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Effect of Polysaccharides and Human Plasma Lipoproteins on the Secretion of Cystatin C by Peritoneal Macrophages from Normal and Tumor Bearing Mice

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Abstract—Intact peritoneal macrophages *in vitro* secreted the cysteine proteinase inhibitor cystatin C. Polysaccharides stimulated cystatin C secretion: lipopolysaccharide < carboxymethylated β-D-glucan < sulfoethylated β-D-glucan. Human plasma low-density- (LDL) and high-density lipoproteins (HDL) are still more potent inducers of cystatin C secretion by macrophages. Peritoneal macrophages from mice with experimental HA-1 hepatoma compared to those from intact mice secreted more cystatin C with maximum polysaccharide-stimulated secretion after 30 min of incubation. LDL and HDL induced cystatin C secretion by tumor macrophages also.

Key words: cystatin C, peritoneal macrophage, lipoprotein, hepatoma HA-1

Cystatins (stefins, cystatins, and kininogens) are natural inhibitors of cysteine proteinases [1]. In tumors, enhanced secretion of proteinases occurs leading to a shift of proteinase/proteinase inhibitor ratio. Decreased level of inhibitors results in uncontrolled tumor progression [2]. When benign murine papilloma transformed into carcinoma, the level of stefin A mRNA decreased [3]. In tumor cell culture, activities of cathepsins B and L were found to increase, whereas the levels of cystatin C and stefin A drastically (4-15 times) decreased [4]. Transfection of carcinoma cells with the cystatin C gene decreased invasive capability of the tumor [5]. The synthetic cysteine-proteinase inhibitor E-64 sensitized bleomycin-resistant carcinoma *in vivo* resulting in persistent and complete inhibition of tumor growth [6]. Another synthetic cysteine-proteinase inhibitor, low-molecular-weight and selective Z-Phe-Arg-fluoromethyl ketone, inhibited pancreatic tumor progression and decreased tumor cell number during *per os* administration to mice [7]. Immunocytochemical studies have demonstrated that cystatin C is synthesized in almost all cell types, mainly in neuroendocrine cells and macrophages [8]. Cystatin C mRNA has been found in macrophages [9].

In this study, our goal was to evaluate the effect of polysaccharides and blood serum lipoproteins on cystatin C secretion in physiological circumstances and upon tumor progression.

MATERIALS AND METHODS

C57BL/6J mice were primed *in vivo* with 4% starch solution 3-5 days before sampling. Macrophages were isolated from the peritoneal lavage of the mouse with 5 ml of RPMI-1640 culture medium. The lavages from 3-4 mice were pooled and plated 2 ml on 35-ml Costar Petri dishes ($(1.5\text{-}2.0)\cdot10^6$ cells per dish) [10]. Cell monolayer (250-300 μg protein) was incubated for 1 h at 37°C and washed twice with RPMI-1640 for removal of unattached cells. The macrophages were stimulated *in vitro* using polysaccharides from bacteria (*Escherichia coli* lipopolysaccharide (LPS), Sigma, USA) and yeasts (carboxymethylated (CMG) and sulfoethylated β-D-glucan (SEG) provided from the Institute of Chemistry, Slovak Academy of Sciences, Bratislava). LPS, CMG, and SEG were applied at 10 μg/ml; low-density- (LDL) and high-

Abbreviations: LPS) lipopolysaccharides; LDL) low density lipoproteins; HDL) high density lipoproteins; CMG) carboxymethylated glucan; SEG) sulfoethylated glucan.

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density-lipoproteins (HDL) at 1 mg/ml protein. Cells were then incubated for 2 h at 37°C.

Hepatoma HA-1 (differentiated hepatocarcinoma) derived from male A/Sn(A) mouse liver was induced with *o*-aminoazotoluene (Institute of Cytology and Genetics, Siberian Division, Russian Academy of Sciences, Novosibirsk) and used as a tumor model. Male A/Sn(A) mice were used at the age of 3-4 months, weighing 20-24 g. After a series of subcutaneous passages, the ascitic form of the tumor was prepared. Peritoneal macrophages were isolated from exudate as mentioned above. Tumor or ascitic cells remaining in the ascitic fluid after the macrophage adhesion were centrifuged at 2000 rpm for 20 min. The pelleted cells were resuspended and incubated for 2 h in the presence of CMG or SEG.

The cystatin C concentration was determined in incubation medium of macrophages, in ascitic fluid, and in incubation medium of ascitic cells by ELISA using a kit purchased from KRKA (Slovenia). A sandwich-ELISA protocol was used [11]. Horseradish peroxidase and 3,3′,5,5′-tetramethylbenzidine were used as indicator enzyme and substrate, respectively, and the reaction products were determined on a multichannel spectrophotometer Star 30 Plate Reader (Kenstar, USA) at 450 nm.

Human blood serum lipoproteins were isolated by flotation in KBr solutions [12] on an Optima L-90K ultracentrifuge (Beckman-Coulter, Austria) using a 70.1 Ti rotor. Proteins secreted by macrophages into the medium were separated by SDS-PAGE by the method of Laemmli [13].

Statistical data processing was performed using Student's *t*-test.

