Expression of Phosphatidylserine Receptor and Down-Regulation of Pro-Inflammatory Molecule Production by its Natural Ligand in Rat Microglial Cultures

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Abstract. Exposure of phosphatidylserine (PS), an aminophospholipid normally sequestered in the inner leaflet of plasma membrane, is one of the crucial steps in the recognition and ingestion of apoptotic cells by macrophages. The recognition of PS on apoptotic cells by peripheral macrophages is mediated by a phosphatidylserine-specific receptor (PtdSerR), which has recently been cloned. In spite of the important role of apoptosis in the CNS, the process of apoptotic neuron recognition by microglia is poorly understood. Because recent studies suggest that engagement of PS with a not yet characterized microglial receptor is necessary for apoptotic neuron uptake, we investigated the expression of PtdSer-R and its functional role in neonatal rat brain microglial cultures. Semi-quantitative RT-PCR analysis revealed that PtdSerR mRNA was detectable in unstimulated cultures and enhanced in LPS activated microglia. The presence of PS-liposomes strongly reduced the release of pro-inflammatory molecules such as nitric oxide, interleukin-1β, and tumor necrosis factor-α by LPS-activated microglia. At variance, the immunoregulatory cytokines interleukin-10 and transforming growth factor-β1 were moderately decreased or unaffected. The activity of PS-liposomes was mimicked by the PS head group phospho-L-serine, but not by phosphatidylcholine-containing liposomes. Our data suggest that, as for peripheral macrophages, PS through its receptor can modulate microglial activation toward an anti-inflammatory phenotype.

Key Words: Apoptosis; Brain macrophages; Cytokine; Interleukin; Microglial activation; Nitric oxide.

INTRODUCTION

Phosphatidylserine (PS) is an aminophospholipid predominantly found in the inner leaflet of plasma membrane of virtually every cell of the body. The asymmetric distribution of PS is maintained by the cells through an ATP-dependent activity and is rapidly lost when cells undergo apoptosis (1). The externalization of PS is a crucial event in the recognition and phagocytosis of apoptotic cells by macrophages and non-professional phagocytes, including fibroblasts and epithelial cells. The removal of apoptotic bodies is of functional importance for eliminating unwanted cells, thereby avoiding tissue inflammation and risks of uncontrolled cell lysis. Indeed, the autodigestion events that characterize apoptosis, such as degradation of DNA and specific proteins, cytoplasmic blebs, and apoptotic body formation, occur after apoptotic cells have been safely engulfed by phagocytes. Autodigestion without phagocytosis would leave the apoptotic process incomplete and ineffective.

The discovery of the role of PS in the uptake of apoptotic cells has prompted an intense search for the molecules expressed by phagocytes that are responsible for PS recognition and apoptotic cell uptake. Recently, a gene coding for a novel protein that appears to mediate specific recognition of cells exposing PS on the plasma membrane has been cloned (2). The gene, which was cloned from a human brain library (3) and first reported as a gene of unknown function, is highly conserved throughout phylogeny. The predicted protein is a type II protein, which does not fall into any of the known receptor families and, as such, denominated phosphatidylserine-specific receptor (PtdSerR). The PtdSerR has been found in macrophages, fibroblasts, epithelial cells, and dendritic cells, but is not expressed by circulating cells such as neutrophils, lymphocytes, monocytes, and red blood cells (2, 4).

In addition to PtdSerR, many different types of receptors are involved in the uptake of apoptotic cells, including class A and class B scavenger receptors, CD68, CD14, selected integrins, and C1q (2, 5). Nonetheless, increasing evidence indicates that the interaction of PS with its receptor has a key role in the clearance of apoptotic bodies by professional phagocytes. The seminal studies by Fadok et al have shown that this interaction is not only involved in the recognition and engulfment of apoptotic cells, but also mediates the suppression of pro-inflammatory cytokine production, inducing macrophages to acquire an anti-inflammatory and immuno-suppressive phenotype (2, 6).

