IRREVERSIBLE ATYPICAL BILE DUCTS (CHOLANGIOFIBROMA ?) INDUCED BY 0.1% ETHIONINE IN CHOLINE DEFICIENT DIET IN RATS

Masami Hiruma, Akihiro Ono, and Azusa Seki

Tsukuba Laboratories, Experimental Biomedical Research Inc. (JISSEIKEN)

Takeshi Kurihara

Department of Gastroenterology, Aoyama Hospital, Tokyo Women's Medical College

Abstract: Atypical bile ducts induced by 0.1% ethionine in choline deficient diet (ECD) were biologically and histologically examined in rat liver. At 1 week after administration, enlargement of nucleolus and central lobular fatty change were observed in hepatic cells. At 2 weeks, there were diffuse and tubular proliferation of oval cells in periportal and along sinusoidal regions. The fatty droplets were large in size and expanded throughout the lobules. At 4 weeks oval cells became more conspicuous, occasionally formed duct structures (bile duct proliferation), and atypical bile ducts were observed with obvious border and secretion of mucin. The neoplastic nodules were first recognized at this time. At 8 weeks the oval cells increased more prominently, and there were many atypical bile ducts with fibrosis. At 12 weeks (group 50% ECD), atypical bile ducts and neoplastic nodules were more conspicuous. In the recovery period, the number of oval cells was much less than during administration, and neoplastic nodules were hardly seen. The atypical bile ducts, however, showed large nodular expansions in the periportal region, being much more conspicuous than those during administration. Macroscopically, the bile duct lesions could be seen as transparent small and white nodules and could be readily distinguished from neoplastic nodules which were milky white in color. The cell proliferation activity of the bile duct lesions showed a higher value in recuperating rats than in administered ones.

From the above findings it was confirmed that the atypical bile ducts are induced in association with oval cell proliferation, and that the cell proliferation activity and covering area did not show reduction by removal of the exciting agent, and that the lesion has the ability of autonomous development. (*J Toxicol Pathol* 8 : 101~106, 1995)

Key words: Ethionine, Choline deficient, Cholangiofibrosis, Bile duct tumor, Histopathology, Rat

Introduction

It has been known that ethionine and choline deficient diet were used as means of making fatty liver models¹, and that hepatic cellular neoplasm was also induced by their single administration^{2,3}. In the past Popper *et al.* (1953)⁴ reported tumor-like nodules induced by ethionine contained diet in rats. Subsequently, Farber has reported liver

carcinoma and detailed carcinogenesis with ethionine⁵. Farber has described that ethionine-induced liver cancer is of the hepatocellular type, and that cholangiofibrosis is irreversible but not neoplastic. On the contrary, cholangiofibrosis is considered to be a precarcinogenic change of cholangiocarcinoma induced by Azo-dye in rat⁶. As in these reports, cholangiofibrosis appears to have either a reactive or neoplastic character according to the kind of carcinogen administered. Expression of the term "cholangiofibrosis" should be used for non-neoplastic lesions, and if the lesions are neoplastic, they should be called "cholangiofibroma" or "cholangioma"⁷. We previously reported that glandular structures were

蛭間正巳 小野晃弘 関あずさ 栗原 毅

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Mailing address : Masami Hiruma, Tsukuba Laboratories, Experimental Biomedical Research Inc. (JISSEIKEN), 8-5-1, Chu-ou, Ami-machi, Inashiki-gun, Ibaraki 300-03, Japan.

observed at an early stage by administration of ECD diet⁸. However, it is still unclear whether the glandular structures are neoplastic or non-neoplastic, in other words cholangiofibrosis or cholangiofibroma.

This paper describes the presence of autodevelopment, cell proliferating activity, and histogenesis of atypical bile ducts.

Materials and Methods

Animals

Sixty 3-week-old male rats of the Sprague Dawley Strain were purchased from Charles River Japan Inc. (Kanagawa, Japan). The animals were kept in a room, which was maintained at a temperature of $24 \pm 2^\circ\text{C}$ and humidity of $55 \pm 10\%$. The room was ventilated nine times per hour and provided with 12 hours of light (from 8:00 to 20:00). Then eight days were allowed for the adjustment of animals to laboratory conditions.

