Immunohistochemical and Ultrastructural Study of Ito Cells (Fat-Storing Cells) in Response to Extrahepatic Bile Duct Ligation in Broiler Chickens

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ABSTRACT. The Ito cell (fat-storing cell) lies in perisinusoidal space of liver and has a variety of functions. We investigated the immunohistochemistry and ultrastructure of Ito cells in normal and cholestatic livers of broiler chickens. Immunohistochemistry demonstrated that Ito cells expressed HHF35 muscle actin, vimentin, desmin, glial fibrillary acidic protein (GFAP), neuron-specific enolase (NSE), chromogranin A and cytokeratins in normal livers. These cells were diffusely scattered throughout the lobules. Livers treated with extrahepatic bile duct ligation (BDL) showed cholestasis, fibrosis, proliferation of biliary ductules and Ito cells. The Ito cells were frequently found in fibrotic areas and were larger in size with more extensive immunoreactivity than those of normal livers. Ultrastructural study demonstrated that Ito cells were closely associated with the production of collagen fibers in BDL livers. These findings suggest that Ito cells actively react against hepatocytic injuries and play a major role in the hepatic fibrogenesis of cholestatic livers of chickens.

KEY WORDS: bile duct ligation, broiler, immunohistochemistry, Ito cell, ultrastructure.

The occurrence of enlarged livers has been reported in broiler chickens at slaughterhouses [9, 15, 16]. These lesions are grossly characterized by enlargement, an irregular surface, discoloration, and consolidation, often associated with thickening of the walls of the gall bladder and bile ducts distended with bile. This condition has histologically been described as fibrosing hepatitis accompanying biliary ductule proliferation and infiltration of heterophils, and a clostridial infection with bile stasis has been suggested as the cause of the disorder [15, 16].

Similar hepatic lesions composed of intrahepatic biliary ductule proliferation and massive fibrosis with few hepatocytes remaining were experimentally produced by ligation of both extrahepatic bile ducts [14]. Some experimental and clinical studies have documented biliary ductule proliferation as the initial morphologic manifestation of liver pathology after extrahepatic bile duct obstruction [2, 8, 22].

The number of Ito cells (fat-storing cells) increases periportal and around the central veins in reactive biliary hepatitis [6] and this increase has been suggested to be related to hepatic fibrosis in humans and other mammals [7, 10]. In the normal human liver, the cells can be identified by the presence of lipid droplets and expression of α-smooth muscle actin [7]. However, the biological nature and the roles of Ito cells are not sufficiently clarified in those hepatic disorders of birds. The purposes of the present study were to investigate the immunohistochemical and ultrastructural features of the Ito cells in normal and bile duct-ligated livers of broiler chickens, and to clarify the relationship of Ito cells with fibrogenesis in birds.

MATERIALS AND METHODS

**Chickens**: Twenty 1-day-old broiler chicks from a commercial hatchery were housed in our experimental facility. The room temperature was consistently controlled at 22°C, and feed and water were supplied ad libitum.

**Experimental design**: Twelve 3-week-old broiler chicks had both extrahepatic bile ducts surgically ligated under anesthesia with xylazine (0.86 ml per kg body weight/BW) and ketalar (1.32 ml per kg BW). Eight control birds were untreated. At 3 weeks after the bile duct ligation (BDL), three birds of the treated group and two control birds were necropsied. The same numbers of birds in both groups were necropsied at 5, 7, and 9 weeks after BDL. The liver, gall bladder, extrahepatic bile ducts and duodenum of each necropsied bird were subjected to pathologic examination.

