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Experimental Neurobiology

Original Article

Role of TGF-β in Survival of Phagocytizing Microglia: Autocrine Suppression of TNF-α Production and Oxidative Stress

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Microglia are recognized as residential macrophageal cells in the brain. Activated microglia play a critical role in removal of dead or damaged cells through phagocytosis activity. During phagocytosis, however, microglia should survive under the harmful condition of self-producing ROS and pro-inflammatory mediators. TGF-β has been known as a classic anti-inflammatory cytokine and controls both initiation and resolution of inflammation by counter-acting inflammatory cytokines. In the present study, to understand the self-protective mechanism, we studied time-dependent change of TNF-α and TGF-β production in microglia phagocytizing opsonized-beads (i.e., polystyrene microspheres). We found that microglia phagocytized opsonized-bead in a time-dependent manner and simultaneously produced both TNF-α and TGF-β. However, while TNF-α production gradually decreased after 6 h, TGF-β production remained at increased level. Microglial cells pre-treated with lipopolysaccharides (a strong immunostimulant, LPS) synergistically increased the production of TNF-α and TGF-β both. However, LPS-pretreated microglia produced TNF-α in a more sustained manner and became more vulnerable, probably due to the marked and sustained production of TNF-α and reduced TGF-β. Intracellular oxidative stress appears to change in parallel with the microglial production of TNF-α. These results indicate TGF-β contributes for the survival of phagocytizing microglia through autocrine suppression of TNF-α production and oxidative stress.

Key words: microglia, LPS, phagocytosis, TNF-α, TGF-β, ROS

INTRODUCTION

Macrophages play an important role in the innate and adaptive immune responses, and produce inflammatory mediators during infection. Inflammatory reaction is necessary to pathogen removal, but sustained inflammation can damage the tissue [1].

Microglial cells are recognized as residential macrophages in the CNS and involved in pathogen removal and inflammatory responses depending on pathophysiological conditions [2]. When microglial cells are activated, phagocytosis occurs together with the release of cytokines including tumor necrosis factor (TNF), interleukin-1β (IL-1β) and other inflammatory mediators such as reactive oxygen species and nitric oxide (NO) [2-4]. In contrast, when removing apoptotic cells or myelin debris, microglia release anti-inflammatory factors such as IL-10, TGF-β, prostaglandin E2, and platelet-activating factor (PAF) [5, 6]. However, uptake of apoptotic cells by microglia down-regulate pro-inflammatory

cytokines such as TNF-α, IL-12, IL-1β and other inflammatory mediators [6, 7].

TGF-β is a regulatory molecule with diverse effects on cell proliferation, differentiation, migration, and survival that affect multiple biological processes, including development, carcinogenesis, fibrosis, wound healing, and immune responses [8, 9]. Especially, the critical role of TGF-β in the immune system is to maintain tolerance via the regulation of lymphocyte proliferation, differentiation, and survival. However, TGF-β controls both initiation and resolution of inflammation by regulating cell differentiation and inflammatory cytokines and costimulatory molecules [10, 11]. It seems that TGF-β acts initially as pro-inflammatory cytokines, but when inflammation is ongoing with other proinflammatory mediators, TGF-β acts as anti-inflammatory cytokines [9, 12]. It has been reported that phagocytosis of apoptotic cells leads to TGF-β secretion in macrophage, which inhibits the production of inflammatory cytokines and chemokines including IL-1β, TNF-α, GM-CSF, and IL-8 [6, 13]. *In vitro*, TGF-β inhibits the expression of several LPS-induced inflammatory mediators such as TNF-α and MMP-12 as well as chemokines including MIP-1α and MIP-2 [14, 15]. Reactive oxygen and nitrogen species produced by activated macrophages are important mediators against invading microbes. TGF-β downregulates the production of nitric oxide (NO) and superoxide ion and inhibits the expression of inducible NO synthase (iNOS) in activated macrophages [16]. Interestingly, however, phagocytizing microglia survive despite of the marked production of pro-inflammatory cytokine and reactive oxygen or nitrogen species. In the present study, we investigated the autocrine regulation those harmful mediators in phagocytizing microglia.

MATERIALS AND METHODS

Materials

Minimum Essential Medium (MEM), trypsin/EDTA, penicillin/streptomycin and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Polystyrene microspheres (PS) (1 μm,diam) (Polysciences Inc.), Bovine serum albumin (BSA.), Rabbit Anti-bovine serum albumin antibody (ICN) and LPS were purchased from Sigma. TNF-α and TGF-β ELISA kits were purchased from R&D Systems (Minneapolis, USA). ED-1 and GFAP were purchased from BD Pharmingen.

