Apoptotic PC12 Cells Exposing Phosphatidylserine Promote the Production of Anti-Inflammatory and Neuroprotective Molecules by Microglial Cells

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Abstract. The interaction of phosphatidylserine (PS), exposed on the surface of apoptotic cells and with its specific receptor (PtdSerR) expressed by microglia, is a crucial event in the recognition and clearance of apoptotic neurons. Here, we extend our previous studies in which PS-liposomes mimicking apoptotic cells were used to investigate the functional role of PS-PtdSerR interactions on microglial functional state. Purified rat microglial cells were either incubated with PC12 cells maintained in complete medium (healthy), exposed to staurosporine or serum deprivation (apoptotic), or treated with hydrogen peroxide (necrotic). After 24 hours, supernatants from co-cultures and single cell type cultures were analyzed for nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), prostaglandin E₂ (PGE₂), transforming growth factor-β1 (TGF-β1), and nerve growth factor (NGF). When lipopolysaccharide (LPS)-activated microglia was cultured with apoptotic PC12 cells, NO and TNF-α levels significantly decreased, IL-10 was not affected, and PGE₂ levels were substantially increased. In addition, TGF-β and NGF syntheses increased when resting microglia was cultured with apoptotic but not healthy or necrotic PC12 cells. We proposed that upon interaction with PS-expressing apoptotic neurons, microglia no longer act as a promoter of the inflammatory cascade and that the specific microglial functional state induced by PS-PtdSerR may be relevant for the final outcome of neurodegenerative diseases.

Key Words: Apoptosis; Brain macrophages; Cytokine; Neurodegeneration; Nerve growth factor; Nitric oxide; Prostaglandin E₂.

INTRODUCTION

Apoptosis is a process of programmed cell death that is essential in the shaping of organs during development and in the maintenance of tissue homeostasis in adult life (1). A main feature of apoptotic cell death is the efficient and fast removal of dying cells by macrophages and non-professional phagocytes. This process ensures the uptake of death-destined cells before their membrane lysis. The release of intracellular contents containing substances that disturb tissue remodeling or homeostasis is thus prevented (2).

The mechanisms involved in the recognition and phagocytosis of apoptotic cells in the peripheral immune system are well documented. Several molecules on the surface of apoptotic cells have been shown to be involved in the recognition and ingestion of apoptotic cells by macrophages (3, 4). Among these, phosphatidylserine (PS), a phospholipid normally found in the inner leaflet of the plasma membrane and externalized during the early phase of the apoptotic process, has received great attention after the identification of a specific macrophage receptor (PtdSerR). This receptor is involved not only in the recognition and ingestion of apoptotic cells, but also in downstream signaling processes leading to the acquisition of an anti-inflammatory and immunosuppressive phenotype by peripheral macrophages (5).

Indeed, the interaction of PS-expressing apoptotic cells or PS-liposomes with PtdSerR induces macrophages to produce transforming growth factor-β1 (TGF-β1) and prostaglandin E₂ (PGE₂), a pleiotropic cytokine and a lipid mediator, respectively, sharing immunomodulatory properties. Moreover, the synthesis of inflammatory molecules such as interleukin-1β (IL-1β) and tumor necrosis factor (TNF-α), typically released by activated macrophages, is prevented (6).

In the CNS, apoptosis plays an important role during development while it is a primary pathogenic mechanism in several adult neurodegenerative disorders. A rapid and effective phagocytosis of apoptotic neurons by microglia, the resident brain macrophages, is necessary in order to protect the surrounding tissue from an undesirable inflammatory reaction, which could exacerbate the neuronal loss. The process of apoptotic neuron recognition by microglia is poorly understood, although experimental evidence suggests that PS externalization is necessary for microglial recognition and phagocytosis of the apoptotic neurons (7). Recently, we demonstrated that microglial cultures express mRNA for PtdSerR, and that the interaction of PtdSerR with PS-liposomes, used to mimic apoptotic neurons, strongly reduces the release of inflammatory molecules such as nitric oxide (NO), IL-1β, and TNF-α (8).

In the present study, we have used as experimental model co-cultures of rat microglial cells and healthy, apoptotic, or necrotic PC12 cells to further support our hypothesis that the interaction of microglia with apoptotic cells promotes the synthesis of anti-inflammatory and neuroprotective molecules.
The experimental setup was chosen because PS exposure is a well-documented change during the early phase of apoptosis in PC12 cells (9), and PC12 cells represent a widely used model to mimic neuronal cells (10).