**Histology and immunohistochemistry**: For light microscopic examination, tissue samples preserved in 10% neutral buffered formalin fixative were routinely processed, embedded in paraffin-wax, and sectioned at 3–5 μm. These sections were stained with hematoxylin and eosin (HE), Masson trichrome, and reticulin silver impregnation. Additional serial sections were prepared for immunohistochemistry by the streptavidin–biotin complex (SAB) immunoperoxidase method. The primary antibodies employed were the following: mouse anti-muscle actin/HHF35 (Enzo Diagnostic Inc., New York, U.S.A.), mouse anti-swine vimentin (Dako Corp., Glostrup, Denmark), mouse anti-human desmin (Dako Corp., Carpinteria, U.S.A.), rabbit anti-factor VIII-related antigen (Dako, Carpinteria), rabbit anti-cow glial fibrillary acidic protein (GFAP) (Dako, Glostrup), rabbit anti-neuron specific enolase (NSE) (Dako, Carpinteria), rabbit anti-human chromogranin A (Dako, Carpinteria), mouse anti-human cytokeratin MNF116 (Dako, Carpinteria), anti-cytokeratin

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high molecular weight (HMW) (Enzo) and anti-cytokeratin low molecular weight (LMW) (Enzo). The primary antibody was omitted in the negative control sections. The immune reaction was evaluated semi-quantitatively as follows: −, no positive cells; +, a small number of positive cells; ++, a moderate number of positive cells; ++++, many to numerous positive cells.

Electron microscopy: Liver tissues were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at 4°C for 48 hr, washed with 0.1 M phosphate buffer and post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer. These tissues were dehydrated through graded-ethanol and embedded in Epon (Quetol 812; Nissin EM Co., Tokyo). Ultrathin sections were stained with uranyl acetate and lead citrate, then examined with a transmission electron microscope (JEM-100SX; JEOL, Tokyo).

RESULTS

Three weeks after extrahepatic BDL, the livers were enlarged with irregular surfaces, mildly consolidated and discolored pale green. The gall bladder and both extrahepatic bile ducts showed mild to severe dilation with edematous thickening of the walls. Five weeks after BDL, enlargement of the livers was more prominent. They were firm and tan, with multiple white foci. The spleens were moderately swollen and the bone marrow was red. Seven and nine weeks after BDL, the livers were severely consolidated and tan, with multiple white foci diffusely scattered throughout the livers. The lesions in the spleens and bone marrows were similar with the lesions before, in 3 and 5 weeks after BDL. There were no significant lesions in the other tissues, including duodenum, kidney, lung and heart of the BDL-treated birds.

Histologically, the hepatic lesions of the BDL group were characterized by diffuse fibrosis, proliferation of biliary ductules (Fig. 1), heterophilic and lymphocytic aggregates, cholestasis and multifocal necrosis of hepatocytes. Three weeks after BDL, fibrosis and heterophilic infiltrates were prominently found in and around portal areas, with mild dilatation of interlobular bile ducts. Periportal fibrosis extended into the parenchyma at 5 weeks after BDL treatment. Collagen fibers proliferated around the portal areas and central veins. In Masson trichrome-stained sections, thin collagen bundles diffusely proliferated, encircling the hepatic cords and the proliferating biliary ductules. Occasionally, small foci of coagulative and lytic necrosis of hepatocytes were scattered throughout the parenchyma. Cholestasis characterized by bile plugs within dilated biliary canaliculi and bile pigments in hepatocytes was evident. Three weeks after BDL, the cholestasis was restricted to the centrilobular zone, and the distribution of bile plugs extended to the perilobular zone at 5–9 weeks after BDL. Hypertrophy of the tunica muscularis was observed in the gall bladder and extrahepatic bile ducts. In addition, BDL birds showed reticular cell and histiocytic hyperplasia in the spleen and hyperplastic marrow with increased numbers of erythroblasts.

Ito cells (fat-storing cells): In control livers, immunohistochemical study demonstrated that Ito-cells were diffusely scattered throughout the hepatic lobules. They were ordinarily located in perisinusoidal areas. These cells were positive for HHF35, NSE, chromogranin A, and cytokeratins, and faintly positive for vimentin, desmin and GFAP. Ito cells were usually small and spindle- or star-shaped with one or several long processes, and had distinct vesicular oval nuclei.

