0014-4754/96/070710-06$1.50 + 0.20

Experimental liver fibrosis induced in rats receiving high doses of alcohol and alternating between regular and vitamin-depleted diets

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Received 7 April 1995; received after revision 13 December 1995; accepted 5 March 1996

Abstract. Liver fibrosis was induced in rats by simulating human alcoholic eating and drinking patterns. Alcohol addiction was established by gradually increasing the ethanol concentration in the drinking water; salts were added at the terminal stage. The hepatocytes of rats receiving alcohol concentrations exceeding 50% (v/v) (similar to vodka) exhibited alcoholic hyaline (Mallory bodies). Alcoholic liver fibrosis was induced by alternating between regular and autoclaved (vitamin-depleted) diets, simulating the irregular eating habits of human alcoholics. In the livers of rats receiving 70% (v/v) ethanol (comparable to absinthe) with 25% saline and fed the alternating diets, pericellular fibrosis was induced. No significant difference in calorie intake between control and alcohol rats was detected except when rats underwent drinking bouts (heavy drinking phase). This indicates that neither a high-fat diet nor a choline-depleted diet is necessary to induce the alcoholic fibrosis seen in human alcoholics.
Key words. Vitamins; alcoholic hyaline; alternative diet; fibrogenesis.

The mechanisms underlying human alcoholic liver cirrhosis have been investigated. However, hepatitis B, C[1,2] and other viruses have been found to coinfect livers from alcoholic humans, and these virus infections complicate histological findings in alcoholic cirrhosis. Lieber et al.[3] and Rubin and Lieber[4] produced experimental liver fibrosis in primates given alcohol. Efforts have been made to develop animal models that are cheaper and require less time for the induction of alcoholic liver fibrosis, and rats represent a good alternative. Rat studies have used lipotropic factor (choline and methionine) deficient diets[5,6], high-fat diets[7], and the delivery of alcohol into the stomach via tubes connected to a pump. However, these techniques tend to induce fatty liver with vacuoles and without Mallory bodies in the hepatocytes rather than liver fibrosis.

Mallory bodies are frequently observed in the cytoplasm of liver cells from American Indians with childhood cirrhosis and patients with alcoholic hepatitis, or alcoholic cirrhosis[8,9]. Studies in patients with alcoholic hepatitis strongly suggest a hit-and-run effect of alcohol, whereas patients with other chronic liver diseases show evidence of a gradual increase in the number of Mallory bodies as the severity of hepatic pathology increases. Mallory bodies are globularly assembled filaments of intermediate diameter found in cells that contain intermediate filament components (e.g. cytokeratins). They are observable by conventional light microscopy or immunohistochemical methods, and are identical in structure irrespective of the initiating factors or putative pathogenesis. Although three morphological types can be identified in samples from diseased livers under electron microscopy, with the fibrillar structures parallel, random or absent, the presence of Mallory bodies does not guarantee pathogenesis and the biological significance of Mallory bodies remains to be investigated[10].

The question arises as to why Mallory bodies and fibrosis tend to be absent in experimental models of rat alcoholic liver fibrosis. Are there unknown mechanisms that are at work in humans but not in animals? Is a choline-deficient or high-fat diet required for the induction of experimental animal liver fibrosis? We succeeded in training rats to imbibe alcohol ad libitum and found that Mallory bodies developed in the course of stellate and pericellular fibrosis induction.

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Materials and methods

Animals. Male Sprague Dawley rats received a regular diet (CE-2: Clea Japan Inc.) or a nutrient-depleted diet (autoclaved for 30 min at 120°C). A group of 12–18 four-week old rats was maintained under the conventional conditions used in vitamin experiments. The rats were kept in individual cages that allowed their excretions to fall through a wide mesh wire in the bottom of the cage to prevent them ingesting faeces or urine. The rats were fed and housed according to university guidelines, and they were killed after ether anesthesia. For histopathological examination of resected livers, we prepared hematoxylin and eosin (H&E) sections or Azan Mallory sections, and used the Silver impregnation method on 15% formalin-fixed, paraffin-embedded tissues.

Training. Ethanol (99.5%) was diluted with distilled water to obtain alcohol concentrations from 12–70% (v/v) (we used volume/volume % for the ethanol concentration)[11]. The alcohol concentration was increased by 3%/week in three 1% increments. The alternating diets were started upon reaching a 50% alcohol concentration; at that point, salts (0.2% NaCl and 0.02% KCl) were added to the liquid. In normal growth controls, the drinking water was supplemented with 0.2% NaCl and 0.02% KCl at the point where alcohol-drinking rats reached an alcohol concentration of 50%.