MATERIALS AND METHODS

Reagents

All cell culture reagents were from Gibco (Grand Island, NY) and virtually endotoxin-free (less then 10 EU/ml as determined by the manufacturer). Rat tail collagen type I was from Upstate Biotechnology (Lake Placid, NY), LPS (from Escherichia coli, serotype 026:B6) and staurosporine were obtained from Sigma. TACS™ AnnexinV kit was obtained from Trevigen (Gaithersburg, MD). ED-1 monoclonal antibody was from Serotec (Oxford, UK). ELISA-kits for rat TNF-α and IL-10 were from En-dogen, Inc. (Woburn, MA). ELISA-kit for human TGF-β1 was from DRG Instruments GmbH, (Marburg, Germany). ELISA-kit for NGF was from Promega Italia (Milano, Italy).

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 (11) and in accordance with the European Communities Council Directive N. 86/609/EEC. Microglial cells harvested from the mixed primary glial cultures by mild shaking were resuspended in Basal Eagle’s Medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and 100 µg/ml gentamicin, and seeded on 24-well uncoated plates at a density of 1 × 10⁴ cells/cm². Cells were allowed to adhere for 20 min and then washed to remove nonadhering cells. After 24 hours of incubation the medium was replaced with fresh medium supplemented with 1% FCS. Cell viability was greater than 95%, as tested by Trypan Blue exclusion. The purity of microglial cultures was then confirmed by immunocytochemistry using the monoclonal antibody ED1. Cells were fixed in 4% paraformaldehyde, permeabilized with acid alcohol (5% acetic acid, 95% ethanol), and then exposed to primary antibody followed by FITC-conjugated goat anti-mouse IgG (F(ab')₂ fragment). Coverslips were mounted in PBS:glycerol 1:1 and examined using a Polyvar (Reichert) ultramicroscope equipped with interference contrast and fluorescence optics. Ninety-nine percent of the cells were positive for ED-1.

The rat pheochromocytoma cell line, PC12, was propagated in flasks coated with rat tail collagen at 37°C (5% CO₂ atmosphere) and maintained in RPMI-1640 medium supplemented with 5% heat-inactivated fetal bovine serum and 10% heat-inactivated horse serum 100 U/ml penicillin, 100 µg/ml of streptomycin, and 2 mM L-glutamine. The cells were plated in 24-well plates, covered with collagen at a density of 1.5 × 10⁴ cells/cm². Experiments were carried out 48 hours after cells were seeded.

Induction of Apoptosis and Necrosis in PC12 Cells

Apoptosis was induced either by 24 hours of serum deprivation or by exposing the cells to 100 nM of staurosporine (prepared as 1 mM stock in dimethyl sulfoxide and stored at −20°C) for 2 hours (12). Necrosis was induced by exposing the cells to 1 mM of H₂O₂ (freshly prepared from a 30% stock solution, prior to each experiment) for 3 hours.

Flow Cytometric Analysis of External PS Exposure with the Annexin V/PI Assay

The redistribution of PS to the outer leaflet of the plasma membrane was revealed by labeling the cells with FITC-conjugated Annexin V according to the manufacturer’s instructions. Cells were analyzed cytofluorometrically using a FACSsort flow cytometer (Becton Dickinson, Sunnyvale, CA). Propidium iodide (PI), which was used to assess plasma membrane integrity, was added to cells to 1 µg/ml immediately before cytofluorometric analysis. This staining allows the distinction among living cells (Annexin V⁻/PI⁻), early apoptotic cells (Annexin V⁺/PI⁻), late apoptotic (Annexin V⁺/PI⁺), and necrotic cells (Annexin V⁺/PI⁺).

Co-Cultures of Rat Microglia and the PC12 Cells

After specific treatments, PC12 cells (8 × 10⁶ cells) were washed twice to remove the death-inducers and cellular debris and seeded onto a microglial layer (2 × 10⁶ cells). PC12 cells were co-cultured with rat microglia in the presence or in the absence of LPS for 24 hours. Supernatants were then recovered and centrifuged at 500 × g for 5 min at 4°C to remove particulate debris and stored at −80°C until use.