Experimental design

Three groups, ECD group contained 0.1% DL-ethionine in choline deficient diet, 50% ECD group, 0.1% ethionine in choline diminished diet (50 mg/100 g), and negative control group were

Table 1. Composition of the ECD and 50% ECD Diet

Vitamin-free casein	8.000 (g)
Lard	37.950
Sucrose	48.375
Salt mixture ¹⁾	4.000
Vitamins ²⁾	1.050
L-Cystine	0.625
DL-Ethionine	0.100
Choline chloride (Group ECD)	0
„ (Group 50% ECD)	0.067
Total Group ECD	100.100
Group 50% ECD	100.167

¹⁾ Composition (mg/100 g): vitamin (V) A 3.125; V.D₃ 25.00; V.B₁ 3.125; V.B₂ 5.00; V.B₆ 3.125; V.K₃ 3.125; V.E 36.00; d-calcium pantothenate 1.250; niacin 1.250; cellulose powders 96.90.

²⁾ Composition (g/100 g): CaHPO₄·2H₂O 0.017; KH₂PO₄ 1.372; NaCl 1.002; Fe-citrate 0.025; MgSO₄·7H₂O 0.399; ZnCl₂ 0.001; MnSO₄·4~5H₂O 0.005; CuSO₄·5H₂O 0.006; CaCO₃ 1.172.

Fig. 1. Experimental design.

prepared. The components of the ECD and the 50% ECD diet (Oriental Yeast Co. Ltd.) are shown in **Table 1**.

Rats of group ECD were fed ECD for 8 weeks, and subsequently were fed a normal diet (MF: Oriental Yeast Co. Ltd.) as recovery periods for 4 weeks. Those of group 50% ECD were fed 50% ECD for 12 weeks, subsequently MF as recovery periods for 4 weeks. Control rats were fed MF over the experimental periods (Fig. 1).

Examination methods

During the periods, 2 cases of group ECD and 1 case of group 50% ECD were dead or killed due to poor prognosis. The other rats were sacrificed after 1, 2, 4, 8, 12, and 16 weeks of administration, and the liver was examined histopathologically. The liver was cut from two portions of the left and right lateral lobes, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with hematoxyline and eosin (HE). A part of the sections were immunohistochemically observed using monochronal antibodies (DAKO) against proliferating cell nuclear antigen (PCNA)⁹ and SAB (streptavidin-biotin) staining kit (Nichir-ei). The area of the cholangial lesion during administration period was compared with that at recovery using an image analyser (Luzex F: Nireko Co. Ltd.). The mean area of atypical bile duct was counted in randomly-selected five different foci from a rat, and then compared with each group. Measurement of PCNA was counted in randomly-selected ten different ducts from a focus, and the mean value was counted from five foci in a rat. These values were compared between administered rats and recuperating ones.

Results

Dead rats

During the experimental period, 3 cases (ECD: 2, 50% ECD: 1) were dead or killed because of poor prognosis. They were autopsied on day 48, 54, and 78 of administration. Macroscopic and microscopic examination revealed commonly hemorrhagic necrosis and suppuration of the kidneys, atrophy of thymus, pancreas, and genital organs, and calcification of systemic organs.

Sacrificed rats according to experimental schedule

Clinical findings

Roughness and looseness of hairs were observed from the following day after administration, and gradually progressed in all rats except the controls. Retardation of growth was in proportion to the degree of hair looseness.

Fig. 2. Liver of recuperating rat sacrificed at 16 weeks after administration of 50% ECD. Many white nodules are visible on the surface of the visceral side (A) and on the cut-surface (B).