RESULTS

Intact macrophages secreted cystatin C into the incubation medium. A small amount of the inhibitor secreted was observed already after 30 min of incubation increasing fourfold in 2 h (Table 1). Polysaccharide increased the cystatin C level in the incubation medium with maximum effect of CMG and SEG: the former increased the inhibitor level 3.7-fold and the latter 4.8-fold for 30 min of incubation. The secretion continued to increase for 2 h: 1.5- and 1.8-fold, respectively, as compared with non-stimulated macrophages.

Figure 1 shows the electrophoretic separation of proteins secreted by macrophages into the incubation medium. Apoproteins with known molecular weights (ApoA-I, 28.3 kD; and ApoE, 34.2 kD) we purified at the Institute of Biochemistry were used as standards. One can see the stimulating effect of polysaccharides on the secretion of proteins differing in their molecular weight. Newly appearing protein bands changing in color depth are visible on the plot.

Table 1. Effect of polysaccharides and low- and high-density lipoproteins on cystatin C secretion by murine peritoneal macrophages

Specimen	Cystatin C, pM	
	30 min	2 h
Control (without additions)	31 ± 3.8	121 ± 9.7
LPS	54 ± 3.7	147 ± 11.6
CMG	115 ± 7.0*	178 ± 13.4*
SEG	148 ± 7.9*	213 ± 13.6*
LDL	242 ± 20.5*	585 ± 53.9*
LPS and LDL	304 ± 23.6	720 ± 71.8
CMG and LDL	1489 ± 116.7**	1425.3 ± 113.41**
SEG and LDL	1531 ± 117.9**	1522 ± 123.6**
HDL	245 ± 20.5*	1126 ± 123.2*
LPS and HDL	291 ± 33.6	1050 ± 171.8
CMG and HDL	622 ± 60.8**	2340 ± 198.6**
SEG and HDL	1437 ± 137.4**	2777 ± 223.3**

* $p < 0.01$ (compared to macrophages without additions).
** $p < 0.01$ (compared to macrophages in presence of corresponding lipoproteins).

Incubation of macrophages with LDL and HDL resulted in a significant increase in cystatin C secretion, more profound compared to polysaccharides. LDL led to 8-fold increase in the inhibitor concentration for 30 min and 5-fold increase for 2 h compared with control. The amount of inhibitor increased 2.4-fold from 30 min to 2 h of incubation. HDL also had a stimulating effect on the production of cystatin C; the level compared with control increased 8-fold for 30 min and 9.4-fold for 2 h of incubation. Still higher secretion of the inhibitor was observed when LPS and LDL were added simultaneously. The level of cystatin C increased 6-fold compared with control when macrophages were incubated with LPS and LDL for 2 h; this effect is 23% higher than the effect of LDL only. CMG or SEG added together with LDL compared to LDL only resulted in more than 6-fold increase in the inhibitor concentration after 30 min of incubation; the prolongation of incubation to 2 h did not result in further changes.

Figure 2 displays the data of electrophoretic analysis of proteins secreted by macrophages after their incubation with LDL + CMG and LDL + SEG. A significant increase is observed in secretion of proteins with molecular weights of about 8-17 kD. We suppose that macrophages exposed to the stimulators secreted not only

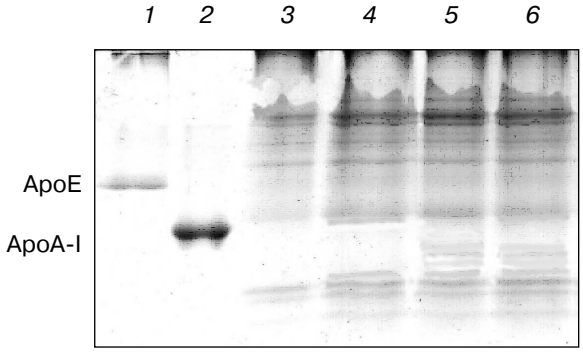

Fig. 1. Proteins secreted by murine macrophages and the stimulating effect of polysaccharides on the protein secretion (proteins were separated from the culture medium by electrophoresis in 12.5% polyacrylamide gel with SDS). Lanes: ApoE (*1*), ApoA-I (*2*), culture medium of macrophages in absence (control) (*3*) or presence of LPS (*4*), CMG (*5*), or SEG (*6*).

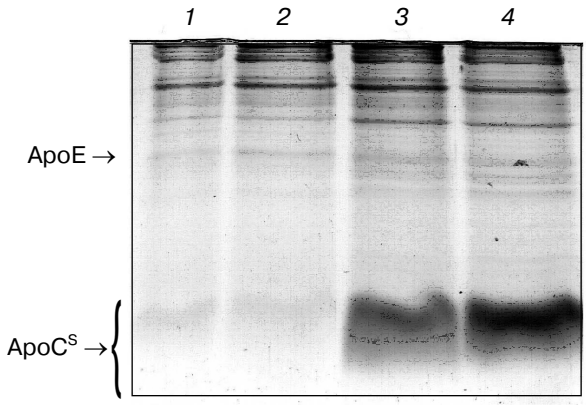

Fig. 2. Enhanced protein secretion by macrophages incubated for 2 h with lipoproteins and polysaccharides (proteins were separated from the culture medium by electrophoresis in 12.5% polyacrylamide gel with SDS). Lanes: LDL (*1*); LDL + LPS (*2*); LDL + CMG (*3*); LDL + SEG (*4*). Arrows indicate the bands with molecular weights of 34 kD (ApoE) and 8-12 kD (ApoC^S).

cystatin C, but also other low-molecular-weight proteins including cytokines.