Whereas the process of apoptotic cell uptake by macrophages in the periphery is well documented, the mechanisms regulating the phagocytosis of apoptotic neurons by microglia (the brain resident macrophages) are still poorly understood. In the CNS, apoptosis occurs during development and is an important mechanism of neuronal death in various neurodegenerative diseases (7). A rapid and effective phagocytosis of apoptotic neurons by microglia is necessary to protect the surrounding tissue from...
an unwanted inflammatory reaction that could exacerbate the neuronal loss. Recent studies suggest that externalization of PS on the outer leaflet of plasma membrane is necessary for microglial recognition and phagocytosis of the neuronal cell line HN2–5 undergoing apoptosis (8). Furthermore, Witting et al have shown that at least 3 classes of receptors are involved in apoptotic cerebellar granule recognition by microglial cells (9). Indeed, the uptake of apoptotic neurons was reduced by N-acetylgalactosamine or galactose, RGDS peptide, or by PS-enriched liposomes, suggesting the involvement of asialoglycoprotein-like lectins, vitronectin receptor, and PtdSerR, respectively. The microglial receptors were, however, not further characterized.

In order to demonstrate the presence of PtdSerR in microglia and to gain more information on its functional role, we have used microglial cultures from neonatal rat brain and investigated by RT-PCR the expression of messenger RNA for PtdSerR. Furthermore, we studied the biological consequences of the interaction of PtdSerR with its ligand PS on some important microglial functions, such as the production of the pro-inflammatory molecules nitric oxide (NO), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and the immunoregulatory cytokines interleukin-10 (IL-10) and transforming growth factor-β1 (TGF-β1).

MATERIALS AND METHODS

Reagents

All cell culture reagents and recombinant rat IFN-γ (specific activity: 4 x 10^6 U/mg) were from Gibco (Grand Island, NY). All cell culture reagents were virtually endotoxin-free (less than 10 E.U./ml as determined by the manufacturer). BCA protein assay was from Pierce Chemical Company (Rockford, IL). ELISA-kits for rat IL-1β, TNF-α, and IL-10 were from Endogen Inc. (Woburn, MA). The ELISA-kit for human TGF-β1 was obtained from DRG Instruments GmbH (Marburg, Germany). Purified inducible NO synthase (iNOS) (from mouse macrophages) and specific anti-iNOS antibody were obtained from Cayman Chemical Company (Ann Arbor, MI). ED-1 monoclonal antibody was from Serotec (Oxford, UK). Western blot enhanced chemiluminescence (ECL) detection system was from Amersham International (Buckinghamshire, UK). All other chemicals, including LPS (from Escherichia coli, serotype 026:B6) L-α-phosphatidylserine (from bovine brain), L-α-phosphatidylcholine (from egg yolk), and O-phospho-L-serine were from Sigma Chemical (St. Louis, MO). RAW 264.7 cells were from American Type Culture Collection (Manassas, VA).

Cell Cultures

Microglial secondary cultures were prepared from 10- to 14-day mixed primary glial cultures obtained from the cerebral cortex of 1-day-old rats, as previously described (10), and in accordance with the European Communities Council Directive N. 86/609/EEC. Microglial cells were harvested from the mixed primary glial cultures by mild shaking, resuspended in Basal Eagle’s Medium (BME) supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 µg/ml gentamicin, then plated on uncoated plastic wells at a density of 1.25 x 10^4 cells/cm². Cells were allowed to adhere for 20 min and then washed to remove non-adhering cells. After a 24-h incubation period, the medium was replaced with fresh medium containing the substance(s) under study. Cell viability was greater than 95%, as tested by Trypan Blue exclusion. Immunostaining, performed as previously described (11), revealed that cultures consisted of ≥99%-positive cells for the microglia/macrophage marker ED 1.

RAW 264.7 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were plated at a density of 6 x 10^4 cells/cm² and incubated for 24 h before collecting for RNA extraction.