Analyses. The serum alcohol concentrations were determined by alcohol dehydrogenase (Sigma diagnostics). Histopathological analyses were performed by qualified pathologists in two independent laboratories to minimize subjectivity. Pathological analysis was performed by Drs K. Hirata (Department of Surgery) and Dr T. Hamada (Department of Surgical Pathology) who did not communicate with each other in the course of these experiments. The regular and autoclaved diets were analyzed according to the provisions of the Japanese Food Sanitation Law, under the supervision of the Environmental Health Bureau, Ministry of Health and Welfare.

Results and discussion

More than 80% of human alcoholics surveyed reported going on drinking bouts (drinking heavily all day for several days)[12]. Most of the heavy drinkers had started drinking when they were less than 20 years old. The quantity and concentration of their alcohol intake increased over the years. Before hospitalization, several of the alcoholics surveyed drank the equivalent of 300–500 ml of absolute ethanol (5–8 ml of ethanol/kg of body weight). Our survey also showed that most of the heavy drinkers did not eat fresh vegetables and fewer than half ingested salty food at the terminal stage. Shortly before hospitalization, they drank almost only alcohol and seldom ate nutritious food (data not shown).

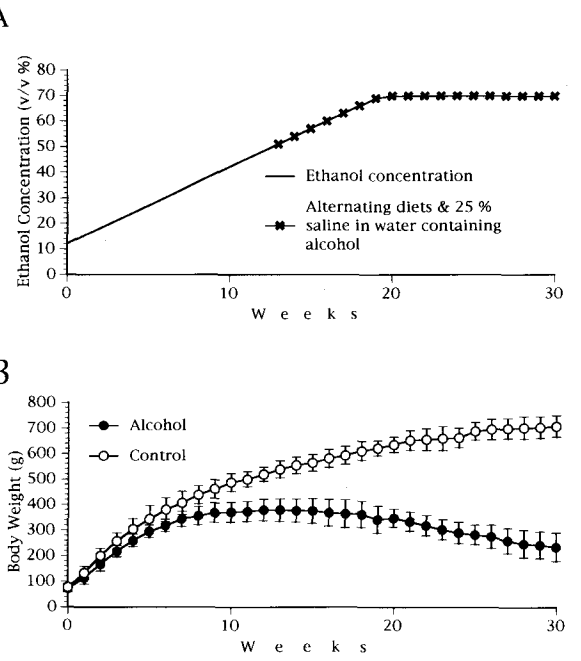

Figure 1. Feeding plan simulating the eating patterns of human alcoholics. *A*) The alcohol concentration was increased by 3%/week in three 1% increments until it reached 70%. Upon reaching a 50% alcohol level, the alternating diets were started and salts were added to the liquid until the end of the experiment. The alternating diets consisted of autoclaved (vitamin-depleted) pellets and regular pellets provided for one week at a time ad libitum. Details of the experiment are described in table 2. *B*) Time course of body weight and S.D. (standard deviation): -○-○-: Mean body weight of control rats (alternating diet, no alcohol), -●-●-: Mean body weight of alcohol-drinking rats.

We simulated these alcoholic eating and drinking patterns in our rats. A low (12%) concentration of ethanol was chosen to induce initial alcohol dependency; at that stage the rats received a regular diet. The 12% ethanol concentration is approximately equivalent to the amount of Japanese sake that was reportedly preferred by Japanese alcoholics at the beginning of their alcoholic history. We increased the alcohol concentration imbibed by our rats by 3% per week in three 1% increments.

In rats receiving more than 50% ethanol, salts (0.2% NaCl, 0.02% KCl) were added to the liquid because almost half of the alcoholics surveyed reported a preference for salty food and/or drink. Another reason for using salts was to prevent dehydration, and to decrease mucosal irritation of the digestive system.

At the same time, rats that imbibed more than 50% ethanol in the salt-supplemented liquid received diets that alternated at weekly intervals (fig. 1). For one week these rats received pellets that had been autoclaved for 30 min at 120°C to reduce their nutritional value (table 1); the next week they received the regular diet. This feeding plan simulates the irregular eating habits and

Table 1. Nutritional value of the diet (CE-2) before and after autoclaving.

