Possible involvement of cathepsin E in free radical production by activated microglia

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Abstract
Cathepsin E (CE) is a major nonlysosomal, intracellular aspartic proteinase. Immunohistochemical and immunoblot analyses revealed that CE was most abundant in microglia cells. Microglia cells are believed to be important in modulating neural function and to have macrophage-like functions in the central nervous system. CE was accumulated in microglia cells may be involved in the process of digestion of damaged neurons and may play a role in immune response in brain tissue through the endosomal/lysosomal system. Objective: The aim of this study is to elucidate the possible function of CE in microglia by examine phagocytic activity and generation of oxygen-derived free radicals of microglia cells by exposure to variety of agent. Methods: Microglia cells were isolated from mixed primary cell cultures of the cerebral cortex from 3-day old male Wistar rats. To detect the phagocytic activity, isolated microglia then were added by LPS and IFN-γ after 2 days in culture, the cell were incubated with opsonized zymosan at 37°C Celsius for 1 hour. Generation of oxygen-derived free radicals of microglia cells was measured by Chemiluminescence (CL) response. The statistical analysis was done by "student t test". Results: Both control and IFN-γ + LPS treated cells exhibited the intense phagocytic activity against zymosan particles. Control cells exhibited a slight CL response upon stimulation with opsonized zymosan. However, the CL response by IFN-γ +LPS, pepstatin A and bafilomycin A treated microglia was markedly enhanced compared to control, but not leupeptin. The synergistic effect was observed between pepstatin A and IFN-γ +LPS. Conclusion: This study shows that Cathepsin E in microglia may play an inhibitory role in free radical production by activated microglia during the process of phagocytosis.

Key words: Cathepsin E, Phagocytic activity, (CL) Chemiluminescence response

Introduction
Cathepsin E (CE) is a major non lysosomal, intracellular aspartic proteinase that localized in various cellular compartments such as the plasma membrane and endoplasmic reticulum (ER). CE is the pepsin superfamily which is highly homologous to the lysosomal aspartic proteinase cathepsin D(CD). Differing from CD, CE has a limited tissue distribution and localizes in various cellular compartments such as the plasma membrane, the endosome-like organelles and the endoplasmic reticulum. However, the detailed function of CE in relation to its appropriate cellular localization is currently unknown. In the present work, the microglia cells are chosen as a predominant cell type expressing CE. Immunohistochemical and immunoblot analysis revealed that CE was most abundant in microglia among various brain cell types. Microglia are believed to be important in modulating neuronal functions and to have macrophage-like functions in central nervous system (CNS).
Materials and Methods

Cell culture

Microglia were isolated from mixed primary cell cultures of cerebral cortex from 3 days-old male Wistar rat according to the method described by Nakajima et al (1992)\(^{17}\). The cerebral cortex was dissected, meninges and blood vessels removed, soaked in Ca\(^{2+}\)- and Mg\(^{2+}\)-free phosphate-buffered saline (CMF-PBS) (GIBCO, Gaithersburg, MD, USA), and minced with a razor blade. Then the tissue was enzymatically dissociated by incubation twice for 15 min at 37\(^\circ\)C in CMF-PBS containing papain (90 U/ml), DNase (Worthington; 2,000 U/ml), D, L-cysteine-HCl (2.23 mg/ml), bovine serum albumin (2 mg/ml) and glucose (50 mg/ml). After termination of the reaction by adding fetal calf serum (FCS), the tissue fragments were isolated by centrifugation and resuspended in the culture medium consisting of Dulbecco’s modified Eagle’s medium (DMEM), 0.3 % NaHCO\(_3\), 50 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, and 10% FCS. The tissue fragments were then dissociated by gentle passage through plastic tips with three different diameters. The mechanically dissociated cells were filtered through a cell stainer with a 70 m pore size (Falcon, Franklin lakes, NJ, USA). The dissociated cells were maintained at 37\(^\circ\)C in a 10 % CO\(_2\)/90% air atmosphere. Subsequent medium replacement was performed every 3 days. After 10-14 days in culture, floating cells layer were isolated by gentle shaking of the flask for 3-5 min. The resulting cell suspension was transferred to plastic dishes and allowed to adhere at 37\(^\circ\)C. Unattached cells were removed after 30 min; microglia were isolated as strongly adhering cells. About 90 % of these attached cells were positive for OX 6 (Serotec), marker for macrophage/microglia cell types.
Immunocytochemistry
To detect phagocytic activity, isolated microglia were transferred to the chamber slides (Nunc Inc., Naperville, IL), unattached cells were removed after 30 min and strongly attached microglia were resuspended in the culture medium consisting DMEM, 0.3 % NaHCO3, 50 units/ml penicillin, 100 µg/ml streptomycin and 10 % FCS. Then IFN-γ (genzyme diagnostic, USA) (100 U/ml) and LPS (Sigma Chemical Co) 1 µg were added as activator of microglia. The culture was maintained at 37°C in 10% CO2/90% air atmosphere. After 2 days in culture, the cells were incubated for 1 h at 37°C with zymosan A (20 mg/ml) that was opsonized with fresh serum. After two time washes with PBS, the cells were incubated with Giemsa’s staining solution (Nacalai Tesque Japan) for 30 min at room temperature and observed with a bright-field microscope (Haga et al. 1993). To investigate the morphological change of microglia, the cells after 2 days of culture were washed with PBS two times and incubated with biotinylated GSA-I-B4 (10 µg/ml at 4°C for overnight. After washing with PBS, the cells were processed with the avidin-biotin-peroxidase complex (ABC) method and visualized with 3′, 3-diaminobenzidine (DAB).