Cell cultures

Primary culture of mixed glial and microglia were prepared as previously described [17]. In brief, cells were cultured from the prefrontal cortices of 1- to 2–day-old Sprague-Dawley rat pups. Mixed glial cells were dissociated by mild trypsinization (for 8 min at 37°C, with 0.1% trypsin-containing HBSS) and passed through sterile nylon sieves (130 μm, pore size) into MEM (Minimum Essential Medium) containing 10% heat-inactivated FBS, penicillin and streptomycin 10 ml. They were then plated onto poly-D-lysine (2 μg/ml)-coated 75-cm^2 culture bottles and maintained in MEM supplemented with 10% FBS (7-8 days in culture). The mixed glial cells were then trypsinized, washed, and plated in growth medium onto poly-L-lysine-coated 48-well plates. Microglia-enriched cultures were prepared from primary mixed glial cell cultures as described [18]. To obtain microglial cells, the flasks were shaken at 200 r.p.m. for 1 min at 37°C on day 8 of the mixed glia cultures. The medium containing detached microglial cells was collected and centrifuged at 2000 r.p.m. for 200 s. The medium after centrifuge was taken as microglial conditioned medium. Cells were res-uspended, counted and plated at a density of 1.05×10^6 cells/mm^2 in 48- or 6-well plates in conditioned medium. After 12
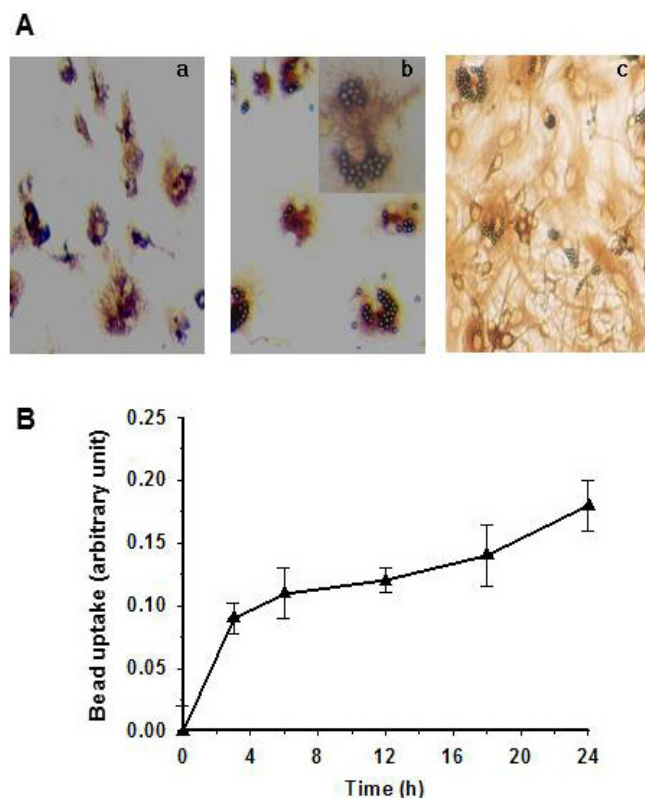

Fig. 1. Phagocytosis of polystyrene microspheres by glial cells. (A) Mixed glial cells were treated with vehicle (a) or polystyrene microspheres (b, c) for 3 h. Cells were fixed and immunostained with antibodies against ED-1 (a, b), or GFAP (c), as described in the Materials and Methods section. (B) Time-dependent uptake of polystyrene microspheres by glial cells. Representative images were obtained from 6 separate experiments.

h, microglia were used for experiments.

Phagocytosis assay

To opsonize particles with anti-BSA antibody, particles were initially associated with BSA [19]. To allow nonspecific adsorption of BSA onto particles, polystyrene microspheres (PS) (diameter=1 μm) were incubated with 10 mg/ml BSA for 1 h at 37°C and washed by centrifugation. To conjugate BSA to PS, PS were incubation with 0.5% glutaraldehyde overnight, then washed by centrifugation, incubated with BSA 10 mg/ml for 2 h at room temperature. Particles with associated albumin were opsonized for 30 min with rabbit anti-BSA antibodies in PBS with a dilution ratio of 1:25. The PS washed with PBS and used in phagocytosis assay.