Preparation of Liposomes

Liposomes (small unilamellar vesicles) were prepared as previously described (12). In brief, lyophilized phospholipids (L-α-phosphatidylserine, PS, or L-α-phosphatidylcholine, PC) were resuspended in PBS to obtain a 5-mM concentration and vortexed for 5 min. The liposome solutions were subsequently sonicated for 5 min in an Elgasonic instrument on the low setting with an output of ~0.42. After a freeze-thaw cycle, lipid suspensions were sonicated for 3 min as before, further diluted in PBS, and added to the cell cultures at different final concentrations.

Cytokine and NO Determination

At the end of the incubation, cell supernatants were collected, centrifuged, and stored at −70°C until tested. The levels of IL-1β, TNF-α, IL-10, and TGF-β1 were assayed by specific ELISA, following the manufacturer’s instructions. For TGF-β1, supernatants were acid-activated before the assay according to the manufacturer’s instructions. The ranges of determination were 10 to 1,000 pg/ml for IL-1β, 31 to 2,500 pg/ml for TNF-α, 8 to 500 pg/ml for IL-10, and 10 to 600 pg/ml for TGF-β1.

The production of NO was determined by measuring the content of nitrite, one of the end-products of NO oxidation, by a procedure modified by Tracey (13), based on the diazotization of nitrite by sulfanilic acid (Griess reaction), as previously described (14). Briefly, 40 µl of 5 mM sulfanilamide, 10 µl of 2 M HCl, and 20 µl of 40 mM N-(1-naphthyl)-ethylenediamine were added to 150 µl of culture medium. After 10 min of incubation in the dark, the absorbance at 490 nm was measured by a microplate spectrophotometer. A standard nitrite curve (0–50 µM) was generated in the same fashion using a 10-mM solution of NaNO₂. The detection limit was 0.25 µM.

Western Blot Analysis

Cell lysates were prepared as previously described (15) and protein concentration measured by BCA protein assay. Equal amounts of protein (25 µg) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 10% non-fat milk and incubated with monoclonal anti-iNOS (1:2,500) antibody for 1 h at 25°C. Horseradish peroxidase conjugated anti-rabbit IgG (1:5,000, 1 h at 25°C) and ECL reagents were used as the detection system. Purified iNOS was used as standard control (0.5 µg/lane). The optical density
Fig. 1. Semi-quantitative RT-PCR analysis of PtdSerR mRNA expression in cultures of the mouse macrophage cell line RAW 264.7 and in mouse and rat tissues. A: The mouse macrophagic cell line RAW264.7 was used as a positive control for the expression of mRNA for PtdSerR (right lane). Expression of GAPDH is shown as internal control (middle lane). The correct size of all PCR products was confirmed by comparing with a DNA standard on agarose gel (left lane). B: Representative semi-quantitative RT-PCR analysis of PtdSerR mRNA in mouse and rat tissues. In the lower panels the amount of PtdSerR mRNA is expressed as the ratio of densitometric measurement (OD) of the sample to the corresponding internal standard (GAPDH).

Fig. 2. Semi-quantitative RT-PCR analysis of PtdSerR mRNA expression in purified rat microglial cultures. Upper panel: representative semi-quantitative RT-PCR analysis of PtdSerR mRNA in microglia cultures treated with control medium (ctr) or LPS (10 ng/ml) for 8 or 24 h. No contamination of genomic DNA was present as shown in the lane -rt (PCR negative control, see Material and Methods section). Lower panels: amount of PtdSerR mRNA expressed as the ratio of densitometric measurement (OD) of the sample to the corresponding internal standard (GAPDH). Data are mean values ± SEM (n = 4). *p < 0.05

RNA Extraction and RT-PCR Analysis

The presence of PtdSerR mRNA in microglia cultures was established by RT-PCR. Total RNA was isolated from purified microglial cultures, mouse macrophage cell line RAW 264.7, adult mouse and 1-day-old rat spleen and brain tissues using the SV total RNA isolation kit from Promega (Promega Italia, Milano, Italy), following the manufacturer’s protocol. The RNA aliquots were stored at −80°C until use. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA previously denatured at 70°C for 10 min in the presence of 0.5 μg Oligo (dT)₂₀⁻₁₈ primer. To optimize reproducibility of cDNA synthesis, a master mix solution containing 15 Units of AMV reverse transcriptase (Promega), 10 Units RNasin ribonuclease inhibitor, and 1 mM dNTP in reaction buffer (10 mM Tris-Cl pH 9.0; 50 mM KCl, 0.1% triton X-100, 5 mM MgCl₂) was used. Each sample was incubated at 42°C for 1 h and the reaction was terminated with a further incubation at 95°C for 5 min. Synthesized cDNA was diluted 1:5 and stored at −20°C until use.

