Brief Definitive Report

CONSTITUTIVE SECRETION OF CYSTATIN C (γ-TRACE) BY MONOCYTES AND MACROPHAGES AND ITS DOWNREGULATION AFTER STIMULATION

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Cystatin C (γ-trace) is a basic protein with a molecular weight of 13,000–14,500, which is present as a constituent of normal plasma, cerebrospinal fluid, urine, and seminal fluid (1). Recently, a variant of cystatin C was found to be the major constituent of the amyloid fibrils in vessel walls of brains of patients with the Icelandic form of hereditary cerebral hemorrhage with amyloidosis (HCHWA-I) (2). At the time these latter observations were made, independent studies were conducted on the regulation of lysozyme (LZM) secretion of mononuclear phagocytes (3). Since LZM is also a constitutively secreted 14,500 mol wt cationic protein, and since it had been suggested by others (4) that macrophages (Mϕ) may be among many other cells containing cystatin C, the possibility that cystatin C secreted into the culture medium by Mϕ could comigrate with LZM on SDS-PAGE of Mϕ supernatants was entertained. The question was of particular interest to us in the context of our studies on the downregulation of selected Mϕ functions during chronic inflammatory states (3, 5, 6). Since cysteine proteinases are released during inflammation, it seemed conceivable that cystatin C, an inhibitor of cysteine proteinases, may be modulated during the inflammatory response. In this article, data are presented showing that, indeed, cystatin C is secreted by monocytes/Mϕ and that its secretion is downregulated when the cells are stimulated.

Materials and Methods

Reagents. IFN was rat IFN-γ (AMGen Biologicals, Thousand Oaks, CA). Descriptions of other reagents and additional procedures are given in references (3, 7).

Peritoneal Mϕ. Resident (Res)- and thioglycollate (TG)-elicited peritoneal Mϕ were obtained from C57BL/6J mice, and cultured as described (3). After incubation at 37°C in an atmosphere of 10% CO_2, the cells were washed with warm HBSS and then cultured either with medium only, DME, plus 0.2% lactoalbumin hydrolysate (LAH), with LPS or IFN-γ for 24 h at 37°C. After the incubation period, the conditioned medium (CM) was removed from the dishes and prepared for SDS-PAGE (3).

Human Monocytes and Lymphocytes. Mononuclear cells were separated from heparinized bloods of healthy donors by Ficoll/Hypaque gradient centrifugation (5). Aliquots of the

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interphase cells were plated on 35-mm plastic petri dishes (Falcon Labware, Oxnard, CA) containing cover slips in concentrations of 10^6 cells/ml of RPMI supplemented with 10% heat-inactivated autologous serum. The cells were incubated for a minimum of 5 h in a 5% CO_2 atmosphere. For some experiments, the nonadherent cells were removed and designated lymphocyte preparations.

Cell Lines. The following cell lines were used in these studies: Rob me, SK-CO-1, HT-29, HM-54, U937, J774, P388D.1, and human fetal fibroblasts. Conditioned media were prepared from cell lines by culturing 10^6 cells in 1–2 ml of DME containing 0.2% LAH for 24 h in 35-mm tissue culture dishes maintained at 37°C in an atmosphere of 10% CO_2. The CM were then processed as described earlier.

Brain Cells. Astroglial and microglial cells were isolated from newborn C57BL/6J mice as described (8). The CM were prepared as described above.

Gel Electrophoresis, Western Blots, and Protein Determination. Details of these procedures have been published (3). The primary antibody for the Western blotting was rabbit antiserum to purified amyloid protein monomer of amyloid fibrils obtained from the leptomeninges of patients with HCHWA-I (2), and the secondary antibody was goat antibody to rabbit IgG peroxidase (E-Y Laboratories, Inc., San Mateo, CA).

Detection and Quantitation of Cystatin C Secretion. The relative amounts of cystatin C secreted into the medium by the variously treated macrophages were quantitated by scanning densitometry of the Western blots or by a radioactive procedure. After washing off unbound anti-HCHWA-I from the Western blots, the blots were incubated with ^{125}I–protein A (New England Nuclear, Boston, MA), washed with buffer several times, and then autoradiographs were prepared. The autoradiographs were used as templates to locate and excise the bands in the Western blots corresponding to the cystatin C. The removed bands were counted in a gamma counter (model 4000; Beckman Instruments, Inc., Fullerton, CA). The data were normalized to counts per milligram of cellular protein.