To study phagocytotic activity, cells were incubated with opsonized-bead at 37°C for 3, 6, 9, 12 and 24 h and then washed with PBS thrice to remove serum and unbound beads [20]. Then cells were lysed by DNA fragment lysis buffer containing 10 mM Tris-HCl, 10 mM EDTA, 0.5% Triton X –100, and 0.2% SDS [21]. The beads were washed twice with PBS, and the absorbance at 550

nm was determined using a microtiter plate reader (Molecular Devices, USA).

Determination of TNF-α and TGF-β

TNF-α and TGF-β levels were determined by ELISA kit (Bio-Source Camarillo, CA) [22]. To analyze TNF-α and TGF-β levels, 3×10^5/ml microglia were incubated overnight. After a washing step with 1% FBS medium, beads were added to each well for various times and then supernatants were collected for determination of TNF-α and TGF-β levels. The sensitivity of the assay was 15.6 pg/ml.

Immunocytochemical staining

Cells grown on 48 well plates were fixed for 10 min at room temperature with 100% ethanol [23]. To inactivate endogenous peroxidase, cells were incubated with PBS containing 3% H_2O_2 for 5 min at room temperature. Nonspecific staining was blocked by incubating cells with PBS containing 8% bovine serum albumin (BSA) for 30 min at room temperature. Cells were then incubated with primary antibody for 2 h at room temperature. The following
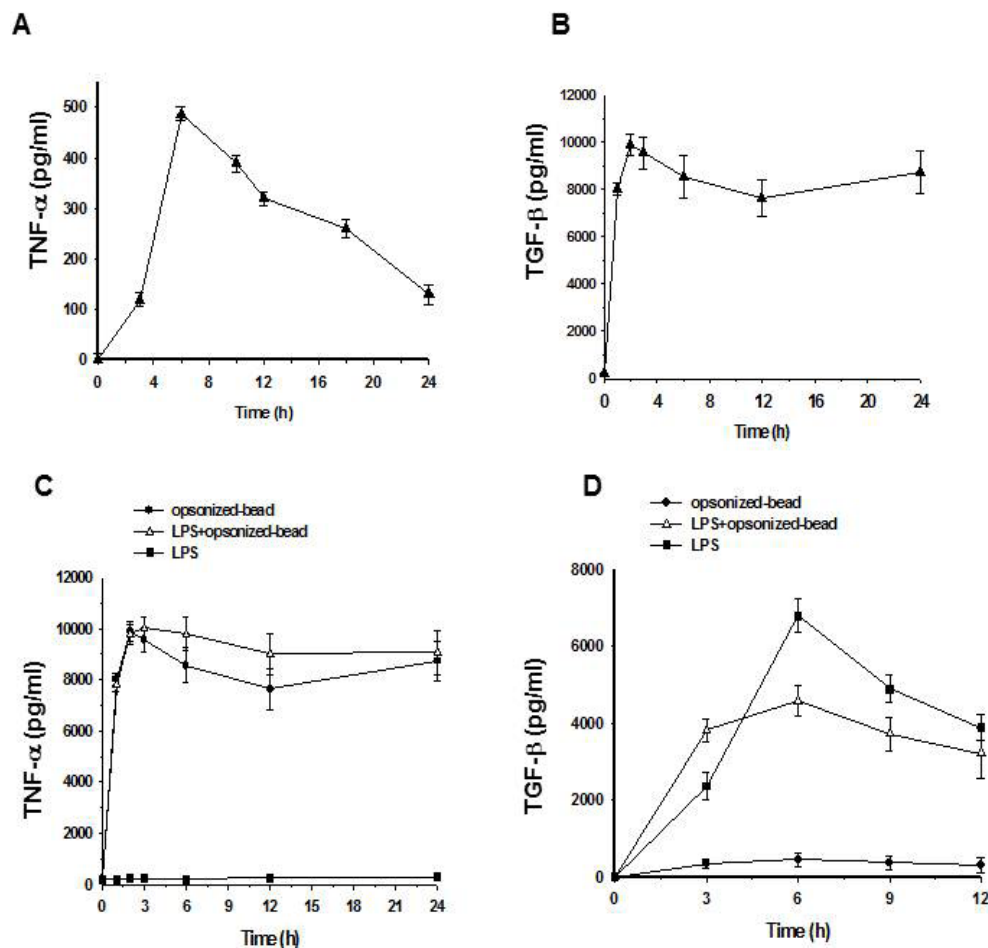