Oligonucleotide primers with similar Tm were designed to generate a PCR fragment of 199 bp for the GAPDH and of 322 bp for the PtdSerR and the PCR conditions (number of cycles and cDNA and primer concentration) that ensure the data to be obtained within the exponential phase of amplification of each template were carefully assessed. The PtdSerR oligonucleotide primers were chosen on the basis of the recent published sequence (gene bank accession number AF304118). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligonucleotide primers were chosen on the basis of sequence published on the gene bank with accession number AF106860.
Fig. 3. Effect of PS-liposomes, OPS, or PC-liposomes on nitrite accumulation and iNOS expression in rat microglial cultures. A: Microglial cells were subcultured for 24 h in 10% FCS-containing medium, which was replaced with fresh medium before stimulation with increasing amounts of PS-liposomes, OPS, or PC-liposomes in the presence or in the absence of 10 ng/ml LPS. Supernatants were collected after 24 h and analyzed for nitrite accumulation. Data, expressed as percentage of inhibition of LPS-induced nitrite accumulation, are mean values ± SEM (n = 11 for PS-liposomes and n = 3 for OPS and PC-liposomes). *p < 0.05 and **p < 0.0005 vs LPS. B: Proteins from microglial cultures incubated for 24 h with increasing concentrations of PS-liposomes, with or without 10 ng/ml LPS, were analyzed by Western blot (25 μg/lane) using specific polyclonal anti iNOS antibodies. One representative experiment of 3 performed is shown.

Five and 15 μl of diluted cDNA were amplified for GAPDH and PtdSerR, respectively. The cDNA was mixed with 5 μl 10x buffer, 200 mM dNTPs, 1.5 mM MgCl₂, 2.5 Units of Taq DNA polymerase (Promega), 3.12 pmol of GAPDH (5’CACCCACCATGGGAGAAAGGCC3’; 5’GATGGATGCTTTGGCCAGG3’) or 12.5 pmol of PtdSerR (5’CGGAACCAGAAGTTCAAGTG3’; 5’CCCTGAACTAAGGCAATTCCA3’) primers in a final volume of 50 μl. A sample containing all reaction reagents except cDNA was used as PCR negative control in each experiment. The absence of genomic DNA was verified using 15 μl of diluted cDNA that was reverse transcribed without the enzyme, used as a further PCR negative control (−rt). The PCR conditions for both couples of primers were as follows: initial denaturation at 95°C for 5 min followed by 28 (GAPDH) or 30 cycles (PtdSerR) at 95°C for 30 s, 59°C for 40 s, 72°C for 40 s, and an additional cycle with extension at 72°C for 7 min.

Twenty μl of each amplified product was electrophoresed in a 1.8% (w/v) agarose gel, stained with ethidium bromide, and photographed. Transcript levels were analyzed by Fluor-S™ Multimager Analyser (Bio-Rad). For each experiment, the ratio between optical density (arbitrary units) of bands corresponding to PtdSerR and GAPDH (used as internal standard) was calculated to quantify the level of the transcript for PtdSerR mRNA.