Immunoprecipitation of Cystatin C From Macrophage-conditioned Medium. Human histiocytic lymphoma cells U937 were incubated with [^{35}S]methionine in methionine-free RPMI 1640 for 24 h. The radiolabeled proteins in the CM were immunoprecipitated with anti-HCHWA-I. The immunoprecipitates were treated with protein A–Sepharose, the complexes were washed several times, prepared for SDS-PAGE, electroblotted onto nitrocellulose paper, and then autoradiographs were prepared.

Fluorescent Microscopy. Details of these procedures have been published (8).

Results and Discussion

As evident from several reports, many cell types contain cystatin C (4, 9). Among human peripheral blood cells, indirect immunofluorescence proved monocytes to be strongly positive for this protein, whereas lymphocytes were negative, even when cultured for 3 d (Fig. 1). This observation was substantiated by immunoblotting culture supernatants of Mϕ from four different sources with an antibody to HCHWA-I (Fig. 2). Faint higher molecular weight bands were also seen, which were due to aggregation of cystatin C (2). The cystatin C detected in Mϕ CM was shown to be synthesized by these cells (Fig. 3). This was demonstrated by radiolabeling U937 cells for 24 h with [^{35}S]methionine and then immunoprecipitating the cystatin C from the CM with anti-HCHWA-I. Autoradiographs prepared from electroblots of the immunoprecipitates revealed a major band identical to that identified by Western blots of CM as cystatin C (Fig. 3). Table I summarizes the results of the examination for cystatin C secretion by other cells that were selected at random from our tissue culture collection. Thus, the release of cystatin C is not unique to monocytes/macrophages, but is a property shared by many cell types, as has been reported by others (4, 9). However, the present study focused primarily on monocytes/Mϕ because of the

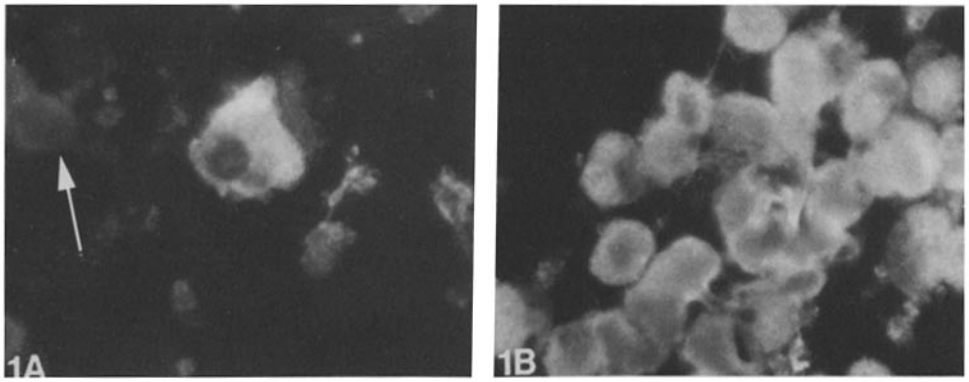

FIGURE 1. Immunofluorescence of human peripheral blood monocytes stained with anti-HCHWA-I. (*A*) 18-h culture. Arrow indicates negative lymphocyte. (*B*) Monocytes cultured for 1 wk. Nonadherent cells and platelets are no longer present.

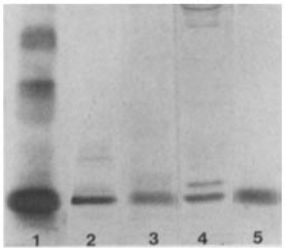

FIGURE 2. Secretion of cystatin C into CM as detected by means of a Western blot, using antibody to HCHWA-I. (*1*) Purified human urinary cystatin C; (*2*) CM from untreated resident mouse peritoneal macrophages. (*3–5*) CM from 10^6 histiocytic lymphoma cells of J774, P388D.1, and U937 cell lines, respectively.

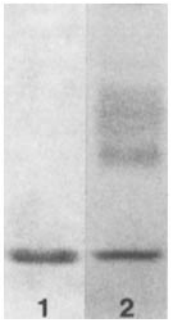

FIGURE 3. Electroblot of conditioned medium from U937 cells demonstrates Cystatin C synthesis and secretion by these cells. Lane 1: Western blot of Cystatin C from the CM of [^{35}S]methionine labeled cells reacted with anti-HCHWA-I. Lane 2: Autoradiograph of anti-HCHWA-I immunoprecipitates obtained from the same sample run in parallel.

