Cholesterol-Induced Stimulation of Postinflammatory Liver Fibrosis

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 145, No. 6, pp. 638-641, June, 2008
Original article submitted May 10, 2007

We studied the effect of high-cholesterol diet and factors inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase on the development of liver fibrosis in C57Bl/6 mice with CCl_4- or zymosan-induced hepatitis. Feeding a high-cholesterol diet led to a sharp increase in collagen content in the liver tissue of animals with CCl_4-induced or zymosan-induced hepatitis. Atorvastatin and calcitriol produced less pronounced fibrogenic effects. Mevalonate partially prevented the development of cholesterol-induced fibrogenesis. High-cholesterol diet led to accumulation of oxysterols, cholesterol esters, and triglycerides and increased the expression of transforming growth factor-β_1 mRNA in liver tissue. Cholesterol-induced potentiation of the fibrogenic response is probably associated with transforming growth factor-β_1 induction due to accumulation of lipids and oxysterols in the liver.

Key Words: *liver fibrosis; hepatitis; cholesterol; mevalonate; 3-hydroxy-3-methylglutaryl coenzyme A reductase*

Macrophages (MP) play the major role in the fibrogenic response to inflammation. These cells are the main source of cytokines with fibrogenic activity. Transforming growth factor-β (TGF-β) is a key cytokine during hepatitides. Published data show that inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG reductase, a key enzyme suppressing the mevalonate biochemical pathway) induce the expression and production of TGF-β in MP [6].

Here we studied the postinflammatory fibrogenic response under conditions of inhibition of the mevalonate pathway.

MATERIALS AND METHODS

Experiments were performed on male C57Bl/6 mice weighing 22-25 g and feeding a standard or isocaloric cholesterol diet (CH, 2.5%). HMG reductase inhibitors atorvastatin (Lipitor, Pfizer) and calcitriol (1alpha,25-dihydroxyvitamin D3, Rocaltrol, F. Hoffman-La Roche Ltd.) in daily doses of 5 and 0.5 μg/kg, respectively, were administered intragastrically to some mice receiving a standard diet. Mevalonic acid lactone (Sigma) in a daily dose of 50 mg/kg was injected intraperitoneally to prevent inhibition of HMG reductase. The granulomatous inflammatory response in the liver was induced by intravenous injection of zymosan A suspension (50 mg/kg, Sigma). The samples were taken after 2.5 weeks. The diffuse inflammatory response in the liver was induced by intragastric administration of 0.1 ml 10% oil solution of CCl_4 (2 times a week, 6 weeks).

The animals were divided into 3 groups. Group 1 mice received one of the HMG reductase inhibitors (atorvastatin and calcitriol) or dietary CH to inhibit the enzyme. The inflammatory response in group 2 mice was induced after 4-day treatment

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0007-4888/08/1456-0692

with atorvastatin, calcitriol, or CH. Mevalonate was administered to group 3 mice over 3 days before and during treatment with the test substances (period of the inflammatory response). Each subgroup included at least 7 animals.

Control animals received water or physiological saline instead of the test substance (vegetable oil in experiments with CCl_4).

Total lipids were routinely extracted from aqueous homogenate of the liver tissue. The lipid-containing phase was dried under nitrogen flow and dissolved with chloroform. The content of CH, CH esters, triglycerides, and oxysterols (transcriptional inhibitors of HMG reductase) was measured in samples.

The major classes of lipids, including phospholipids, CH, fatty acids, triglycerides, and CH esters, were separated by thin-layer chromatography. CH esters, CH, and triglycerides were routinely assayed in bands.

Oxysterol concentration was measured after soft saponification of lipid extracts [2]. The sterol fraction was extracted, concentrated, and purified by thin-layer chromatography. Oxysterol bands were extracted from silica gel with chloroform. The extracts were studied by reversed-phase high-performance liquid chromatography.

Expression of TGF-β mRNA was studied by the standard method of reverse transcription polymerase chain reaction (PCR). Total RNA was isolated from the liver. Reverse transcription was performed in a reaction mixture containing 1 μg total RNA, 0.5 μg oligo $(dT)_{12\text{-}16}$, buffer, and 200 U M-MULV reverse transcriptase (Biosan). The reaction was conducted at 37°C for 1 h.