	Before	After
Vitamin B_1	1.73 mg (100%)	0.35 mg (20%)
Vitamin C	19 mg (100%)	9 mg (47%)
Retinol	0.36 mg (100%)	0.19 mg (53%)
Vitamin B_{12}	4.9 μg (100%)	2.6 μg (53%)
Inositol	163 mg (100%)	109 mg (67%)
Pantoic acid	2.85 mg (100%)	2.21 mg (78%)
Vitamin B_6	1.26 mg (100%)	0.99 mg (79%)
Niacin	15.9 mg (100%)	14.0 mg (88%)
Vitamin B_2	1.37 mg (100%)	1.21 mg (88%)
Vitamin E	11 mg (100%)	10 mg (91%)
Folic acid	0.15 mg (100%)	0.15 mg (100%)
Biotin	47.8 μg (100%)	48.1 μg (101%)
Choline	250 mg (100%)	260 mg (104%)

Nutrient contents in 100 g of the regular and autoclaved diet. The autoclaved diet is usually given to SPF mice.

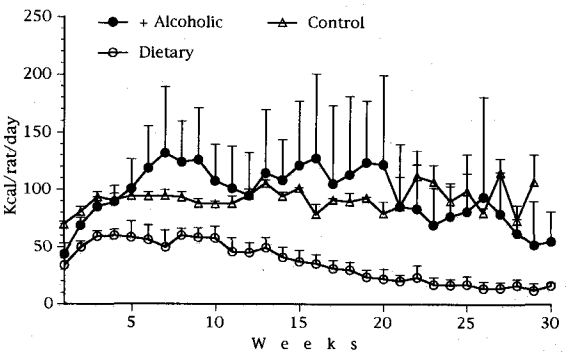

Figure 2. Calculated calorie intake depicting the wide range of standard deviation, indicating the effect of drinking bouts on individual alcoholic rats. -△-△-: Mean calorie intake of control rats. -●-●-: Mean total calorie intake of alcohol-drinking rats, -○-○-: Mean dietary calorie intake of alcohol drinking rats. The regular and autoclaved CE-2 diet contained 3.5 kcal/g (Clea Japan, Inc.), and ethanol contains 7.1 kcal/g[14].

the decreased intake of fresh vegetables encountered in the advanced stage of human alcoholism[13]. Nutrients such as protein (24.3 g/100 g), fat (4.5 g/100 g), cellulose (34 g/100 g), ashes (6.8 g/100 g) and metals (Ca, P, Mg, K, Na, Mn, Fe, Cu, Zn) were similar in the regular and autoclaved diets.

Figure 2 depicts the calorie intake[14] of alcohol-drinking rats and control rats eating the same alternating diet. The mean total (dietary and alcoholic) calorie intakes of rats trained from an early age (4 weeks) to drink alcohol was almost the same as in the control rats. The standard deviation in the total calorie intake of alcohol-dependent rats was very wide, resulting from the drinking bouts of individual rats. Most of the rats engaged in these bouts at 5–10, 15–20, and 25–30 weeks, but it was impossible to synchronize these bouts in our experimental group. The total calorie intake, however, was similar to that of the control rats except during the drinking bout.

Rats that received increased alcohol concentrations from 50 to 70% and the alternating diets were killed at week 25. Fibrosis was present in almost all areas of the liver. Liver fibrosis was noted in the 2 rats that were maintained at an alcohol concentration of 73% (fig. 3B). The other 4 rats stopped eating and died within 3 weeks, and no sections were examined. Autopsies revealed that portions of their intestines were ischemic and filled with liquid that suggested gastrointestinal toxicity. Therefore we reduced the maximum ethanol concentration to 70% (equivalent to absinthe). Typically, animals in this group occasionally stood on their hind legs and held the liquid dispenser with both front paws. After drinking, they fell down slowly and slept with their front legs extended. Upon waking, they repeated this manoeuvre. This activity was reminiscent of the behaviour of humans in the far advanced stages of alcoholism.

Figure 3A shows typical histopathological features of livers obtained at week 18 from rats imbibing up to 63% alcohol. Hematoxylin and eosin staining revealed pericellular fibrosis, swelling of the liver cells, and Mallory body formation. All the liver sections obtained at week 30 from rats imbibing up to 70% alcohol showed pericellular fibrosis, centrilobular fibrosis accompanying alcoholic steatosis, central fibrosis, and stellate fibrosis originating from the Glisson sheath (fig. 3D). In the terminal stage, the rats drank but seldom ingested food. Their liver sections manifested marked pericellular fibrosis.

