GENETICS

Effects of Hydroxystyrene on the Expression of the Inflammatory Cytokine Genes and Their Content in Macrophages Tolerant to Endotoxin

M. I. Dushkin, E. I. Vereshchagin*, A. Yu. Grebenshchikov, A. F. Safina***, and Ya. Sh. Shvarts**

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 127, No. 1, pp. 71-74, January, 1999
Original article submitted March 30, 1998

Preincubation of resident macrophages with 25-hydroxycholesterol decreased the lipopolysaccharide-induced expression of mRNAs of tumor necrosis factor and interleukin-1, while 7-ketocholesterol stimulated the expression of the tumor necrosis factor mRNA. Tolerant macrophages characterized by decreased tumor necrosis factor secretion in response to lipopolysaccharide have increased (in comparison with resident macrophages) content of 25- and 27-hydroxycholesterol.

Key Words: *hydroxystyrenes; cytokines; endotoxin tolerance; macrophages*

Oxidized cholesterol derivatives (hydroxystyrenes) have wide-spectrum biological activity and effectively modulate the immune functions of macrophages and lymphocytes [2,8]. 25-Hydroxycholesterol (25-hydroxyCS) and 7-ketoCS inhibit the early stages of T lymphocyte activation [10] and reactions participating in the macrophage-lymphocyte responses [7]. Recent experiments on macrophage cultures have shown that 25-hydroxyCS stimulates the production of interleukin-8 (IL-8) that regulates chemotaxis of polymorphonuclear lymphocytes [11] and inhibits the binding of the nuclear factor kB to DNA involved in the regulation of expression of tumor necrosis factor (TNF) and IL-1 genes in endotoxin-activated cells [15]. Intraperitoneal injection of 7-25-hydroxyCS to mice induced monocyte and neutrophil migration and decreased the endotoxin-induced expression of I(a)-antigens on the surface of peritoneal macrophages [13]. Unusually high concentrations of hydroxystyrenes were detected in atherosclerotic plaques and foam cells of macrophagal origin, overloaded with CS [9]. Atheroma and granulomatous inflammation develop in a similar way, which allows us to regard atherosclerosis as a form of chronic inflammation involving macrophages transformed into foam cells [4]. The tolerance status, which was simulated by reinjections of endotoxin in low doses, is an important factor in the development of chronic inflammation associated with a low level of TNF and IL-1 production in macrophages [14]. The contribution of hydroxystyrenes to macrophage tolerance and chronization of the inflammatory process has not been investigated. We examined the effects of two typical hydroxystyrenes 25-hydroxyCS and 7-ketoCS on the expression of TNF and IL-1 genes and analyzed the content of endogenous hydroxystyrenes in resident and endotoxin-tolerant peritoneal macrophages of rats.

Institute of Therapy, Siberian Division of Russian Academy of Medical Sciences; *Medical Institute; **Institute of Clinical Immunology, Siberian Division of Russian Academy of Medical Sciences; ***Institute of Clinical and Experimental Lymphology, Siberian Division of Russian Academy of Medical Sciences

MATERIALS AND METHODS

Experiments were carried out on Wistar rats weighing 200-250 g. Endotoxin tolerance was induced by 4

0007-4888/99/0001-0063 $22.00

intravenous injections of *E. coli* 0127:B8 endotoxin (Sigma) in a dose of 500 μg/kg every 24 h for 4 days. Control animals were injected with an equal volume of normal saline according to the same protocol. Peritoneal macrophages were isolated by lavage in Hanks' solution and subsequent 2-h incubation in RPMI-1640 with 10% fetal calf serum and 50 μg/ml gentamycin at 37°C in an atmosphere with 5% CO_2, followed by washing of the macrophage monolayer from lymphocytes and neutrophils [3]. The monolayer of cells from intact rats was incubated for 20 h in the presence of 5 μg 25-hydroxyCS, 7-ketoCS, and CS (Sigma), washed in Hanks' solution, incubated with and without 5 μg/ml endotoxin for 2 h, and the intracellular concentrations of TNF and IL-1 mRNA were then measured. The total RNA was extracted by guanidine isocyanate [6]. The concentrations of the TNF and IL-1 mRNA were determined by dot-blot hybridization. The RNAs were denatured by formaldehyde, transferred on nitrocellulose filters by capillary transfer, and incubated with ^{32}P-labeled cDNA obtained by the nic-translation method [1]. Radiograms were scanned in an Ultrascan device (LKB).