Cytokine and NO Determination

The levels of TNF-α, IL-10, and TGF-β1 were assayed by specific ELISA according to the manufacturer’s instructions. For TGF-β1, supernatants were acid-activated before the assay according to the instructions provided with the kit. The ranges of determination were as follows: 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)-ethylene-diamine were added to 150 µl of culture medium. After a 10-min 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.

Statistical Analysis

Data are expressed as mean ± SEM, with the number of independent experiments (run in duplicate) indicated in parenthesis. Comparison between treatment groups was made by Student t-test. A 2-tailed probability of less than 5% (p < 0.05) was taken as statistically significant. Analysis of variance (ANOVA) for repeated measures of TGF-β1 was followed by post-hoc comparison according to Tukey test.

RESULTS

Characterization of Apoptotic and Necrotic Process in PC12 Cells

In order to evaluate the extent of the apoptotic process in PC12 cells induced by 2 experimental paradigms, such

Fig. 1. Characterization of serum deprivation- or staurosporine-induced apoptosis and necrosis in PC12 cells. A representative experiment of FITC-Annexin-V (Ax-V) and propidium iodide (PI) staining used to detect PS externalization in PC12 cells and to quantify the process of cell death is shown. Cells were untreated (A), exposed to 100 nM staurosporine for 2 hours (B), withdrawn from serum for 24 hours (C), or treated with 1 mM of H₂O₂ for 3 hours (D). The relative distribution (in percentage) within each population of early (Ax-V⁻/PI⁻), late apoptotic (Ax-V⁻/PI⁺), and necrotic cells (Ax-V⁺/PI⁻) is indicated.

as staurosporine treatment and serum deprivation, we analyzed PS externalization by staining the cells with a combination of Annexin V (Ax-V) and PI. Preliminary time course and/or dose-response studies were performed to obtain the experimental conditions giving the highest proportion of PC12 cells in the early phase of apoptosis (Ax-V⁺/PI⁻). In our study, optimal conditions were achieved when PC12 cells were exposed to 100 nM of staurosporine for 2 hours or serum-deprived for 24 hours. As shown in Figure 1A, very few Ax-V and PI-positive cells (less than 3%) were present in untreated cultures, suggesting a low constitutive level of dying cells. Upon staurosporine addition, the cells rapidly displayed a striking increase in Annexin binding. The percentage of early apoptotic cells was between 30% and 40% (Ax-V⁺/PI⁻, lower right quadrant), whereas that of late apoptotic cells ranged between 8% and 20% of the total cell population (Ax-V⁻/PI⁻, upper right quadrant). Dead cells (Ax-V⁻/PI⁻, upper left quadrant) represented about 7% of the population (Fig. 1B).

Apoptosis induced by 24-hour serum deprivation was clearly associated with an increase in Annexin binding, although there was a lower percentage of early and late apoptotic cells when compared to staurosporine treatment (Fig. 1C). Indeed, cells positive for Annexin V represented about 8% to 15% (early apoptotic), whereas that of late apoptotic cells ranged between 15% and 20% of the total cell population. Necrotic cells (Ax-V⁻/PI⁺, upper left quadrant) represented about 10% of the population.

Necrosis was induced by exposing PC12 cells to 1 mM of H₂O₂ and quantified by Ax-V⁺/PI⁻ staining and Trypan Blue exclusion. The percentage of necrotic cells (Ax-V⁻/PI⁻) ranged between 50% and 70% of the total cell population. In addition, about 10% of the population was Ax-V⁻/PI⁺ and no early apoptotic cells (Ax-V⁻/PI⁻) were detected (Fig. 1D). Trypan Blue exclusion assay confirmed the range of necrotic cells found with the Ax-V⁻/PI⁻ staining (50%–70% of total population).

In the experiments presented, apoptotic (either exposed for 2 h to 100 nM of staurosporine or serum-deprived for 24 hours), or necrotic PC12 cells were added to microglial cells prepared as described in material and methods, in a ratio of 4:1 and allowed to interact for 24 h.
Effects of PC12 Cells on Pro-inflammatory Molecule Secretion by Resting or LPS-Activated Microglia

To determine whether PC12 cells would influence microglial activation, the production of pro-inflammatory molecules such as NO and TNF-α was analyzed. For this purpose, healthy, apoptotic, or necrotic PC12 cells were added to resting or LPS-activated microglia.

PC12 cells did not secrete detectable amounts of NO either in basal condition or after LPS stimulation. In resting microglial cultures, the levels of nitrite were barely detectable. As expected, a significant increase in nitrite production was induced by 10 ng/ml LPS.