We found that for the induction of Mallory bodies, the intake of 50 μl of ethanol/day/g body weight (alcohol concentration 40–60% ethanol) was sufficient. However, for the induction of fibrosis, hepatocyte injury or necrosis was also required. The serum alcohol concentration in these rats at 18 and 30 weeks was 0.15 and 0.40%, respectively, as determined by alcohol dehydrogenase. We found that the liver sections of rats imbibing low concentrations of ethanol (12–25%) manifested neither hepatocyte injury nor fibrosis.

Alcohol dehydrogenase is activated in the stomach and small intestine by orally administrated ethanol[15–17]. Chronic ethanol feeding alters the microvillus membrane component only insignificantly and does not alter membrane fluidity. Acute intoxication, however, does produce changes[18]. Therefore, we chronically administered slowly increasing ethanol concentrations to rats. Most of the rats who regularly imbibed 70% alcohol after receiving increasing alcohol concentrations from 12 to 70%, survived until the formation of liver fibrosis, and had neither diarrhoea nor constipation.

We found that feeding of the alternating diet induced liver fibrosis efficiently in rats imbibing high alcohol levels. Fibrogenesis may be induced by alternating be-

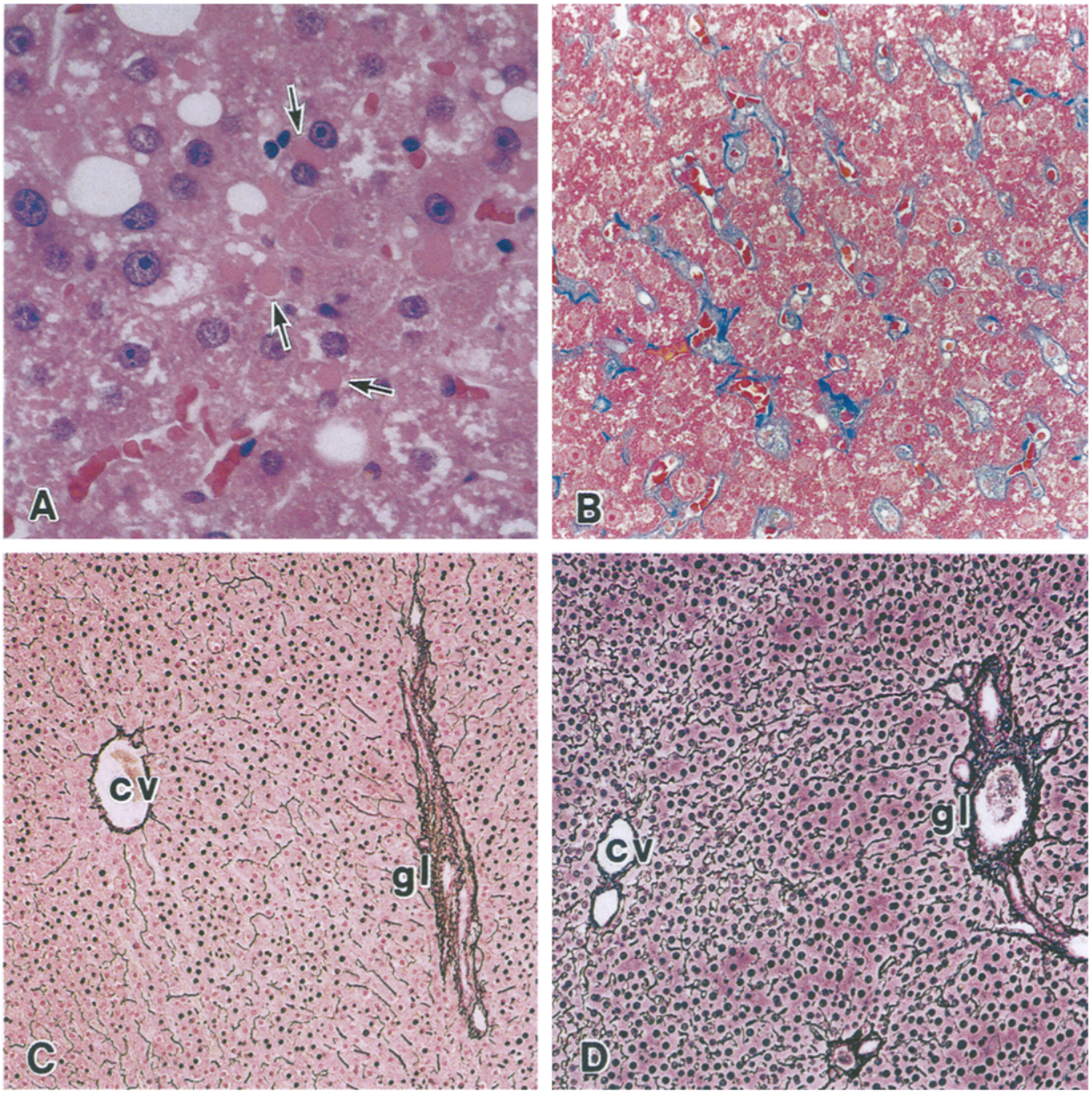

Figure 3. Histopathological features of livers from alcohol-drinking rats were processed for collagen staining using the silver impregnation or Azan Mallory staining. *A*) Hematoxylin and eosin section from a liver obtained at week 18 (alcohol concentration administered, 63%: table 2 Al-1, line 6*) (×400). The arrows indicate Mallory bodies. *B*) Azan Mallory section (×200) of a liver obtained at week 36 (table 2: Al-4 group, lines 13 and 14). *C*) and *D*): Silver impregnated sections obtained at week 30 (×100). *C*) (control): table 2 Con-1 lines 11 and 12*. *D*) (alcohol concentration, increased to, and maintained at 70%) table 2 Al-1, lines 11 and 12*.
*For details, see table 2. cv: central vein, gl: Glisson sheath.