Macroscopic findings

The liver showed slight fatty change at 1 and 2 weeks after administration, white nodules from 8 weeks, and two types of white nodules at 12 weeks. The nodules in one type varied in size were milky white in color and scattered. The other one was small transparent nodules of millet size, which tended to be numerous observed near the porta hepatis at the visceral side (Fig. 2). The former nodule was so-called "neoplastic nodule" consisting of hepatic cells, and the latter consisted of many atypical bile ducts (choangial nodule) from histological examination. The choangial nodules were recognized in all rats of 50% ECD at 16 weeks after administration.

Microscopic findings

1. Period of administration (ECD: 1 to 8 weeks, 50% ECD: 8 & 12 weeks)

On the first week after administration, hypertrophy of nucleolus was observed in many hepatic cells, and there was already atypical cell morphology accompanied by slight central lobular fatty depositions. On the second week, oval cells diffusely proliferated in the periportal and along

Fig. 3. Liver at 4 weeks after administration of ECD. Note oval cell hyperplasia and bile ducts proliferation. HE, $\times 200$.

the sinusoidal regions. The vacuolar fatty droplets became more large in size and diffusely spread in the lobule. Hyperplasia of oval cells became more conspicuous at 4 weeks, and bile duct proliferation and glandular structures became apparent for the first times (Fig. 3). The bile duct proliferation consisted of duct forming oval cells, and the glandular structures consisted of atypical bile duct, which had irregular lumen with fibrosis, clear border, and mucous secretion. Neoplastic nodules were also observed at this time. At 8 weeks, there was marked proliferation of oval cells and atypical bile ducts. The atypical bile ducts showed nodular proliferation in periportal area and in lobule, and consisted of a layer of flat or columnar lining cells, which were occasionally piled up and showed mitosis and basophilic cytoplasm. Cell debris was present within the irregular lumen and these ducts were encircled by slight fibrosis. At 12 weeks (50% ECD), atypical bile

Fig. 4. Liver at 12 weeks after administration of 50% ECD. Note many atypical bile ducts with fibrosis, and there is cellular debris within the lumen. Arrow shows mitosis of the lining cell. HE, A: low magnification, $\times 100$. B: high magnification, $\times 200$.

Fig. 5. Recuperating rat liver at 16 weeks after administration of 50% ECD. The atypical bile ducts show dense proliferation and have irreversible characteristics. HE, A: low magnification, $\times 100$. B: high magnification, $\times 200$.

ducts and neoplastic nodules became more conspicuous (Fig. 4).

2. Recovery period

The oval cells decreased in number, and were only present in the periporal regions in ECD and 50% ECD. The neoplastic nodules could hardly be recognized. Atypical bile ducts, however, were more extended than those during administration (Fig. 5). The area covered by the atypical bile ducts was obviously larger than that during administration, and did not become smaller by removal of the inciting agent (Table 2).

3. PCNA immunohistochemistry

PCNA positive ratio during administration and recuperating period was compared (Table 3). In group ECD it was 24.6% at 8 weeks after administration but 34.0% in the recuperating periods. In group 50% ECD it was 39.5% at 12 weeks, 47.9% in the recuperating period (Fig. 6). From the above results, PCNA positive ratio was higher during

Table 2. Covering Area of Atypical Bile Duct Foci at Final Administration and Recuperation Period (μm^2)

No. of rat Examined	ECD		50% ECD	
	8W ¹⁾	12W (Recovery)	12W	16W (Recovery)
1	143101.2 $\pm$ 101984.6 ²⁾	1920509.2 $\pm$ 1608222.7	607604.2 $\pm$ 657843.4	859243.6 $\pm$ 460993.5
2	125264.2 $\pm$ 49303.7	760373.6 $\pm$ 486536.0	605972.0 $\pm$ 233989.4	762321.2 $\pm$ 583129.1
3	27781.7 $\pm$ 16673.6	429424.6 $\pm$ 291853.3	119672.4 $\pm$ 80273.7	77088.8 $\pm$ 78017.6
4	56065.5 $\pm$ 20183.9	191371.0 $\pm$ 115195.0	477499.3 $\pm$ 401408.4	1169020.6 $\pm$ 867156.1
5	54962.5 $\pm$ 28459.0	273518.7 $\pm$ 189732.6	394623.2 $\pm$ 167638.2	1528404.0 $\pm$ 1152167.1
6	—	—	122402.3 $\pm$ 44151.5	—
Mean	81435.0 $\pm$ 44602.3	715039.4 $\pm$ 633396.8	387962.2 $\pm$ 202621.8	879215.6 $\pm$ 482266.13