In the BDL-treated livers, the number of Ito cells diffusely increased, and they were occasionally enlarged and markedly positive for HHF35. These cells proliferated in periportal and severely fibrotic areas as well as around cords of atrophic hepatocytes adjacent to congested sinusoids (Fig. 2). Immunostaining of serial sections demonstrated that Ito cells were also positive for vimentin, desmin, GFAP (Fig. 3), NSE, chromogranin A, and cytokeratins (Table 1) and these reactivities were stronger than those of the normal Ito cells. Spindle-shaped cells closely resembling myofibroblasts morphologically were scattered among the HHF35 muscle actin-positive cells. They encircled cords of atrophic hepatocytes and biliary ductular structures, giving an “onion-like” appearance [24]. These cells were positive for HHF35 muscle actin as well as vimentin, chromogranin A and cytokeratin, but were negative for desmin, GFAP and NSE.

Proliferation of biliary ductules: The biliary ductule proliferation was prominent from 3 weeks after BDL. Epithelial cells of intrahepatic bile ducts including proliferating biliary ductules in BDL livers showed intense reactivity for NSE (Fig. 4). The epithelial cells of interlobular ducts were also positive for NSE. In contrast, NSE was faintly and restrictedly expressed by the interlobular bile ducts of the control livers. At 7 and 9 weeks after BDL, the proliferation of biliary ductules was more prominent than before. Cholangiocytes of the control and BDL livers were negative for MNF116, cytokeratin HMW and cytokeratin LMW.

Vascular proliferation associated with BDL: Neovascularization was observed around the portal triads and central veins in the BDL group. The endothelial cells lining the proliferating vessels were positive for factor VIII-related antigen. These endothelial cells were negative for other antibodies used in this study. In the control chickens, endothelial cells positive for factor VIII-related antigen were restricted to the portal vessels and central veins.

Electron microscopy: Ultrastructural study of the BDL livers revealed the presence of Ito cells in the space of Disse. These cells were elongated, with or without lipid droplets in the cytoplasm (Fig. 5). They had abundant rough endoplasmic reticulum in their cytoplasm indicating that they had active proteogenesis. Small amounts of collagen fibers arranged in bundles were recognized in the vicinity of the Ito cell from 3 weeks after BDL (Fig. 6).
Fig. 1. Liver, 7 weeks after extrahepatic BDL; characterized by fibrosis and proliferation of biliary ductules (arrows). HE stain. Bar=33 µm.

Fig. 2. Liver, 3 weeks after extrahepatic BDL; distribution of HHF35-positive reactivity is noted in Ito cells (arrows) surrounding a cord of atrophic hepatocytes. HHF35 immunostaining, hematoxylin counterstain. Bar=34 µm.

Fig. 3. Liver, 3 weeks after BDL treatment; GFAP-positive reactivity is recognized in the cytoplasm of Ito cells (arrows), located in perisinusoidal areas. GFAP immunostaining, hematoxylin counterstain. Bar=33 µm.

Fig. 4. Liver, 3 weeks after extrahepatic BDL; epithelia of proliferated biliary ductules (arrows) and Ito cells (arrowheads) are positive for NSE. NSE immunostaining, hematoxylin counterstain. Bar=33 µm.
DISCUSSION

The Ito cell, also referred to as fat-storing cell, hepatic stellate cell or lipocyte, is one of the sinusoidal-constituent cells that play multiple roles in liver pathophysiology. The function of the cell is expanding from a fat-storing site to a center of extracellular matrix metabolism and mediator production in the liver [8]. These cells were detected in the perisinusoidal area of the normal liver in humans and mammals [8, 13, 23, 25]. Our study demonstrated that Ito cells were diffusely scattered throughout the lobules, located in the spaces of Disse in the liver in normal chickens, and showed positive reactivities for HHF35 muscle actin, vimentin, desmin, GFAP, NSE, chromogranin A and cytokeratins. In BDL livers, proliferating Ito cells markedly expressed HHF35 muscle actin. This finding was in accordance with previous reports in mammals [5, 8], in which the number of α-smooth muscle actin-positive Ito cells increased following biliary obstruction.