PCR was conducted in a reaction mixture (20 μl) of 3 μl cDNA, 0.2 mM each dNTP, 0.7 U Taq polymerase, PCR buffer (Biosan), 500 nM direct primer, and 500 nM reverse primer. The primers were selected using IDT PrimerQuest software. AC CGCAACAACGCCATCTAT and GCAACAATT CCTGGCGTTAC served as the direct and reverse primers of mouse TGF-β_1, respectively. TGTGA TGGTGGGAATGGGTCAG and TTTGATGTCA CGCACGATTTCC served as the direct and reverse primers of β-actin, respectively. The product was distilled in 1.8% agarose gel and stained with ethidium bromide. Densitometry involved Total Lab. software.

Gene expression was calculated as the staining ratio for gene-specific and β-actin-specific bands and expressed in relative units.

Collagen content in the liver was estimated morphometrically after staining of histological sections with Sirius red and fast green (Lopez-de Leon method).

The results were analyzed by standard statistical methods. The significance of differences was evaluated by Student's t test ($p<0.05$).

RESULTS

Feeding the high-CH diet and development of zymosan- or CCl_4-induced inflammatory process were followed by a significant increase in the contents of CH, CH esters, triglycerides, and oxysterols in the liver tissue. The combination of the inflammatory process and high-CH diet did not result in further increase in the contents of CH, CH esters, and triglycerides, while oxysterol concentration increased under these conditions (Table 1). Liver inflammation in mice receiving high-CH diet was accompanied by an increase in the contents of CH, neutral lipids, and transcriptional inhibitors of HMG reductase (oxysterol).

Morphometry of histological sections from control animals showed that high-CH diet has little effect on the content of collagen in the liver tissue.

TABLE 1. Contents of Free CH, CH Esters, Triglycerides, and Oxysterols in the Liver of Mice with Zymosan-Induced or CCl_4-Induced Hepatitis Receiving High-CH Diet ($M\pm m$)

Group	CH, μg/mg protein	CH esters, μg/mg protein	Triglycerides, μg/mg protein	Oxysterols, ng/mg protein	
				25-OH-CH	7-keto-CH
Control	9.0±0.8	7.5±1.1	24.7±1.3	17.1±1.7	—
CH	21.0±1.6***	18.4±1.2***	32.6±2.4*	32.8±4.4**	8.0±0.6
Zymosan	12.2±1.4	9.0±0.7	30.9±1.5*	25.1±3.6	6.1±1.1
CH+zymosan	17.3±1.9**	13.3±1.5**	34.2±3.6**	64.9±5.2***	12.6±2.7
CCl_4	11.5±1.3	14.3±1.7**	45.6±2.7***	23.2±2.0	25.0±1.2
CH+CCl_4	16.7±0.9**	12.6±0.9**	49.0±3.6***	52.5±3.9***	42.4±3.7

Note. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared to the control.