LPS added simultaneously with HDL did not alter their effect on the inhibitor secretion, whereas CMG and SEG potentiated this effect increasing the cystatin C concentration 2.5- and 5.9-fold for 30 min and 2.4- and 2.6-fold for 2 h, respectively. It is remarkable that the maximum effect of HDL, independently of whether or not polysaccharides were present, was observed after 2 h of incubation and about 2-fold exceeded that of LDL.

Macrophages from the murine hepatoma HA-1, compared with intact mice, secreted cystatin C even more intensively (Table 2). Polysaccharides had no effect on the inhibitor secretion, which even was decreased after 2 h of incubation. We suppose that in tumor progression macrophages had been already stimulated, so additional stimulation does not result in elevation of the inhibitor concentration. However, the same cells responded to the presence of lipoproteins in the medium by enhanced secretion of cystatin C. In particular, LDL or HDL added to the incubation medium of "tumor" macrophages resulted, respectively, in 1.8- and 1.7-fold increase of the cystatin C level in the medium. Unlike intact murine macrophages, LDL and HDL revealed similar effect in this case.

Table 2. Effect of polysaccharides and low- and high-density lipoproteins on cystatin C secretion by peritoneal macrophages from hepatoma HA-1-bearing mice

Specimen	Cystatin C, pM	
	30 min	2 h
Control (without additions)	218 ± 20.7**	210 ± 19.7**
LPS	187 ± 13.6	120 ± 11.8*
CMG	200 ± 20.8	117 ± 10.6*
SEG	228 ± 21.4	131 ± 12.3*
LDL	389 ± 25.7*	—
HDL	363 ± 35.5*	—

* $p < 0.01$ (compared to macrophages without additions).
** $p < 0.01$ (compared to intact macrophages).

It is worth noting that the ascitic fluid (without tumor cells and macrophages) of murine hepatoma HA-1 also contained a significant amount (1580 ± 117 pM) of cystatin C. Like macrophages, ascitic hepatoma cells also secreted the inhibitor, whose level, however, was low (120 ± 13 pM) and did not depend on the presence of various polysaccharides.

DISCUSSION

Data exist on the effect of macrophage stimulators as a broad spectrum of biologically active substances secreted by macrophages [14]. Cystatin C is an endogenous inhibitor responsible for the extralysosomal regulation of lysosomal cysteine proteinase activities [1]. Moreover, endogenous inhibitors are involved in many physiological processes, such as coagulation, digestion, and immunity [15]. Cystatin C was shown to block *in vitro* the replication of *Herpes simplex* virus [16].

A few publications have been recently devoted to the synthesis and secretion of cystatin C by macrophages [9],

but there is no data on a separate or combined influence of effectors such as polysaccharides and lipoproteins on these processes. In the present study, we used a series of macrophage stimulators, both well known (LPS) and poorly investigated (CMG and SEG). We have demonstrated that intact macrophages secrete a cysteine proteinase inhibitor. Polysaccharides enhance the secretion, SEG and CMG being the most potent.

At the present time, the regulatory effect of blood lipoproteins on cellular immunity is well understood. In particular, very-low-density lipoproteins (VLDL) and LDL enhanced proliferation of both T-lymphocytes and NK-cells (Natural Killer). HDL increased twofold the proliferation of large granular lymphocytes [17]. HDL altered the secretion of cytokines by NK-cells, so that the secretion of interleukins 2 and 8 and γ-interferon increased [18]. The main protein component of HDL, ApoA-I, inhibited the IgG-induced activation of neutrophils [19]. When added to activated neutrophils, ApoA-I decreased by 60% the granule release and superoxide production. It has also been demonstrated that LPS stimulate the macrophagal endocytosis of LDL and HDL particles [20, 21].

Both LDL and HDL appeared to increase significantly the cystatin C secretion by macrophages, and polysaccharide stimulators when added simultaneously with lipoproteins significantly potentiated this effect. Macrophages isolated from mice bearing the experimental hepatoma HA-1 compared with intact macrophages secreted cystatin C more actively, as is evident from high level of cystatin C in ascitic fluid. Lipoproteins increased this secretion, but to less extent compared with their effect on the macrophages of intact mice. Moreover, polysaccharide stimulators demonstrated even slight decrease of cystatin C secretion throughout incubation.

A distinct mechanism of the effect of lipoproteins on the functional activity of macrophages, which we have demonstrated in our study, is still unclear. We suppose that this effect is connected with the ability of lipoproteins to enhance protein synthesis in macrophages [20, 21]. Lipo- and polysaccharides potentiate this effect; however, it is not yet understood how polysaccharides operate themselves.

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