Electrophoresis and immunoblotting
Immunoblot analyses of cell extracts of control and IFN-γ (100U/ml) and LPS 1 µg treated microglia by discriminative antibodies specific for cathepsin E and cathepsin D. protein were separated on 5-12 % polyacrylamide gels under reducing conditions according to the method of Laemmli18 and subsequently blotted onto nitrocellulose membranes and immunostained as described previously19,20. The density of band from Immunoblot analyses of control cell and IFN-γ and LPS treated cell then were determined computerized by densitometric scanning.

Measurement of luminol-dependent Chemiluminescence (CL)
Microglia cells were cultured for 2 days in the absence (control) or present of the following agents: IFN-γ (100U/ml) + LPS (1 mg/ml), pepstatin A (10-100 µM), batflomycin A1 (0.5 µM), and leupeptin (100 µM) were washed with Hank’s balanced salt solution (HBSS), and resuspended in HBSS at a final cells concentration of 1 X 10^5 cells/ml. Zymosan A (Sigma) suspended in PBS (20 mg/ml) was boiled for 10 min and washed prior to being opsonized to reduce clumping. The zymosan suspension was incubated with an equal volume of rat serum at 37°C for 30 min. The particles were washed twice with PBS and suspended in the original volume in PBS. The cuvette for the reaction mixture consisting of 0.1 ml of freshly diluted luminol solution (0.2mM), 0.1 ml of the microglia suspension (1 X 10^5 cells/ml), and 0.1 ml of the opsonized zymosan (20 mg/ml) was maintained at 37°C in a six channel BIOLUMAT (“berthold” Multi-Biolumat LB 9505 C). The intensity of light emitted in the cuvette was recorded automatically. The CL response is expressed by the peak of chemiluminescence response (cpm X 10^3).

Statistics
Multiple experiment results were collected for each measurement and the results were expressed as means ± SEM. A student’s t-test was used for analysis of the differences. The level required for statistical significance was P<0.05.

Results
Changes in Morphology and Phagocytic Activity
Morphologically, most of the isolated microglia cells had ameboid- and rod-shaped cell bodies with no or thick processes (Fig. 1 A). Most of these cells became ameboid-like cells and showed a number of vacuoles in the cytosol when cultured in the presence of IFN-γ (100U/ml) and LPS 1 µg (Fig. 1 B). Both control and IFN-γ (100U/ml) and LPS 1 µg treated cells exhibited the intense phagocytic activity against zymosan particles (Fig.1 C, D).
Fig. 1. Morphological change and phagocytic activity of cultured rat microglia. Isolated microglia were cultured in the absence (A) or presence of IFN-γ and LPS for 2 days and then stained with B4-Isolectin. Note that control cells (A) showed rod-or ameboid-shaped cell bodies, but most of the IFN-γ LPS treated cells became ameboid-like cells (B). Both control (C) and IFN-γ+LPS treated cells (D) showed a similar phagocytic activity against zymosan particles. Bar = 20 µm.