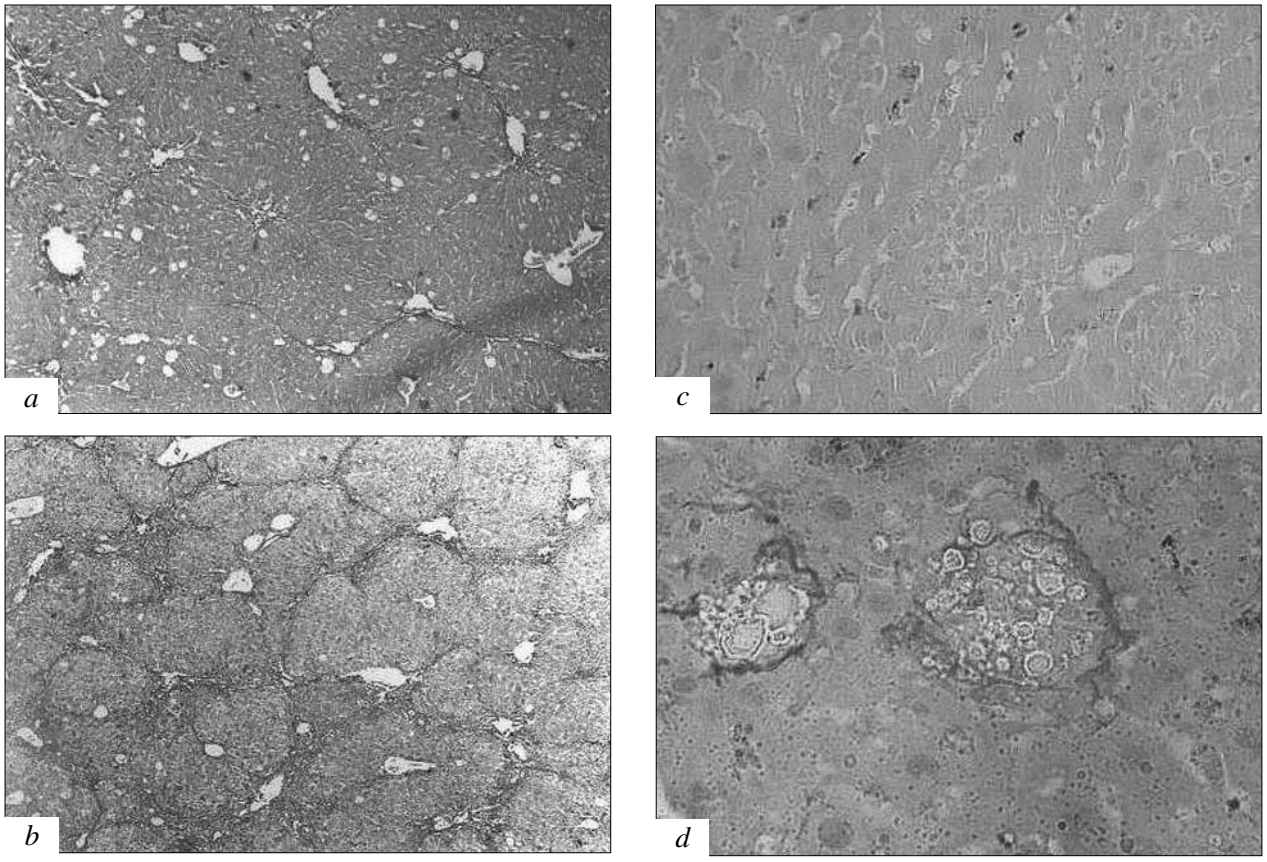

Fig. 1. Typical histological signs of liver tissue in C57Bl/6 mice with CCl_4-induced and zymosan-induced hepatitis receiving standard or high-CH diet. CCl_4 (*a*); CCl_4+CH (*b*); zymosan (*c*); and zymosan+CH (*d*). Staining with picrosirius red and fast green. ×100 (*a*, *b*); ×400 (*c*, *d*).

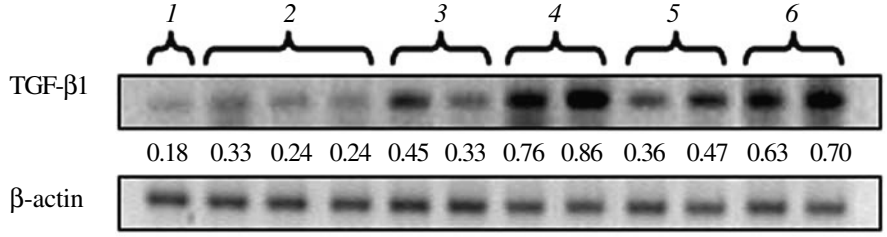

Fig. 2. Effect of 2.5% CH diet on TGF-β mRNA expression in the liver tissue of C57Bl/6 mice with zymosan- or CCl_4-induced inflammation. Intact animals (*1*); CH (*2*); CCl_4 (*3*); CH+CCl_4 (*4*); zymosan (*5*); and CH+zymosan (*6*). Band numbers: optical density ratio for expression of TGF-β_1 and β-actin.