possible role cystatin C may play in inflammation. The ubiquitous distribution and constitutive secretion of this protein is reminiscent of other proteinase inhibitors secreted by Mϕ. For instance, although the α_1 proteinase–inhibitor, as well as the α_2-macroglobulin, are functionally important products of monocytes/Mϕ (10, 11), other cells secrete these inhibitors as well (12, 13). Cystatin C is a potent inhibitor of cathepsin B, an enzyme contained in lysosomes and released during phagocytosis and inflammation (14, 15). Therefore, it is judicious to assume that this inhibitor serves to limit the acute inflammatory reaction. In line with this hypothesis, it seemed of interest to determine whether, or how, an inflammatory stimulus would affect the constitutive secretion of this proteinase inhibitor by Mϕ. Accordingly, resident mouse peritoneal Mϕ were incubated for 24 h in the presence of either medium alone, medium containing LPS, or

TABLE I
Detection of Cystatin C by Western Blotting of the Culture Media from Various Cells

Cell type*	Detection of cystatin-C
Adenocarcinoma, colon (HT 29)	+
Adenocarcinoma, colon (SK-CO-1)	+
Melanoma (Rob Me)	+
Melanoma (HM-54)	+
Fetal fibroblasts	+
Astroglial and microglial cells	+
Peripheral blood lymphocytes	−

* All cell lines were of human origin with the exception of the brain cells, which were from newborn mice.

TABLE II
Effect of LPS on Cystatin C Secretion by Resident Peritoneal Macrophages

LPS treatment	Decrease in cystatin-C secretion*
μg/ml	%
None	Control
0.001	6.6 ± 9.3
0.01	25.0 ± 2.2
0.10	30.8 ± 5.2
1.0	46.8 ± 4.3
10.0	48.8 ± 13.8

* Mean ± SD.

TABLE III
Effect of IFN-γ on Cystatin C Secretion by Resident Peritoneal Macrophages

IFN treatment	Decrease in cystatin-C secretion*
U	%
None	Control
1	1.3 ± 12.3
10	18.3 ± 0.7
10^2	23.2 ± 2.3
10^3	44.9 ± 5.2

* Mean ± SD.

medium containing IFN-γ. The relative differences among cystatin C levels in the CM from these cells are shown in Tables II and III. All three types of Mϕ secreted cystatin C. However, the amount of cystatin C secreted by the cells stimulated with LPS or by cells stimulated with IFN-γ was much less than the amount elaborated by untreated cells. The degree of suppression in cystatin C secretion was dose dependent upon the LPS or IFN-γ concentrations. IFN-α and -β treatments have also been shown to decrease cystatin C secretion (16). It was further found that untreated TG-elicited Mϕ secreted 59% less cystatin C than untreated resident Mϕ and that LPS treatment of TG-elicited Mϕ caused a further reduction to 71%. This observation suggests that Mϕ stimulation in vivo with agents like TG which have been demonstrated to stimulate Mϕ and enhance

certain properties (17), may concomitantly downregulate other functions, like the secretion of cystatin C. The present data show that Mϕ stimulated with the same agents that we reported to decrease the constitutive secretion of LZM (3), namely TG, LPS, and IFN, also caused a downregulation of cystatin C production. The significance of a reduced level of LZM secretion during the inflammatory response is difficult to assess at the present time. However, a reduction in the amount of cystatin C production has more obvious pathophysiologic implications. Cystatin C is a cysteine proteinase inhibitor able to neutralize acid hydrolases (14, 15), and recently it has been shown to possess close homologies with the kininogens (2, 18). A decreased level of the antiproteinase-α_1-antitrypsin has been shown to be associated with several chronic disease states (19), and the level of α_2-macroglobulin may also be affected (20, 21). Thus, it is reasonable to postulate that during a chronic inflammatory condition, particularly one dominated by mononuclear phagocytes, downregulation of cystatin C production may lead to tissue pathology attributable to persistence of unopposed proteolysis.

Summary

Cystatin C (γ-trace) was found to be a constitutively secreted protein of isolated human monocytes and mouse peritoneal macrophages, as well as the histiocytic lymphoma cell lines U937, P388D.1, and J774. This proteinase inhibitor is not uniquely secreted by monocytes/macrophages, but was also identified in the conditioned media from several primary cells, including brain cells, and diverse established cell lines. In vitro treatment of resident mouse peritoneal macrophages with either LPS or IFN-γ caused a downregulation in cystatin C secretion. Elaboration of this protein was also diminished by macrophages that had been stimulated by thioglycollate in vivo, and treatment of these cells with LPS led to further decline. It is suggested that, under some inflammatory conditions, downregulation of cystatin C may contribute to tissue pathology.

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