The addition of healthy, apoptotic, or necrotic PC12 cells to resting microglia did not change the basal level of nitrite accumulation (not shown). Similarly, the level of NO produced by activated microglia did not change upon the addition of healthy or necrotic PC12 cells (Fig. 2). Conversely, nitrite accumulation in LPS-activated microglial cells that had interacted with apoptotic PC12 cells was reduced when compared to LPS-activated microglial cells cultured alone or with healthy PC12 cells (Fig. 2). The effect of staurosporine-induced apoptotic PC12 cells on NO production by LPS-activated microglia was higher than that elicited by serum-deprived apoptotic PC12 cells.

TNF-α was not produced by resting microglia and, similar to NO accumulation, the addition of healthy, apoptotic, or necrotic PC12 cells did not induce any TNF-α synthesis. PC12 cells did not release TNF-α either in basal condition or after LPS stimulation (not shown).

Effects of PC12 Cells on PGE_2 and Anti-Inflammatory Cytokine Secretion by Resting or LPS-Activated Microglia

Because of their largely described role as anti-inflammatory and immunoregulatory agents (16), the production of IL-10, TGF-β1, and PGE_2 was analyzed in co-cultures of microglia and PC12 cells as well as in the single cell type cultures.

PC12 cells did not secrete detectable amounts of IL-10 either in basal condition or after LPS stimulation. In resting microglial cultures the levels of IL-10 were barely detectable and were not modified by the addition of healthy, apoptotic, or necrotic PC12 cells. On the contrary, a significant increase of IL-10 production was observed after LPS stimulation. There was a clear increase in IL-10 production after the addition of healthy PC12
cells when compared to pure LPS-activated microglia (584 ± 261 pg/ml vs 91 ± 21 pg/ml, respectively). However, there was no significant difference in microglial IL-10 secretion by LPS-activated microglia cultured alone or with apoptotic or necrotic PC12 cells (Fig. 4), possibly because of the high variability in the levels of LPS-induced IL-10 among independent experiments.

As for IL-10, PGE₂ was almost undetectable in resting microglia, whereas it was upregulated after LPS stimulation (26 ± 5 pg/ml vs 199 ± 68 pg/ml, respectively). PC12 cells did not secrete detectable amounts of PGE₂, either in basal condition or after LPS stimulation (not shown). The addition of staurosporine-treated PC12 cells to LPS-activated microglia significantly increased the PGE₂ production when compared to pure activated microglia cultured alone or with healthy or necrotic PC12 cells (Fig. 5). In contrast to staurosporine-treated PC12 cells, serum-deprived PC12 cells did not affect PGE₂ levels when co-cultured with activated microglial cells (not shown). As before, the addition of the 3 types of PC12 cultures (healthy, apoptotic, or necrotic) had no effect on PGE₂ synthesis in resting microglia (not shown).

As far as TGF-β production is concerned, consistent with earlier reports (8, 16), microglial cells constitutively secreted TGF-β1 that was not further modulated by LPS. Thus, the following experiments were performed using resting microglia. We found that healthy PC12 cells secrete fairly high amounts of total TGF-β1 (active and inactive TGF-β1) and, as expected, the levels of this cytokine were reduced when PC12 cell underwent apoptosis or necrosis (Fig. 6). The levels of total TGF-β1 were also similar in microglial, necrotic PC12 cells, and in microglial/necrotic PC12 cell co-cultures (Fig. 6C).