However, consumption of high-CH diet during zymosan-induced or CCl_4-induced hepatitis was followed by a sharp increase in collagen content (by 2 times, Fig. 1). CCl_4 induced signs of moderate liver fibrosis, including the appearance of small perivascular, periportal, and perivenular collagen deposits and presence of connective tissue septa with underdeveloped porto-portal bridges (Fig. 1, *a*). Feeding of high-CH diet during CCl_4-induced inflammation was accompanied by severe liver fibrosis (massive deposits of collagen and signs of pseudonodular transformation; Fig. 1, *b*). Under the influence of zymosan (standard diet), excessive deposits of collagen were not revealed in zymosan-induced granulomas and intralobular area (Fig. 1, *c*). The influence of zymosan in mice receiving high-CH diet was manifested in the formation of perifocal collagen deposits in granulomas (Fig. 1, *d*). Granulomatous encapsulation was accompanied by the appearance of intralobular collagen deposits (thin separate fibers).

We studied whether the fibrogenic effect of CH is associated with oxysterol accumulation in the liver tissue and inhibition of HMG reductase. Some mice received high-CH diet and zymosan injection during daily treatment with mevalonate. Mevalona-

TABLE 2. Collagen Content in Liver Tissue of Mice with Zymosan-Induced or CCl_4-Induced Hepatitis Receiving High-CH Diet, Atorvastatin, or Calcitriol and Treated or Untreated with Mevalonate (*M*±*m*)

Group	Volume density of collagen deposits in liver tissue, arb. units
Control	25.4±1.0
CH	27.40±1.04
Zymosan	27.60±1.81
Mevalonate	25.60±2.61
Atorvastatin	26.8±3.6
Calcitriol	29.2±1.9
CH+zymosan	47.1±3.2***+
Atorvastatin+zymosan	32.5±2.7*
Calcitriol+zymosan	33.4±2.7*
Mevalonate+CH+zymosan	34.8±3.2**°
Mevalonate+atorvastatin+zymosan	29.34±4.20
Mevalonate+calcitriol+zymosan	29.13±3.0
CCl_4	53.5±8.2***
CH+CCl_4	104.7±10.6ˣ
Mevalonate+CH+CCl_4	86.1±11.5

Note. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared to the control; +$p<0.001$ compared to zymosan; °$p<0.05$ compared to CH+zymosan; ˣ$p<0.001$ compared to CCl_4.

te can prevent the effect of HMG reductase inhibition. We showed that mevalonate significantly decreased the stimulatory effect of high-CH diet on collagen formation. Other inhibitors of HMG reductase had potent, but statistically insignificant fibrogenic effect on mice with zymosan-induced hepatitis. Mevalonate abolished this effect (Table 2).

Feeding of high-CH diet and administration of atorvastatin or calcitriol to animals with CCl_4-induced and zymosan-induced hepatitis did not result in further increase in ALT activity in blood plasma. Probably, the increased induction of fibrogenesis was not associated with the alkylating effect of these substances.

Our results show that feeding of high-CH diet and administration of pharmacological inhibitors of HMG reductase potentiate the fibrogenic response in animals with liver inflammation. Treatment with mevalonate prevents these changes.

High-CH diet resulted in increased expression of TGF-β_1 mRNA in liver tissue (more than 1.5 times). Under these conditions the severity of CCl_4-induced and zymosan-induced inflammation increased by 2.2 and 2.3 times, respectively. The expression increased most significantly during inflammation and consumption of high-CH diet. CH increased the content of TGF-β_1 mRNA in mice with CCl_4-induced and zymosan-induced hepatitis (by 4.5 and 3.7 times, respectively, compared to the intact control; Fig. 2).

We conclude that CH-dependent induction of fibrogenesis during hepatitis is related to increased expression of TGF-β. Published data show that oxysterols significantly increase the expression and production of TGF-β in MP [4]. In our experiments, high-intensity fibrogenesis is probably associated with increased expression of TGF-β due to oxysterol accumulation in MP. We found that mevalonate significantly decreased the CH-induced fibrogenic response. It can be hypothesized that fibrogenic activity of oxysterols is related to inhibition of HMG reductase. This assumption is confirmed by the fibrogenic effect of atorvastatin and calcitriol. Another mechanism for potentiation of the fibrogenic response is associated with accumulation of neutral lipids and fatty acids in the liver. These substances undergo lipid peroxidation and stimulate TGF-β production during inflammation [5,7].

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