Fig. 2. Production of TNF-α and TGF-β by phagocytizing microglial cells. Microglia were incubated with polystyrene microspheres (diameter=1 μm). (A, B) Time-dependent productions of TNF-α and TGF-β were determined in microglia incubated with beads. (C, D) Time-dependent productions of TNF-α and TGF-β were determined in LPS (20 ng/ml) -pretreated or -untreated microglia incubated with opsonized beads. Each point represents the mean +/- standard deviation of TNF-α and TGF-β determination (pg/ml). N=6 (A, B) and 8 (C, D).

primary antibodies were used: anti-GFAP Ab at a dilution of 1:500; anti-ED-1 Ab at a dilution of 1:250. After washing extensively with PBS, cells were probed for 1 h at room temperature with anti-mouse horseradish-peroxidase-conjugated secondary antibody at a dilution of 1:200. The immunoreactivity was visualized with diaminobenzidine in the presence of 0.1% H_2O_2. For negative controls the first antibodies were omitted.

Determination of cell viability: lactate dehydrogenase (LDH) release

Cell injury or death was assessed by morphological examination using a phase-contrast microscope (DM IL, Leica, Germany) or by measuring the amount of lactate dehydrogenase (LDH) released into the culture medium employing a diagnostic kit (Sigma-Aldrich, St. Louis, MO), as previously described [24]. Absorbance readings were measured at 450 nm using a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA) and are expressed as the % of total LDH release, which was derived from sister cultures subjected to repeated freeze/thaw cycles.

Measurement of intracellular oxidative stress: DCF-DA assay

Cells were loaded with 30 μM CM-H_2DCFDA. CM-H_2DCF-DA diffuses through cell membranes and is hydrolyzed by intra-

cellular esterases to the nonfluorescent analog dichlorofluorescein (DCFH). DCFH then reacts with intracellular free radicals such as peroxyl radical, peroxynitrite, or hydrogen peroxide to form dichlorofluorescin (DCF), a green fluorescent dye. Two hours after loading, cells were washed with EBSS buffer containing 0.1% BSA and 2.5 mM probenecid. Fluorescent intensities were then measured at 488 nm of excitation wavelength and 525 nm of emission wavelength employing a fluorescence microscope (DM IL HC Fluo, Leica, Germany) equipped with a digital camera (DFC420C, Leica, Germany). The intensity of fluorescence was quantified by an image analyzer (TOMORO ScopeEye 3.5, Seoul, Korea).

Statistical analysis

Data are expressed as the mean±standard deviation (s.d) and analyzed for statistical significance by using one-way ANOVA followed by Scheffe's test for multiple comparisons. A p value <0.05 was considered significant.

RESULTS

First, we observed the time-dependent phagocytosis of opsonized beads in cultured mixed glial cells (Fig. 1). Opsonized beads were rapidly phagocytized by glial cells, especially microglial cells,
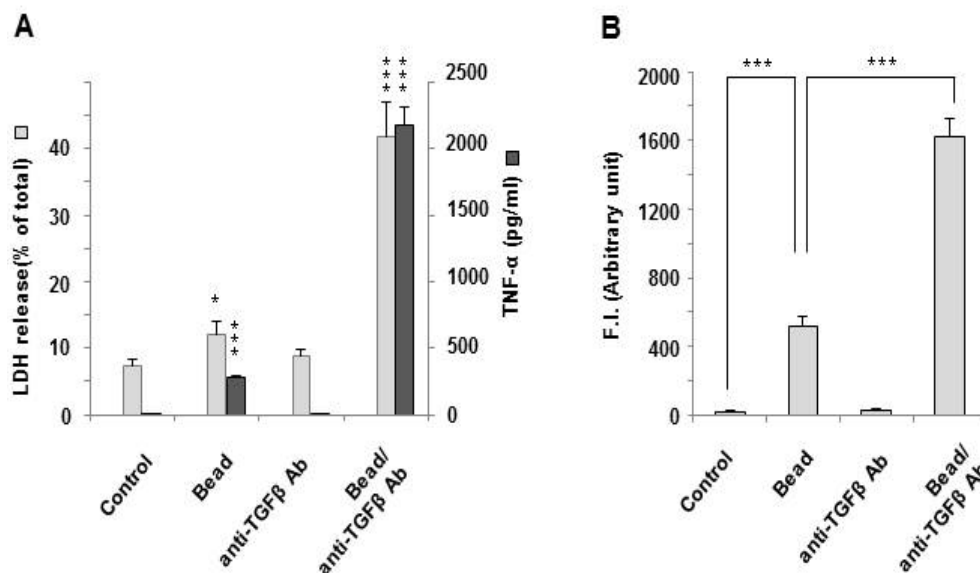