Effect of IFN-γ and LPS on the protein levels of cathepsin E and D in microglia

The cellular protein levels of cathepsin E and cathepsin D were determined by immunoblot analysis before and after treatment with IFN-γ and LPS. The cathepsin E was significantly decreased by treatment with IFN-γ and LPS (Immunoreactivity of control cell represent 100% and IFN-γ and LPS treated cell maximally about 50% of that of the control cells), whereas the cathepsin D level was slightly but not significantly decreased by this treatment (Fig.2). In consistence with this, the total aspartic proteinase activity in control cells was significantly decreased by the IFN-γ and LPS treatment (11.7±3.70 vs 5.38±2.24 units/mg protein), indicating that the decrease in activity was attributable to cathepsin E.
Effects of pepstatin A and IFN-γ and LPS on the CL response of opsonized zymosan-stimulated microglia. The CL response is known to represent the extent of free radical production. As shown in Fig 3 A, control microglia exhibited only a slight CL response upon stimulation with opsonized zymosan. However, the CL response by IFN-γ and LPS treated microglia was markedly enhanced as compared to the non-treated cells (approximately 20 times greater than the control cells). Pepstatin A-treated microglia also showed enhancement of the CL response, although the extent was maximally about 20% of that by IFN-γ and LPS treated cells. When the zymosan – stimulated microglia were treated by combination of IFN-γ and LPS and pepstatin A, the CL response was increased by approximately 3-fold of that by IFN-γ and LPS alone. It is also noted that the onset and peak of the Cl response enhance by this combination were shifted to the left as compared with those induced by each agent (Fig. 3B and Table 1). To determine whether the enhanced CL response by pepstatin A was due to the inhibition of the endosomal/lysosomal system or specific for the inhibition of the aspartic proteinase activity, the effects of bafilomycin A1 and leupeptin were similarly examined bafilomycin A1 is a vacuolar type H+ - ATPase inhibitor to disrupt intracellular acidic compartments such as endosomes and lysosomes. Leupeptin is a potent inhibitor of cysteine proteinases as well as serine proteinases. When microglia were pretreated with Bafilomycin A1 (5nM) for 2 days and then stimulated with opsonized zymosan (20 mg/ml), the elicited CL response was significantly increased (Fig. 3 C). However, leupeptin (100 µM) had little or no effect on the CL response of zymosan-activated microglia.
Fig. 3  Effects of various agents on the CL response of zymosan-activated microglia. Isolated microglia were cultured for 2 days in the absence (control) or present of the following agents: IFN-\(\gamma\) (100U/ml) + LPS (1\(\mu\)g/ml), pepstatin A (10-100 \(\mu\)M), bafilomycin A\(_1\) (0.5 \(\mu\)M), and leupeptin (100 \(\mu\)M). Then the cells were stimulated with opsonized zymosan (20 mg/ml). Left panels (A, B) indicate records of the real time of CL. Response and the right panel represents the mean (+SEM) of the peak CL response. The number in the bracket represent number of sample. *p<0.05; **p<0.01; ***p<0.001 VS control cells

Table 1  Effect of pepstatin A and IFN-\(\gamma\) LPS on onset and half-time of luminol chemiluminescence in zymosan-activated rat microglia cells

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<thead>
<tr>
<th>Priming agent</th>
<th>Chemiluminescence responses</th>
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<tr>
<td></td>
<td>Onset</td>
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<tr>
<td>IFN-(\gamma)+LPS</td>
<td>4.50 ± 0.56*</td>
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<tr>
<td>Pepstatin A</td>
<td>2.12 ± 0.56</td>
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<td>IFN-(\gamma)+LPS + Pepstatin A</td>
<td>2.83 ± 1.10</td>
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Discussion

