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Effects of Cholesterol and Nuclear Hormone Receptor Agonists on the Production of Transforming Growth Factor-β in Macrophages

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We studied the effects of cholesterol, its oxidized derivatives mevalonate, and nuclear receptor agonists LXR, RXR, and FXR on the production of transforming growth factor-β1 (TGF- β1) by macrophages. After recruiting of macrophage monocytes into the focus of inflammation, the production of TGF-β1 increased by 3.5 times in comparison with control macrophages. Cholesterol diet stimulated the production of TGF-β1 by 2.5 times. Cholesterol directly stimulated macrophage production of TGF-β1 *in vitro*, while addition of mevalonate to the incubation medium effectively reduced this induced production. Agonists of nuclear receptor sharply reduced the production of TGF-β1 in recruited macrophages. Under conditions of inflammation, hypercholesterolemia can be a factor of fibrogenesis due to TGF-β1 induction in macrophages, which depends on the products of mevalonate biochemical chain.

Key Words: *transforming growth factor-β; macrophage; cholesterol; nuclear hormone receptors; mevalonic acid*

Chronic inflammatory processes are often associated with hypercholesterolemia. It can promote the development of postinflammatory fibrosis and organ sclerosis. It was demonstrated on the models of nephrosclerosis [2], hepatofibrosis [1], atherosclerosis, and other processes that cholesterol diet sharply stimulated fibrogenic response under conditions of chronic alteration of tissues and inflammatory reaction. This intense fibrogenesis is associated with hyperexpression of TGF-β1, the key cytokine stimulating fibrosis [1,4].

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Mononuclear phagocytes are the main cell producers of TGF-β1 in the kidneys, liver, and arterial wall. However, the mechanisms of TGF-β1 elevation in hypercholesterolemia remain little studied. Presumably, cholesterol (CH) can directly induce the synthesis and release of TGF-β1 in macrophage monocytes.

The effect of CH on macrophages can be explained by inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A-reductase (HMG-CoA reductase), the key enzyme of the mevalonate biochemical chain realizing mevalonate synthesis. The data on the effects of HMG-CoA reductase inhibitors on the production of TGF-β1 are contradictory [10,11]. A possible experimental approach is the use of mevalonate, a direct product of HMG-CoA reductase activity, which abolishes the effects of its inhibition.

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Inhibition of HMG-CoA reductase can be due to not only direct effect of CH, but also to the effects of its oxidized derivatives (hydroxysterols). Hydroxysterols can inhibit HMG-CoA reductase via binding to the LXR nuclear hormone receptors [12]. The ligands of other nuclear receptor (RXR and FXR) are also involved in the HMG-CoA reductase down regulation [8]. It is unknown whether CH effect on TGF-β1 production depends on LXR and other nuclear receptors.

In contrast to the CH effect, the proinflammatory stimuli and cytokines stimulate activity of HMG-CoA reductase and mevalonate chain [6]. The effect of CH on the production of TGF-β1 in inflammation-stimulated macrophages is virtually not studied, and it is unknown, how much this production depends on the nuclear hormone receptors and the mevalonate chain products.

A problem attracting special interest is macrophage capacity to produce TGF-β1 under conditions of the so-called macrophageal hyporeactivity, characteristic of chronic inflammatory response and developing in macrophages during lasting or repeated treatment with LPS or other inflammatory agents. The role of TGF-β1 and, more so, of the mevalonate chain in the development of this condition is virtually not studied.

We studied the effect of CH on the production of TGF-β1 in inflammation-stimulated macrophages and evaluated the relationship between this production and activity of the mevalonate chain and nuclear hormone receptors.

MATERIALS AND METHODS

Mononuclear phagocytes were isolated from male C57Bl/6 mice kept under vivarium conditions on standard laboratory diet with free access to water and food or under the same conditions on isocaloric 2.5% CH diet for 2 weeks. Four days before sacrifice, the experimental mice were intraperitoneally injected with 4% hydrated 1.4-α-glycan; controls received buffered saline (0.9% NaCl, 50 mM phosphate buffer, pH 7.4).

One day before sacrifice, some animals were intraperitoneally injected with 50 μg/kg *E. coli* 0111:B4 LPS. Some animals received the same dose twice: 2 and 1 days before sacrifice. Peritoneal lavage cells were then obtained. Macrophages were isolated by adhesion to the sublayer and cultured at 37°C, 5% CO_2, and 100% humidity, 250,000 cells/well in 24-well plate in serum-free RPMI-1640 with 2 mM HEPES and a mixture of antibiotics for cell culturing (MP Biomedicals). Macrophage monolayers were preincubated for 2 h with CH (5 μg/ml, hydroxysterol 7-keto-CH (5 μg/ml), LXR hydroxysterol ligand 25-hydroxy-CH (5 μg/ml), with RXR ligand 9-*cis*-retinoic acid (5 μM), or with FXR ligand farnesole (10 μM). Mevalonic lactone (2 mM) was then added into some wells and after 2 h 200 ng/ml *E. coli* 0111:B4 LPS was added to some of these wells. The monolayers were incubated for 24 h, the incubation medium was collected, frozen at -20°C, and stored for 1-1.5 weeks before measurement of TGF-β1. Macrophages from animals fed CH diet were cultured without CH or ligands to nuclear hormone receptors in incubation medium.