Ultrastructural study demonstrated newly formed collagen fibers around the Ito cells. Our immunohistochemical data also indicated a positive correlation between the number of Ito cells and the degree of fibrosis. Ito cells are important effectors during fibrogenesis in mammals [4, 12]. The present investigation suggests that Ito cells in the hepatic tissue of chickens can be easily identified by characteristic immunoreactivities, and that they play a major role in the fibrogenesis of BDL chickens.

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GFAP is an intermediate filament first identified in astrocytes. Similar to other intermediate filaments, GFAP does not provide exclusive specificity for glial cells and has been demonstrated in non-glial cells such as human salivary gland duct cells and pleomorphic adenoma cells [1]. In the liver, GFAP is considered to be a specific marker for Ito cells, which might allow distinction between these cells and other fibroblastic liver cells [11, 13, 19]. Our results suggest that these findings in mammals were also applicable to chickens.

The enhancement of Ito cells and myofibroblast-like cells seems to closely follow proliferating biliary ductules, suggesting a crucial role in hepatic tissue remodeling after BDL. The present study showed a greatly increased number of Ito cells, including myofibroblast-like cells in the BDL livers. Both types of cells predominantly surrounded cords of hepatocytes and proliferating bile ductules. In chronic liver disease, Ito cells, that can differentiate into myofibroblast-like cells, have a high fibrogenic capacity and are also involved in matrix degradation [7]. Our results showed that the GFAP-positivity was present in Ito cells, but the myofibroblast-like cells were negative. Although we could not clarify the functional differences between the GFAP-positive Ito cells and the GFAP-negative myofibroblast-like cells, the latter should be considered a phenotype of Ito cells.

We have shown that after BDL in broilers, there were increased numbers of Ito cells and immunoreactivities for NSE and chromogranin A. NSE is a glycolytic enzyme found in neural cells as well as in the cells of the diffuse neuroendocrine system. Regardless of limited specificity, NSE is used as a broad-range marker that reacts with most neuroendocrine cells and neoplasms [17]. Chromogranin A has also been found to be a highly reliable marker for neuroendocrine cells [21, 26]. To our knowledge, however, Ito cells in mammals has not been reported to show neuroendocrine capability in any situation. Therefore, the detail examination is required to clarify the expression of NSE and chromogranin A in Ito cells.

Cytokeratins constitute a diverse group of intermediate filament proteins, expressed as pairs in keratinizing and non-keratinizing epithelial cells. Although the expression of cytokeratins in Ito cells has not been described until now, the present results recognized that Ito cells in the normal and BDL chickens expressed these antigens. In contrast, the cholangiocytes were negative immunoreactivities for cytokeratin markers. It is proposed that during development, cytokeratin expression may be related to the maturation stage of the biliary tree [3] or the cytokeratin antibodies used in the present study did not react it in the liver of chickens.

Based on the immunohistochemistry for anti-factor VIII-related antigen, there were increased numbers of small ves-

Table 1. Immunohistochemistry in normal and BDL livers of chickens

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<td>HHF35 (anti-muscle actin)</td>
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a) Normal livers. b) Bile duct-ligated livers. c) High molecular weight. d) Low molecular weight.
sels in periportal and pericentral areas of BDL livers. In rats, BDL treatment induced hypoxia and caused an increase in periportal vascular endothelial cell proliferation [18] in order to increase oxygen delivery [20].

The all treated birds showed reticular cells and histiocytic hyperplasia in spleen and hyperplastic bone marrow. Although the pathogenesis of this condition has been remained unclear in the present study, it is considered similar with Banti syndrome in mammals [27].

Prolonged obstruction of bile flow leads to marked proliferation of biliary ductules and formation of fibrous tissue. In conclusion, the present study suggests that Ito cells play a major role in the fibrogenesis with enhancement of the immunoreactivity.
REFERENCES