This study is report on the possible involvement of cathepsin E in free radical production by activated microglia. Immunohistochemical and immunoblot analysis revealed that CE was most abundant in microglia among various brain cell types. In microglia, cathepsin E resides intracellularly and only a small amount of the protein is excreted in the culture medium. Differential localization between cathepsin E and cathepsin D in microglia is consistent with previous data showing their different cellular localization in various cell types, such as rat kidney cells and human gastric cells, rat neutrophils. However, a significant amount of cathepsin D has also been shown to localize in endosomes besides lysosomes, in rabbit macrophages, and human hepatoma cell line Hep G2 cells. In microglia cells procathepsin D is membrane-associated in the endosomes and its membrane associated disappeared concomitantly with proteolytic processing of the proenzyme. Therefore, cathepsin E may share some common function with cathepsin D in extralysosomal proteolysis in the same compartment of a few cell types, although neither colocalization nor membrane association of cathepsin E and cathepsin D in endosome-like organelles is evident in rat microglia. Microglia are also proposed to be the mediators of immune response in the brain since the activated microglia express MHC class II antigens to present antigens to T-lymphocytes and to secrete cytokines. Recently, it has also been shown that cathepsin E is closely linked with the proteolytic processing of exogenous antigens. Therefore, the enzyme accumulated in microglia as the mature enzyme may play a role in the immune response in brain tissue. The precise function of cathepsin E in microglia, as well as macrophages is currently unknown. However, since cathepsin E is considered to be synthesized as a catalytically inactive precursor protein and completely processed to the catalytically active mature enzyme in the endosome-like vacuoles of these cells, the enzyme is likely to play a crucial role in the endosomal proteolytic system.

Since microglia also are known to be activated to be the phagocytic macrophage-like cells in response to brain injuries, cathepsin E accumulated in these cells may be involved in the process of digestion of the damaged neurons through the endosomal/lysosomal proteolytic system. Therefore, the enzyme accumulated in microglia as the mature enzyme may play a role in the immune response in brain tissue. As microglia as well as other phagocytic cells can be primed (preactivation) by exposure to a variety of agent including LPS and IFN-γ. Although the molecular basis of the priming mechanism is currently unknown, the involvement of PKC in the activation of NADPH oxidase has been well documented. In Most of these studies LPS and IFN-γ was used to be a priming agent. The results shown most of the isolated microglia cells had ameboid- and rod-shaped cell bodies with no or thick processes (Fig. 1 A). Most of these cells became ameboid-like cells and showed a number of vacuoles in the cytosol when cultured in the presence of IFN-γ 100U and LPS 1µg (Fig. 1 B). Both control and IFN-γ 100U/ml and LPS 1µg treated cells exhibited the intense phagocytic activity against zymosan particles (Fig. C, D).

Activated microglia are known to produce active oxygen species by which invading organisms are killed. During the phagocytosis process, phagocyte undergo remarkable alteration in oxidative metabolism, that generates various species of oxygen radical. The enzyme system responsible for the generation of these oxygen radicals, the neutrophil NADPH oxidase. The activity of the NADPH – oxidase can be measured in number of different assay system. The luminol-amplified chemiluminescence (CL) technique, is very sensitive and simple to perform and widely used to study respiratory burst activity induced in phagocytic cell. The CL response is know to represent the extent of the production of free radical including active oxygen species. The present study presents the first evidence indicating possible the involvement of the endosomal/lysosomal system besides the PK-mediated system, in priming
mechanism of active microglia. Pepstatin A and bafilomycin A1, but not leupeptin, enhanced the CL response of activated microglia stimulated by zymosan (Fig 3). The onset and T half of the peptatin A- induced CL response were apparently shorter than those induced by LPS and IFN-γ (Fig 3, Table 1). Also, the pepstatin A-induced CL response was synergistically enhanced by adding LPS and IFN-γ. PKC unlikely to be involved in the priming mechanism by the endosomal/lysosomal system. Since aspartic proteinases, especially cathepsin E, were significantly decreased in LPS and IFN-γ treated microglia at both protein and activity levels (Fig 2). The CL response induced by pepstatin A, as well as bafilomycin A1, is likely to be attributable to the inhibition of cathepsin E. Therefore, cathepsin E may play an inhibitory role in the priming mechanism through the endosomal/lysosomal system.

Reference


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