Neurotrophic Factor Production by Microglial-PC12 Cell Co-Cultures

We investigated whether the addition of apoptotic cells to microglia could affect the levels of NGF, a neurotrophin known to be produced by microglial cells (17). We found a constitutive NGF secretion in pure resting microglia that increased after LPS stimulation (9.2 ± 1.2 pg/ml vs 13.1 ± 0.9 pg/ml). NGF was also detectable in supernatants of single PC12 cell cultures, which was slightly decreased in cultures undergoing apoptosis or necrosis (Fig. 7). A moderate increase in NGF levels was observed when staurosporine-treated PC12 cells were added to resting microglia. This increase was significant when compared to single cultures as well as to microglia/healthy PC12 cells co-cultures (Fig. 7B). As for PGE₂, NGF levels in the co-culture supernatants from serum-deprived PC12 cells with microglia were similar to those observed in pure microglia and in the single cell type cultures (not shown). The levels of NGF in LPS-activated
Fig. 6. Effects of healthy, apoptotic, or necrotic PC12 cells on TGF-β production by resting microglia. Microglial cells (M) were subcultured for 24 hours in 10% FCS-containing medium, which was replaced with 1% FCS-containing medium before addition of healthy (A), apoptotic (B) or necrotic (C) PC12 cells. Supernatants were collected after 24 hours and analyzed for total TGF-β1 accumulation. Each panel shows the accumulation of TGF-β1 in the co-cultures and, for comparison, in the 2 single cell type cultures. M = microglia; H = healthy PC12; A = apoptotic PC12 cells; N = necrotic PC12 cells. **p < 0.005 vs H and M + H. *p < 0.05 vs M and A (A, B), n = 4; (C) n = 3 independent experiments, run in duplicate). A 2-way ANOVA carried out using as grouping factor the experimental design (healthy vs apoptotic vs necrotic cultures) and as repeated measures the sum of the TGF-β1 level in the single type of culture vs the level in the co-cultures gave a nearly significant (sum of the single type of culture-co-culture experimental design) interaction (F 2,8 5 3.12; p 5 0.0998). Post-hoc comparisons, carried out by Tukey test performed in the presence of a nonsignificant F (51), revealed that the difference between the level of TGF-β1 in the co-cultures was higher than the sum of the TGF-β1 values obtained in the single cell type cultures only as seen in panel (B) and not in (A) or (C).

Microglia were not modified by any of the 3 types of PC12 cells (not shown). The lack of effect of apoptotic PC12 cells on LPS-induced NGF synthesis could reflect a refractory state of activated cells, which may already be at maximal level of NGF induction.

DISCUSSION

Neuronal death by apoptosis occurs in several neurological diseases, including Alzheimer and Creutzfeldt-Jakob diseases (18, 19). A number of studies have shown that neurons dying by apoptosis expose PS on their surface during the early phase of the process. In addition, neuronal PS exposure has been described as one of the mechanism for recognition and phagocytosis by microglial cells (7, 20). However, information on the functional consequences related to this process is still sparse.

We have recently demonstrated that interaction of PS-liposomes (used to mimic apoptotic neurons) with the receptor PtdSerR inhibits the release of several inflammatory molecules by activated microglia favoring the acquisition of an anti-inflammatory phenotype (8). To address the hypothesis that the recognition of apoptotic neurons by microglia specifically affects the functional state of microglia, we have developed co-culture models in which microglial cells interact with apoptotic PC12 cells, those expressing PS on their membranes, or with healthy or necrotic PC12 cells.

We found that the synthesis of the pro-inflammatory molecules NO and TNF-α by LPS-activated microglia was significantly decreased upon interaction with apoptotic PC12 cells. These findings are consistent with our previous studies in which NO and TNF-α production was profoundly inhibited when microglial cells were exposed to PS-liposomes (8), and further support the hypothesis that the interaction of activated microglia with apoptotic cells, through the recognition of PS, prevents the synthesis of pro-inflammatory molecules.

Interestingly, we found that the presence of healthy PC12 cells reduced, although to a lesser extent, the production of TNF-α by activated microglial cells. This finding is consistent with recent in vitro studies on glia-neuron co-cultures demonstrating that neurons, either via soluble factors or by cell-cell contacts, reduced TNF-α production by activated glial cells (21). The inhibition of microglial NO and TNF-α induced by staurosporine-treated PC12 cells was consistently higher than that induced by serum-deprived PC12 cells, with the higher percentage of apoptotic and PS-exposing PC12 cells in staurosporine-treated cultures than in serum-deprived cultures. Although serum deprivation cell death represents a more “physiological” stimulus than that obtained with the bacterial alkaloid staurosporine, the externalization of PS in serum-deprived cells is much more asynchronous than in staurosporine-treated cells, where this change occurs rapidly and in a larger proportion of cells. Nonetheless, we cannot exclude that apoptosis by serum deprivation could be accompanied by a higher proportion of...
secondary necrosis during the co-cultivation with microglial cells, which could counteract the signals triggered by apoptotic cells.

In our co-culture models, the interaction of healthy PC12 cells with activated microglia promoted the release of higher levels of IL-10 compared to microglia cultured alone. Such levels were moderately, although not significantly, reduced by apoptotic cells but not necrotic cells. As before, this observation is consistent with our previous report in which only high doses of PS-liposomes were able to reduce IL-10 production by activated microglia (8).

