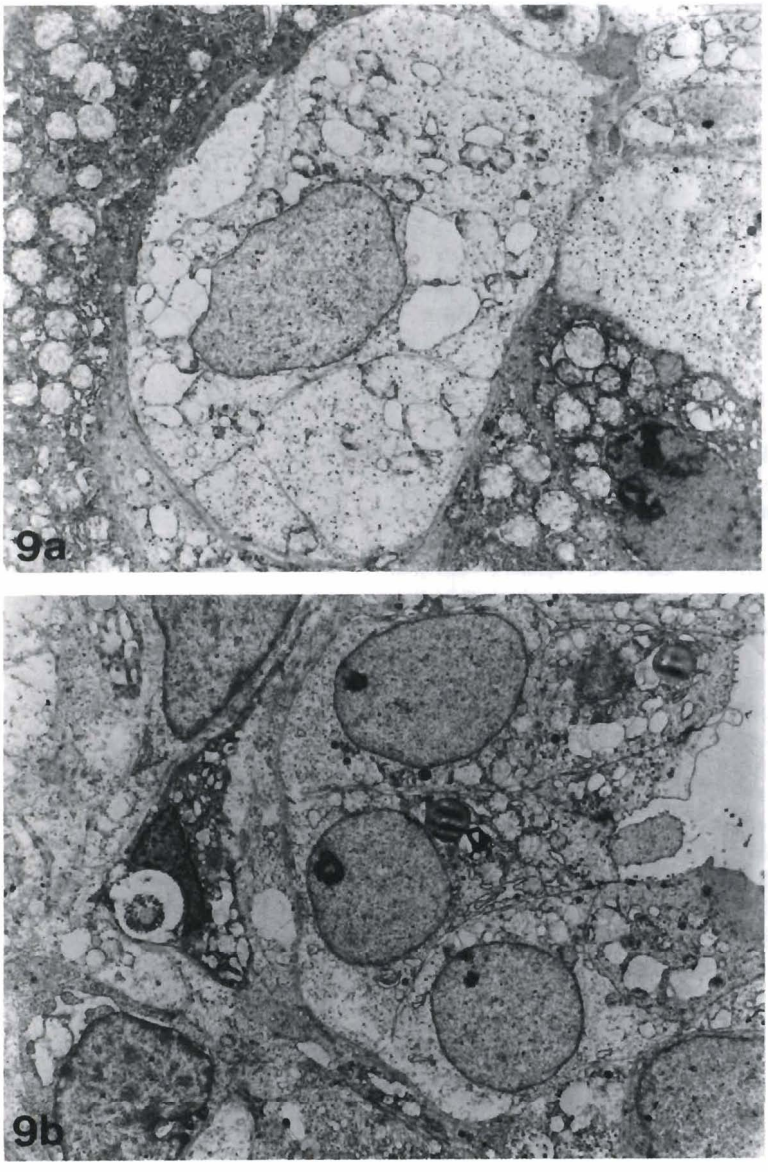


Fig. 9. Small epithelial cells in the liver of a Mini rat at 7 days after GalNAc-injection. The cells have electron-lucent round to oval nuclei and scanty intracytoplasmic organella **(a)**. Intracytoplasmic organella are somewhat more developed in the cells making up a duct-like structure **(b)**. a, x 1,200; b, x 1,500

shown that the hepatic response to partial hepatectomy in terms of both hepatocyte growth factor (HGF) gene expression and DNA synthesis in hypophysectomized rats is accelerated by treatment with GH. Therefore, it is probable that the suppression of GH gene expression in Mini rats would negatively influence the regeneration of hepatocytes and bring about prolonged oval cell proliferation.

The descendants of oval cells are thought to be both bile duct epithelial cells and hepatocytes. In GalNAc-induced acute hepatitis, Dabeva and Shafritz (1993) have suggested that oval cells might proliferate and differentiate into mature hepatocytes, though direct proof of this is still lacking. In the present study, transformation into hepatocytes could not be confirmed. On the other hand, small epithelial cells positive for both cytokeratin 7 and α-fetoprotein made up duct-like structures at 7DAI. This suggests that oval cells differentiated into bile duct epithelial cells.

Intermingled with the oval cell population, many Ito cells were observed in the present study with their positive reaction for α-smooth muscle actin, indicating an activated state (Geerts et al., 1989). Activated Ito cells are well known to enhance the production of matrix components (Takahara et al., 1988) and may thus have been involved in their accumulation around the proliferating oval cells in the present study. This would imply an indirect control of proliferation and differentiation of oval cells through regulation of the extracellular environment.

In the present study, an increase in amounts of fibronectin and laminin along the sinusoidal lining started prior to the proliferation of small epithelial cells. Thereafter, deposition of were these extracellular matrix components progressed with the time and were detected around the proliferating small epithelial cells in the whole hepatic lobules at 7 DAI. This suggests that fibronectin and laminin may play a role in migration of oval cells from the periportal area into the whole hepatic lobule. On the other hand, the deposition of type IV collagen was increased at 7 DAI when duct-like structures appeared, and this suggests that type IV collagen may play a role in making up duct-like structures by oval cells.

Extracellular matrix components are known to affect differentiation and proliferation of parenchymal cells through their close attachment (Bruijin et al., 1988; Reif et al., 1990; Martínez-Hernández et al., 1991), and many cell-matrix interactions are mediated by specific plasmalemmal receptors, like integrins (von der Mark and Kuhl, 1985; Ruoslahti and Pierschbacher, 1986; Tamkun et al., 1986; Urushihara and Yamada, 1986; Dedhar et al., 1987). Although it is not known what kind of adhesion molecules the oval cells express, the present results suggest an intimate association with the extracellular matrix.

In conclusion, prominent proliferation of oval cells was observed even after 7 days in GalNAc-injected Mini rats associated with many activated Ito cells and accumulation of fibronectin, laminin and type IV collagen. The results are in line with the hypothesis that activated Ito cells and their products may be involved in the proliferation and differentiation of oval cells. Further investigations are now in progress to clarify the role of cytokines in this process.

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Accepted March 4, 1997