The tolerance of peritoneal macrophages to endotoxin was tested by their ability to produce TNF in response to endotoxin on L929 cells sensitive to TNF. Endogenous hydroxystyrenes were measured in resident and endotoxin tolerant macrophages. For this purpose, total lipids were extracted from cells with hexane-isopropanol (3:2) [3], concentrated under nitrogen current, and soft saponification was carried out in a methanol:water mixture (5:1) containing 5 M NaOH and butyryl hydroxytoluene (10 μg/ml) for 15 h at 18-20°C [5]. The steroid fraction was extracted with a chloroform:methanol mixture (2:1), and the samples concentrated under nitrogen flow were analyzed by reverse-phase chromatography on a 100×2.3 mm column packed with C18 Nucleosil (5 μ in diameter) with elution by the methanol:water mixture (9:1). The clusters were analyzed with a Gilson Int. ultraviolet detector at a wavelength of 211 nm. The reference samples were 25- and 27-hydroxyCS (Research-plus).

The results were statistically processed using Student's *t* test.

RESULTS

The effects of 24-hydroxyCS and 7-ketoCS on the expression of TNF and IL-1 genes were compared with that of purified CS in resident peritoneal macrophages incubated with and without endotoxin in a dose of 5 μg/ml medium. Without endotoxin induction, the levels of TNF and IL-1 mRNA in macrophages were decreased, markedly increasing after the addition of endotoxin to the incubation medium. No significant changes in the steroid effect on the basal level of TNF and IL-1 mRNA were observed. Preincubation of macrophages with 5 μg/ml 25-hydroxyCS followed by 2-h stimulation of cells by endotoxin led to a significant decrease in the level of IL-1 (Fig. 1, *a*) and TNF mRNA (Fig. 1, *b*) (by 57 and 30%, respectively). By contrast, 7-ketoCS increased the endotoxin-induced level of TNF mRNA but did not affect the expression of the IL-1 gene. 7-ketoCS is often produced during autooxidation of CS and is a predominant hydroxystyrene in oxidized LDL [12]. Our data on increased expression of TNF under the effect of 7-ketoCS may be referred to previously observed anti-inflammatory properties of oxidized lipoproteins. 25-hydroxyXS and similar styrenes hydroxylated in the aliphatic CS series (26 and 27-hydroxyCS) may be the products of intracellular hydroxylases [5]; the concentrations of these steroids increase considerably in foam cells overloaded with CS [9]. Our results indicate that unlike 7-ketoCS, 25-hydroxyCS promotes the tolerance to endotoxin stimulation in peritoneal macrophages. One of the probable mechanisms of macrophageal tolerance to prolonged exposure to endotoxin *in vivo* is intracellu-

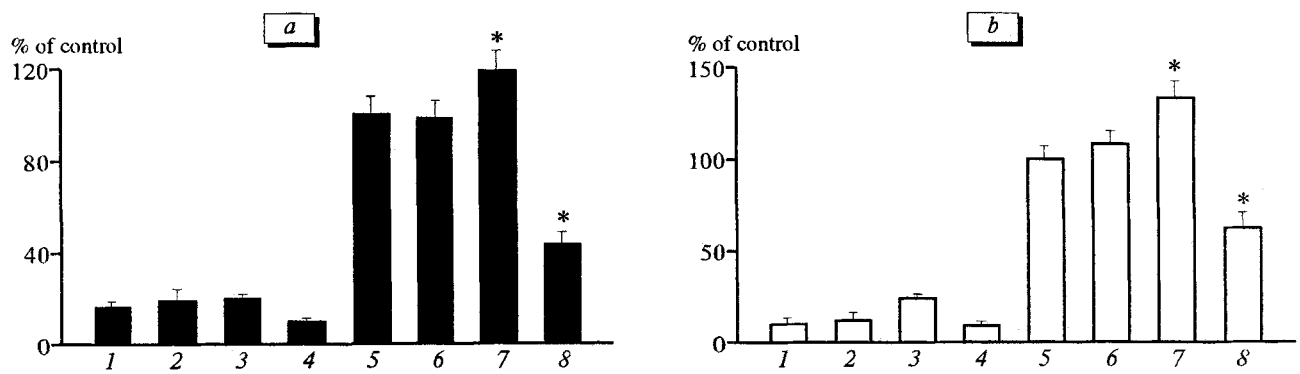

Fig. 1. Effects of cholesterol (CS) derivatives on the content of interleukin-1 (*a*) and tumor necrosis factor mRNAs (*b*) in peritoneal macrophages. *1*) control; *2*) incubation of macrophages with CS; *3*) with 7-ketoCS, 5 μg/ml; *4*) with 25-hydroxyCS, 5mg/ml; *5*) with LPS, 5 μg/ml; *6*) with LPS and CS; *7*) with LPS and 7-ketoCS; *8*) with LPS and 25-hydroxyCS. *$p<0.05$ *vs. 5.*

lar accumulation of hydroxystyrenes. To check up this hypothesis, we measured the content of hydroxystyrenes in macrophages of endotoxin-tolerant and control rats. Incubation of isolated peritoneal macrophages in the presence of 25 μg/ml endotoxin led to an increase in the cellular production of TNF both in tolerant and control animals. However, the production of TNF by macrophages of control rats was 4 times higher in comparison with the macrophages of animals treated with endotoxin (8093±490 and 2044±560 U/10^6 cells, respectively, $p<0.05$), which confirms the development of endotoxin tolerance in rat macrophages after its fractionated injections *in vivo*.