In line with results obtained with peripheral macrophages (6), we found that after interaction with apoptotic PC12 cells, activated microglia secreted a remarkable amount of PGE₂. This lipid mediator is considered a potent pro-inflammatory molecule, being a vasodilator and a mediator of edema, fever, and pain (22). However, an increasing number of in vitro and in vivo studies indicate that PGE₂ is an important modulator of immune and inflammatory response, as it can regulate several macrophage and lymphocyte functions (23). In particular, within the brain, PGE₂ could exert beneficial effects by controlling microglial activation (24, 25) or protecting neurons from noxious stimuli such as NMDA and macrophage-mediated toxicity (26, 27). Nonetheless, at very high concentrations, PGE₂ has been shown to stimulate the release of glutamate and the pro-inflammatory interleukin-6 by astrocytes and to induce neuronal apoptosis (28–30). PGE₂ levels have been found to be increased during the recovery phase in a murine model of multiple sclerosis, suggesting a protective effect of PGE₂ in this pathology (31). Elevated levels of PGE₂ have been found in the CSF of patients affected by Creutzfeldt-Jakob disease and in a murine model of prion disease, where neuronal apoptosis has been described (19, 32), although the role of PGE₂ in degenerative diseases is far from being fully understood (33).

Another metabolite that we found increased in the co-cultures of microglia and apoptotic PC12 cells is TGF-β1, a pleiotropic molecule that, similar to PGE₂, is involved in the modulation of inflammatory and immune response (34). In the CNS, TGF-β1 exerts many biological effects on different cell types, including microglia, astrocytes, and neurons (35). In normal brain, TGF-β1 is minimally expressed, but its expression strongly increases after acute or chronic diseases such as ischemia, Alzheimer, and prion diseases (31, 36, 37).

Recently, a high expression of TGF-β1 and COX-2, the inducible isoform of the first limiting enzyme in PGE₂ synthesis, has been described in an animal model of prion disease (38, 39). Interestingly, in the same model, no evidence for increased synthesis of IL-1β, IL-6, or the expression of inducible nitric oxide synthase was obtained (40), suggesting that in chronic diseases microglial activation is associated with anti-inflammatory rather pro-inflammatory functions. Moreover, several groups have demonstrated that TGF-β could also act as a neuroprotective molecule (41, 42), rescuing hippocampal neurons from apoptosis. Unfortunately, in our cellular model, in which microglial cells are not easily induced to produce TGF-β1 (8, 16), we were not able to ascertain if TGF-β1 secretion was due exclusively to microglial cells or to PC12 cells.

Finally, we found a significant increase in NGF production only when microglial cells were cultured with...
apoptotic PC12 cells. NGF, the best neurotrophin characterized so far, is a crucial factor for the survival or death in several neuronal populations, as well as for the regulation of neuronal phenotype and function (43). In addition, NGF may play a role in the inflammatory response to tissue injury and also acts as pro-apoptotic factor depending on the type of NGF receptor mainly expressed by the cell target (44, 45). During the course of acute as well as chronic neurodegenerative diseases, elevated levels of NGF are present at the site of injury where inflammatory cells as well as dying neurons are present (46, 47). It has been hypothesized that microglial cells, once in contact with degenerating apoptotic neurons become able to release trophic factors to facilitate the functional recovery of the surrounding compromised neurons (48). Supporting this hypothesis are several studies reporting that microglia in co-cultures can either potentiate the survival of cortical neurons or protect apoptotic neurons via soluble factors (43, 49). The present study further supports this view by showing that microglia respond to apoptotic neurons by releasing NGF and suggests that reciprocal interactions between these 2 cell types are necessary to maintain tissue homeostasis.

In degenerative pathologies such as Alzheimer and prion diseases, many neurons die by apoptosis and are phagocytosed by activated microglial cell (18, 19). Thus, it is possible that apoptotic, but not necrotic, neurons promote the synthesis of some microglial products (NGF, TGF-β, and PGE₂), while preventing that of others (TNF-α and NO). From our in vitro studies it is difficult to speculate the possible functional consequence of this specific orientation of microglial phenotype. However, as previously suggested for peripheral macrophages (50), we propose that upon contact with PS-expressing apoptotic cells, microglia no longer act as promoters of the inflammatory-cascade and that this may be important for limiting neuronal loss in several pathologies in which neuronal death by apoptosis has been described.

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