The concentration of TGF-β1 in supernatants was measured by solid-phase EIA with the Biosource kits according to the instruction. Experiments were repeated 3 times in triplexes.

The significance of differences between the means was evaluated using Student's *t* test.

RESULTS

The level of TGF-β1 in culture medium with macrophages isolated from animals receiving no inflammatory stimulus (constitutive secretion of resident macrophages) was 0.96±0.05 pg/10^3 cells. Aseptic inflammation in the abdominal cavity induced by intraperitoneal injection of α-glycan 4 days before sacrifice recruited macrophage monocytes into the abdominal cavity and more than 3.5-fold increased the production of TGF-β1. This increase of TGF-β1 production by peritoneal macrophages is in line with the data on TGF-β1 induction under the effect of eliciting agents [9]. Incubation of recruited macrophages with CH significantly (p<0.05) increased TGF-β1 production. Though induction of TGF-β1 expression in macrophages *in vivo* in hypercholesterolemia is a known fact [4], the direct stimulatory effect of CH on TGF-β1

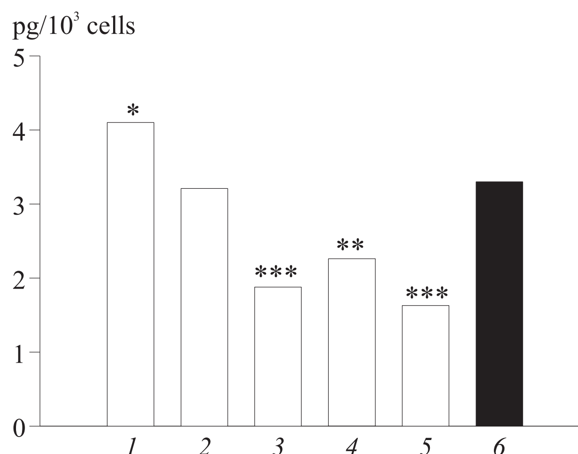

Fig. 1. Effects of CH (*1*), 7-keto-CH (*2*), 25-hydroxy-CH (*3*), 9-*cis*-retinoic acid (*4*), and farnesole (*5*) on the production of TGF-β1 in recruited peritoneal macrophage culture; *6*) production of TGF-β1 in macrophages incubated without CH, 7-keto-CH, and nuclear receptor agonists (control). Here and in Figs. 2, 3: *p<0.05, **p<0.01, ***p<0.001 compared to the control.

production in macrophages was demonstrated for the first time.

In contrast to the effect of CH, incubation of macrophages with nuclear receptor agonists reduced TGF-β1 secretion, elevated in recruiting (Fig. 1). The level of TGF-β1 was most effectively reduced by farnesole and 25-hydroxy-CH, while retinoic acid was less effective. Reduction of TGF-β1 production by the nuclear receptor agonists indicates that stimulation of the nuclear receptor function blocks the growth of not only pro-, but also of antiinflammatory molecules of the cytokine cascade. The production of TGF-β1 was just negligibly reduced by 7-keto-CH hydroxysterol, incapable of reacting with LXR.

Culturing of macrophages with mevalonate reduced the production of TGF-β1 in the control and during incubation with CH, 7-keto-CH, and 25-hydroxy-CH, but had no effect on cells incubated with retinoic acid and farnesole (Fig. 2). Hence, the effects of CH and hydroxysterols on the production of TGF-β1 depend on the intermediate products of the mevalonate biochemical chain, including (presumably) farnesole.

Incubation of macrophages with LPS led to a slight increase in TGF-β1 production. In the presence of nuclear receptor agonists, LPS stimulated significantly the production of TGF-β1. On the other hand, LPS effect in the presence of CH was slight, while in the presence of 7-keto-CH LPS reduced significantly this production. The data on LPS capacity to induce the production of TGF-β1 in macrophage monocytes are contradictory. Some authors observed incapacity of LPS to modify it [13], while others demonstrated a stimulatory effect on TGF-β1 production [5]. Our data indicate that the effect of LPS on the production of TGF-β1 depends critically on the macrophage reactivity, induced by their conditioning, and that CH, 7-keto-CH, and nuclear receptor agonists condition/modify macrophage reactivity differently.