tween normal and vitamin-depleted diets (table 2: (Al-1, lines 11–12) and (Al-4, lines 13–14)). In 70% alcohol-drinking rats maintained on a regular diet (table 2: (Al-2, lines 11–12)) or an autoclaved diet (table 2: (Al-2, lines 9–10)), no significant degree of fibrosis occurred within 30 or 25 weeks, respectively.

Rats in the Al-2 group received an autoclaved diet from week 15 onward. Mallory bodies were noted at 20 weeks, no Mallory bodies or fibrosis was found at 25 weeks. Rats in this group died before week 30, possibly from malnutrition. Rats in the Al-3 group received a normal diet. They exhibited Mallory bodies at 18 and 20 weeks; neither Mallory bodies nor fibrosis were detected at 30 weeks. Upon reaching a 50% alcohol level and until the end of the experiment, rats in all 3 groups received salts (0.2% NaCl and 0.02% KCl) dissolved in their drinking liquid. Rats in the Al-7 group received a normal diet and 12% ethanol. Neither Mallory bodies nor fibrosis were detected at 20, 30 and 36 weeks.

Table 2. Details of the experimental strategy

No.	Alcohol (Al) or control (Con) rats	Score of Mallory body or fibrosis	Al-1	Al-2	Al-3	Con-1	Al-4	Al-5	Con-2	Al-7	Con-3
			12–70%	12–70%	12–70%		12–73%	12–73%		12%	
1	Drinking Training (3%/w)		21 (21)	3 (3)	4 (4)	-	-	-	-	0 (6)	-
2	Drinking Training (2%/w)		-	-	-	-	9 (9)	5 (5)	-	-	-
3	Drinking Bout		21 (21)	3 (3)	4 (4)	-	9 (9)	5 (5)	-	0 (6)	-
4	Mallory body (10w)	o	3 (3)	-	-	0 (3)	-	-	-	-	-
5	Alternative Diet Started (15 w)		18 (18)	Auto	-	14 (14)	-	-	-	-	-
6	Mallory body (18 w)	ooo	4 (4)*	-	1 (1)	0 (4)	-	-	-	-	-
7	Mallory body (20 w)	ooo	2 (2)	1 (1)	1 (1)	-	2 (2)	1 (1)	0 (1)	0 (2)	0 (2)
8	Alternative Diet Started (21 w)		-	-	-	-	7 (7)	-	3 (3)	-	-
9	Mallory body (25 w)	o	1 (3)	0 (1)	-	0 (3)	1 (1)	1 (1)	0 (1)	-	-
10	Pericellular fibrosis (25 w)	(1+)	1 (3)	0 (1)	0 (1)	0 (1)	-	-			
11	Stelle fibrosis (30 w)	(1X)	6 (6)*	-	0 (2)	0 (4)*	-	-	0 (1)	0 (2)	0 (2)
12	Pericellular fibrosis (30 w)	(2+)	6 (6)*	-	0 (2)	0 (4)*	-	-	0 (1)	0 (2)	0 (2)
13	Stelle fibrosis (36 w)	(2X)	-	-	-	-	2 (2)*	-	0 (1)	0 (2)	0 (2)
14	Pericellular fibrosis (36 w)	(3+)	-	-	-	-	2 (2)*	-	0 (1)	0 (2)	0 (2)
15	Died suddenly [total rats used]		2 [21]	1 [3]	0 [4]	0 [17]	4 [9]	3 [5]	0 [4]	0 [6]	0 [6]