¹⁾: Days after administration.²⁾: Values are mean $\pm$ S.D. of five foci.**Table 3.** PCNA Positive Ratio of Lining Cells in Atypical Bile Ducts

No. of focus	ECD		50% ECD	
	8W ¹⁾	12W (Recovery)	12W	16W (Recovery)
1	48/185 (25.9%) ²⁾	59/171 (34.5%)	80/188 (42.6%)	85/144 (59.0%)
2	42/167 (25.1%)	28/152 (18.4%)	81/183 (44.3%)	126/239 (52.7%)
3	34/190 (17.9%)	52/139 (37.4%)	76/236 (32.2%)	88/195 (45.1%)
4	32/124 (25.8%)	64/163 (39.3%)	71/157 (45.2%)	62/156 (39.7%)
5	59/207 (28.5%)	53/128 (41.4%)	69/190 (36.3%)	87/201 (43.3%)
Total	215/873 (24.6%)	256/753 (34.0%)	377/954 (39.5%)	448/935 (47.9%)

¹⁾: Days after administration.²⁾: Number of PCNA positive cells/Number of lining cells in ten tubules.

recovery than that during administration in both ECD and 50% ECD rats.

Discussion

In rat liver ECD and 50% ECD induced not only neoplastic lesions but also severe systemic disorders such as necrosis of kidney and pancreas, atrophy of thymus, pancreas, and genital organs, and calcification of tongue and heart [from our unpublished data]. Therefore, death of rats often seen in the early stage during experimentation is considered not to be associated with hepatic lesions. The cause of rat's death may be due to mainly renal disorders, which grow more severe because of secondary bacterial infection.

Hepatic cellular neoplastic nodules and atypical bile ducts were induced by ECD in rats⁸. The cholangial lesions, in addition to ethionine,

have been reported in rats treated with TAA, 2-AAF, and AAF plus choline deficient^{2,10,11}. Of these chemicals TAA and AAF plus choline deficient progress to cholangiocarcinoma, but there are only a few descriptions of cholangiofibrosis in ethionine. Most of the reports on hepatic carcinogenesis have described hepatic cellular carcinoma, but few have mentioned bile duct changes. It is difficult to differentiate a non-neoplastic cholangiofibrosis from neoplastic cholangiofibroma by only morphological examination. If the lesions regress by removal of inciting agent, they are regarded as non-neoplastic, but on the contrary, if they do not regress they are neoplastic⁷. In the present study it was considered that atypical bile ducts were lesions having probably a neoplastic character, showing conspicuous atypical changes, autonomous development, and oval cell origin. Furthermore, cell proliferating activity as observed

Fig. 6. Recuperating rat PCNA staining at 16 weeks after administration of 50% ECD. Positive reactions are recognized not only at the nuclear portion but also the cytoplasm of the lining cells. Counterstaining with methyl green, $\times 200$.

by PCNA immunohistochemistry was more obvious in recuperating rats than during the course of administration. From these reasons the present atypical bile ducts should be considered as cholangiofibroma rather than cholangiofibrosis.

In liver cancer induced by ethionine, Farber has recognized the existence of irreversible cholangiofibrosis which is not neoplastic⁵. This was because he had not observed progression to carcinoma by long term administration. In Farber's case the bile duct lesion was macroscopically white in color and had a fibrotic appearance, which was undemarcated to the surrounding tissue. On the contrary, that of the present study was nodular and was poorly demarcated from the

surrounding tissue. It was considered that this difference was probably associated with choline, that the choline deficiency perhaps intensified the duct-forming ability of ethionine.

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