Measurements of hydroxystyrenes showed the concentrations of 25- and 27-hydroxyCS in resident and endotoxin-tolerant peritoneal macrophages, increased with the development of tolerance, as estimated per certain number of cells (Fig. 2). In tolerant macrophages the content of 25-hydroxyCS was 4.85 μg/10^7 cells. The concentrations of 25- and 27-hydroxyCS in tolerant macrophages were 90 and 85% higher, respectively, than in resident macrophages. The CS/25-hydroxyCS ratio in tolerant macrophages was 60% lower than in resident cells. Characteristically, the content of 25-hydroxyCS in peritoneal macrophages was higher than that of 27-hydroxyCS. The 25/27-hydroxyCS ratio in the tolerant and resident macrophages was 2.3 and 1.8, respectively.

Our data suggest that intracellular products of CS hydroxylation serve as local factors of steroid nature, regulating the development of the inflammatory response of macrophages.

The study was supported by grants of the Russian Foundation for Basic Research (No. 04-49940) and of a priority program of medicine "Atherosclerosis".

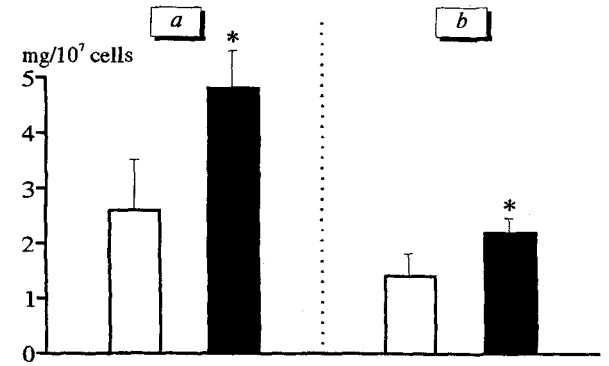

Fig. 2. Concentration of 25-hydroxycholesterol (*a*) and 27-hydroxycholesterol (*b*) in resident (light bars) and tolerant (dark bars) peritoneal macrophages. *$p<0.05$ *vs.* resident macrophages.

REFERENCES

1. V. V. Abramov, A. F. Poveshenko, A. Yu. Grebenshchikov, and V. V. Kozlov, *Byull. Sibirsk. Otdeleniya Rossiisk. Akad. Med. Nauk*, No. 1, 60-64 (1995).
2. M. I. Dushkin, *Uspekhi Sovrem. Biol.*, **111**, No. 6, 845-857 (1991).
3. M. I. Dushkin, E. V. Mandrikova, G. Yu. Lyubimov, *et al.*, *Biokhimiya*, **55**, 1607-1615 (1990).
4. V. A. Nagornev and E. G. Zota, *Uspekhi Sovrem. Biol.*, **116**, No. 3, 320-338 (1996).
5. I. Bjorkhem, O. Anderson, U. Diczylalusy, *et al.*, *Proc. Natl. Acad. Sci. USA*, **91**, 8592-8596 (1994).
6. P. Chomszynsky and N. Sacchi, *Anal. Biochem.*, **162**, 156-159 (1987).
7. M. Dushkin, Y. Shwartz, and A. Grebenschikov, *Atherosclerosis*, **134**, No. 1,2, 230 (1997).
8. F. Guardiola, R. Codony, P. B. Addis, *et al.*, *Food Chem. Toxicol.*, **34**, 193-211 (1996).
9. L. M. Hulter, H. Lindmark, U. Diczfalusy, *et al.*, *J. Clin. Invest.*, **97**, 461-468 (1996).
10. O. Kucuk, J. Stoner-Picking, S. Yachnin, *et al.*, *Lipids*, **29**, 657-660 (1994).
11. Y. Liu, L. M. Hulten, and O. Wiklund, *Arterioscler. Tromb. Vasc. Biol.*, **17**, 317-323 (1997).
12. I. Maor, H. Mandel, and M. Aviram, *Ibid.*, **15**, 1378-1387 (1995).
13. C. Y. H. Moog, C. Waltzinger, B. Luu, and P. Bischoff, *Immunology*, **70**, 344-350 (1990).
14. J. N. Moore, J. A. Cook, D. D. Morris, *et al.*, *Circ. Shock*, **31**, 281-295 (1990).
15. B. G. Ohlsson, M. C. O. Englund, A. Karlsson, *et al.*, *J. Clin. Invest.*, **98**, 78-89 (1997).