Al indicates rats trained to drink alcohol and Con indicates control rats without alcohol. Liver fibrosis was induced in groups Al-1 and Al-4. % indicates the v/v concentration of ethanol[11]. 12–70%: the imbibed alcohol concentration was increased by 3% per week at 1% increments. 12–73%: the imbibed alcohol concentration was increased by 2% per week at 1% increments until it reached 70%, thereafter, it was increased by 1% per week until it reached 73%. Con-1 and Con-2 received the alternating diets (one week regular pellets, the next week vitamin-depleted pellets), the drinking water was supplemented with 25% salts (0.2% NaCl, 0.02% KCl). Con-3 is the growth control group, and these rats received neither alcohol nor salts in their drinking water; they were fed the regular pellets. The numbers in columns Al-1 through Con-3 indicate the number of rats positive for the findings listed in the second column, and adjoining numbers in parentheses indicate the number of rats in the experimental group. Auto indicates a group of rats that received only the autoclaved diet and increasing alcohol concentrations in their drinking water. For scoring the presence of Mallory bodies, three 7 × 7 mm sections were determined from each liver sample. o = One Mallory body-carrying cell was found per field (10 × and 40 × lenses), ooo = More than 3 cells carrying Mallory bodies per field; (1+) = Significant pericellular fibrosis in each section, (2+) = Significant pericellular fibrosis in each field; (1X) = In a few fields, stellate fibrosis originating in Glisson sheath, periportal and perivenular area per sections, (2X) = Stellate fibrosis in the Glisson sheath, periportal and perivenular area per field; - = not examined; *typical features of control and alcohol rats were shown in fig. 3, fig. 3A = group Al-1, line 6, fig. 3B = group Al-4, lines 13 and 14, fig. 3C = group Al-1, lines 11 and 12, fig. 3D = group Con-1, lines 11 and 12.

Extracellular matrix proteins correlate well with fibrosis[19–21]. Using reverse transcription–polymerase chain reaction (RT-PCR), we analyzed the gene expression levels of extracellular matrix proteins in the livers of alcohol-drinking rats at the pre-fibrosis stage. Type IV collagen and laminin gene expression levels increased in parallel with time and increasing alcohol concentration; on the other hand, fibronectin mRNA levels showed an erratic pattern compared to those of β-actin (data not shown). Because we altered the vitamin content of the diets, we need to assess how this change affects the extracellular matrix gene expression levels at a time when the rats are imbibing a high concentration of alcohol 70% (v/v). We are in the process of analyzing these gene expression levels and will report new findings in the near future.

Retinoic acid modulates Ito cell proliferation, collagen, and transforming growth factor β production[22] in rats. Retinol-treated endothelial cell cultures demonstrated an increase in fibronectin[23] gene expression[22]. Gene expression of type I collagen is modulated by vitamin C[24,25] and retinoic acid[22]. In alcoholic liver fibrosis, these vitamins may modulate gene expression of extracellular matrices such as fibronectin, collagens, profilin, and receptor proteins. Other effects of vitamin depletion, of the addition of salts, and of formulaic changes in chemicals in the autoclaved diet need to be investigated.

To induce the formation of alcoholic Mallory bodies in experimental models, the concentration of ethanol should be increased gradually and steadily under a regular diet regimen. To induce alcoholic liver fibrosis, the maximum concentration of ethanol should not exceed 70%. For the rapid induction of fibrogenesis, we found that alternating between the autoclaved diet (low vitamin B_1, C, A, and B_{12}) and the regular diet was successful. We could induce alcoholic liver fibrosis in rats without feeding a low-choline or high-fat diet.

Acknowledgments. We thank Prof. Takashi Mita, Dr Osamu Ohmori, and Prof. Ken Higashi for useful discussion, Prof. Yasuyuki Sasaguri for section analysis, and Clea Japan, Inc. for providing the analysis of the rat food.

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