The identity of amplified fragments was confirmed by Southern blotting using a digoxigenin oligonucleotide probe and the DIG Luminescent Detection Kit (data not shown).
Fig. 4. Effect of PS-liposomes on cytokine production by unstimulated and stimulated rat microglial cultures. Microglial cells were subcultured for 24 h in 10% FCS-containing medium, which was replaced with fresh medium before stimulation with increasing amount of PS-liposomes in the presence or absence of 10 ng/ml LPS. Supernatants were collected after 24 h and analyzed for TNF-α (A), IL-1β (B), and IL-10 (C) production. *p < 0.05, **p < 0.01, ***p < 0.005 vs LPS or vs control (a).
Fig. 5. Effect of PS-liposomes on TGF-β1 production by unstimulated and stimulated rat microglial cultures. Microglial cells, were subcultured for 24 h in 10% FCS-containing medium, which was replaced with fresh medium before stimulation with increasing amounts of PS-liposomes in the presence or absence of 10 ng/ml LPS. Supernatants were collected after 24 h and analyzed for TGF-β1 content.

3A). The inhibition of NO production by PS-liposomes was due to a reduced expression of iNOS, the enzyme responsible for NO synthesis in LPS-activated microglia (Fig. 3B).

In addition, PS-liposomes dose-dependently inhibited the synthesis induced by LPS of the cytokines TNF-α and IL-1β, without affecting their very low basal levels (Fig. 4A, B). The production of TNF-α and IL-1β was inhibited by 70 ± 7% (n = 9) and 73 ± 7% (n = 7), respectively, in the presence of 10 μM PS-liposomes, and virtually abrogated (95 ± 2% and 89 ± 4% of inhibition for TNF-α and IL-1β, respectively) at the highest concentration (100 μM). As for NO synthesis, the cytokines were inhibited by OPS but not PC-liposomes (not shown).

In contrast with the above microglial products, the synthesis of the anti-inflammatory and immuno-suppressive cytokine IL-10 was not modified by 10 μM PS-liposomes, either in the absence or in the presence of LPS. At higher concentration (100 μM), PS-liposomes reduced the LPS-induced IL-10 production by 62 ± 15% (Fig. 4C).

We also examined the effect of PS-liposomes on the synthesis of TGF-β1, another important immunoregulatory cytokine that has been previously shown to mediate the anti-inflammatory phenotype induced by apoptotic cells in peripheral macrophages (2, 6, 16). In line with earlier reports (17, 18), microglial cells constitutively expressed high levels of TGF-β1, which were not further modulated by LPS or PS-liposomes, alone or in combinations (Fig. 5), or by PC-liposomes (not shown).

Effect of PS-Containing Liposomes on IFN-γ-Activated Microglia

To verify that the inhibitory activity of PS-containing liposomes on LPS-stimulated microglia was not restricted to LPS-evoked signals, we treated microglial cultures with IFN-γ, a well-known microglial activator. RT-PCR analysis revealed that the presence of the inflammatory cytokine IFN-γ (100 U/ml) did not significantly affect the expression of PtdSerR mRNA when analyzed at 8 and 24 h (Fig. 6). Consistent with previous data, IFN-γ did not induce the synthesis of TNF-α (19) nor that of IL-10 (not shown). However, as expected, IFN-γ stimulated microglial NO synthesis, which was almost completely prevented by the presence of 10 to 100 μM PS-containing liposomes (Fig. 6).

DISCUSSION

In the brain, apoptosis represents an important physiological as well as pathological event, occurring during development and in the course of acute and chronic neurological disease (7). Detailed understanding of the molecular processes regulating neuronal apoptosis and the clearance of apoptotic bodies by brain macrophages may provide the basis of therapeutic strategies to prevent or limit neuronal loss. Although extensive studies by several groups have largely improved knowledge of the mechanisms of recognition of apoptotic cells by peripheral...
phagocytes—recently reviewed by Schlegel and Williamson (1)—the phagocytic clearance of neurons undergoing apoptosis by microglia is still not entirely understood.