The data obtained on animals receiving CH diet are in general similar to *in vitro* data (Fig. 3). Cholesterol diet led to a significant (p<0.001) increase in TGF-β1 production in macrophages; incubation of these cells with LPS did not modify, and with mevalonate slightly reduced the production of TGF-β1. Simultaneous addition of LPS and mevalonate into the incubation medium also reduced significantly the production of TGF-β1.

A single injection of LPS *in vivo* reduced the production of TGF-β1, while two injections led to an increase of TGF-β1 release (p<0.001). Two and more injections of LPS is a model of LPS tolerance formation in macrophages [3]. Since TGF-β causes macrophage hyporactivity [7], hyperproduction of TGF-β1 seems to be an autocrine mechanism of tolerance formation

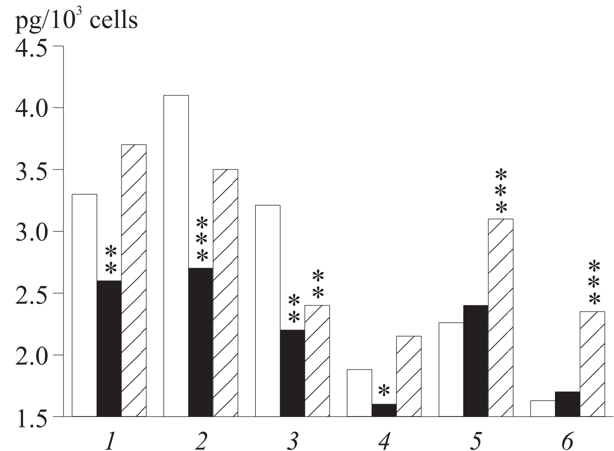

Fig. 2. Effects of mevalonic lactone and *E. coli* 0111:B4 LPS on the production of TGF-β1 in culture of recruited peritoneal macrophages, incubated without (*1*) or with CH (*2*), 7-keto-CH (*3*), or nuclear hormone receptor ligands: 25-hydroxy-CH (*4*), 9-*cis*-retinoic acid (*5*), or farnesole (*6*). Light bars: incubation without mevalonate and LPS (control); dark bars: with mevalonate; cross-hatched bars: with LPS.

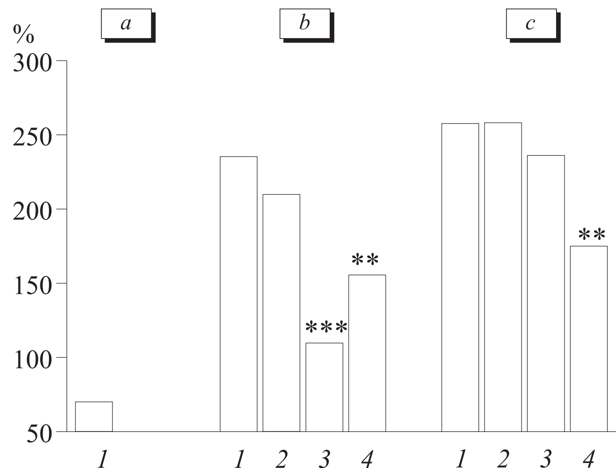

Fig. 3. Effect of intraperitoneal injections of *E. coli* 0111:B4 LPS - single (*a*) or double (*b*), or CH diet (*c*) on the production of TGF-β1 in culture of recruited peritoneal macrophages incubated with mevalonic lactone, LPS, or both. *1*) incubation without mevalonate and LPS (control); *2*) with LPS; *3*) with mevalonate; *4*) with LPS and mevalonate. Production of TGF-β1 by peritoneal macrophages, isolated from intact animals and incubated without mevalonate and LPS, is taken for 100%.

and maintenance. Effective arrest of TGF-β1 hyperproduction with mevalonate in the LPS tolerance model indicates that this status depends on the synthesis of the mevalonate chain products.

Hence, recruiting of macrophage monocytes into the focus of inflammation results in a sharp increase of TGF-β1 production in these cells, and CH diet stimulates this production. This increase of TGF-β1 production can be caused by direct effect of CH on recruited macrophages. The known biological effects of TGF-β1 suggest that under conditions of inflammatory recruit-

ing of macrophages hypercholesterolemia can be a factor of fibrogenesis and attenuation of the inflammatory response severity. Nuclear hormone receptor agonists down regulate the production of TGF-β1 and presumably can be effective antifibrogenic agents. Since mevalonate abolishes the CH-induced increase of TGF-β1 production, this increase is mediated, at least partially, by intermediate products of the mevalonate biochemical chain, but not by nuclear hormone receptors. The use of derivatives of the mevalonate chain products in fibrous processes can be a new approach to the treatment of organ sclerosis.

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