Microglial cells represent the brain resident macrophages. Similar to the peripheral macrophages, they derive from hematogenous precursors that enter the developing CNS (20). In the immature brain, microglia shows a rounded and simple morphology and is thought to be involved in phagocytosis and removal of degenerating cells. During the postnatal period, the morphology of microglia undergoes progressive changes until the cells acquire long, fine, branched processes and a down-regulated and quiescent phenotype, probably as a consequence of the unique CNS microenvironment. Resting microglia can be rapidly activated in response to pathological events and participate in resolution or exacerbation of the tissue damage, depending on the extent of their activation (20, 21). Because of their adaptation to the CNS environment, microglial cells often behave atypically when compared to peripheral macrophage populations (22). Nonetheless, recent studies indicate that some of the molecular events governing recognition of apoptotic cells by peripheral phagocytes may also take place in the brain. PS exposure, known as an early marker of apoptosis, has been reported to occur in neuronal cells and to be an obligatory step for microglial recognition (8, 9, 23). In addition, the interaction of neuronal PS with a microglial-uncharacterized receptor has been suggested on the basis of the ability of PS-enriched liposomes or of the head group OPS to prevent the uptake of apoptotic cerebellar granules by microglia (9).

Here we present evidence for the expression in microglial cells of mRNA for PtdSerR, the specific receptor for PS recently cloned in peripheral macrophages (2). The mRNA for PtdSerR was found in non-stimulated cultures and its expression increased in the presence of LPS, suggesting that the process of activation up-regulates the expression of the receptor. A correlation between the presence of PtdSerR and the state of cell activation has been reported in previous studies in which PS-liposomes inhibited the phagocytosis of apoptotic cells by elicited macrophages, but not by unstimulated macrophages (24, 25). Consistently, the expression of PtdSerR was much lower in unstimulated than in stimulated human monocyte-derived macrophages (2). The presence of well-detectable levels of PtdSerR mRNA in non-stimulated microglial cells may be explained by considering that these purified cultures, probably because of their origin from immature brain or to the manipulations required for their preparation, are more “responsive” than the in vivo “resting” microglia. On the other hand, they do require further activation by LPS or other inflammatory agents to acquire the functions typically associated with their activation, including phagocytosis, cytokine, and free radical production (26).

Neonatal microglial cultures, though not suitable for studying the characteristics of resting microglia, represent a useful model to identify the molecular mechanisms related to their activation. We have taken advantage of this model to study the functional properties of PtdSerR. We stimulated microglial cultures with PS-liposomes to restrict our analysis to signals elicited by this single ligand-receptor interaction. We found that in LPS-activated microglia, PS liposomes specifically inhibited the production of NO, IL-1β, and TNF-α, known as potent pro-inflammatory substances. These findings suggest that the recognition of PS induces microglial cells to phagocytize apoptotic cells without eliciting the synthesis of pro-inflammatory mediators that could promote inflammatory reaction in the surrounding tissue. Such mechanism could be specific for the phagocytosis of apoptotic neurons (8, 9), since apoptotic T cells have been shown to be removed by microglial cells through a PS-independent mechanism (27).

In contrast to the findings of Fadok et al (6), we found that PS-liposomes only moderately reduced the LPS-stimulated synthesis of IL-10, a pleiotropic molecule with anti-inflammatory immunomodulatory functions. This cytokine has been shown to improve the neurological outcome after CNS injury and to be neuroprotective against excitotoxicity, possibly through the inhibition of IL-1β and TNF-α synthesis and by preventing caspase-3 activity (28–30). Therefore, is tempting to speculate that retaining the ability to synthesize IL-10 could promote neuroprotection. At present, the mechanisms by which PS modulates microglial functions remain undefined. However, our data...
indicate that such mechanisms are not restricted to LPS-evoked signals, as we found that PS-liposomes effectively inhibited NO synthesis induced by IFN-γ. Further studies in vitro and in experimental animal models will help clarify the intracellular events evoked by the interaction of PtdSerR with PS exposing neurons and the functional role of PtdSerR in vivo.

Recent work on primary hippocampal cultures indicates that PS exposure by neurons undergoing apoptosis is reversible and independent from the maintenance of genomic DNA integrity, suggesting a “window of opportunity” for the rescuing of neurons not yet committed to suicide (23). If this was the case, a detailed understanding of the molecular mechanisms regulating expression and functions of microglial PtdSerR could provide additional tools to control engulfment of PS expressing neurons and to favor the reversal of the apoptotic process by appropriate therapeutic interventions.

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