Fig. 3. Neutralization of TGF-β increases the LDH release, TNF-α production and intracellular oxidative stress in bead-activated microglia. (A) At 18 h after incubation with beads, microglial injury was assessed by measuring LDH release into the culture media and the culture medium was used for the determination of the level of TNF-α. Data were expressed as % of total LDH release measured in parallel cultures exposed to repeated freeze/thaw cycles. Data were expressed as mean±SD from 6 independent experiments. *p<0.05, ***p<0.001: significantly different between control vs. bead, or bead vs. bead/anti-TGF-β. (B) Intracellular oxidative stress measured by DCF fluorescence. CM$_2$DCF-DA was loaded at 5 h and fluorescence intensities measured at 6 h after incubation with beads. Data were expressed as mean±SD from 7 independent experiments. ***p<0.001: significantly different between indicated groups.

which are immunopositive to ED-1 (Fig. 1A, b), but not by GFAP-immunopositive astroglial cells (Fig. 1A, c). Initially, bead uptake by microglial cells occurred rapidly, and gradually increased later (Fig. 1B).

We further determined the production of TNF-α and TGF-β in microglia phagocytizing beads (diameter=1 μm). Regardless of opsonization, phagocytosis of beads stimulated microglial production of TNF-α and TGF-β (Fig. 2). While the level of TNF-α production decreased gradually after 6 h, TGF-β release remained high (Fig. 2A, B). However, opsonized bead increased much more production of TNF-α and TGF-β in microglia (Fig. 2C, D). However, there was no synergistic increase of TNF-α and TGF-β production between oponized bead and LPS treatment (Fig. 2C, D). While the level of TNF-α production (Fig. 2A) remained high, TGF-β (Fig. 2B) release was gradually decreased (Fig. 2C, D).

Since bead-activated microglia release TGF-β and down-regulate production of TNF-α (Fig. 2), we neutralized TGF-β by using anti-TGF-β Ab to study whether down-regulation of TNF-α was caused by co-produced TGF-β (Fig. 3). Expectedly, neutralization of TGF-β using anti-TGF-β Ab augmented the LDH release, TNF-α production and intracellular oxidative stress in bead-activated microglia.

DISCUSSION

In the central nervous system, resident microglial cells are major inflammatory cells responding to various kinds of brain injury. Activated microglia potentiates damage to BBB integrity and exert neurotoxic functions through the production of reactive oxygen species (ROS), cytokines (IL-1β, IL-6, TNF-α), and MMP-9. Activated microglia are also known to remove dead cells or other debris in brain lesions. In turn, however, microglial cells can be damaged by those biologically active molecules produced by themselves. To understand the self-protective mechanism, in the present study we studied the temporal changes of production of pro- and anti-inflammatory cytokines as well as ROS in phagocytizing microglia.

TNF-α is a pro-inflammatory cytokine mediating an array of inflammatory response, including activation of leukocytes, production of cytokines, chemokines and adhesion molecules, and stimulation of proteases in neutrophils and endothelial cells [25]. TGF-β is generally thought to have anti-inflammatory effects, but it may have both pro- and anti-inflammatory effects depending on the context in which it is acting [9]. Previous reports also showed that TGF-β might play an important role in early steps of inflammation by inducing monocytes to improve their survival, migration and stimulatory capacity and by attracting both CD4+

and CD8+ T cells to sites of inflammation [26, 27].

When phagocytosis of opsonized-bead induced microglial activation in our experiments (Fig. 2A, B), TGF-β as well as TNF-α are simultaneously released, demonstrating that TGF-β participates in early steps of inflammation. TNF-α production increased steadily at first, but it decreased 6 h later, whereas TGF-β level lasted high for longer than 1 d (Fig. 2A, B). From these results, we can speculate that autocrine secretion of TGF-β from activated microglia results in a gradual decrease of inflammatory TNF-α production. It also consistent with the result that direct addition of TGF-β to LPS-stimulated macrophages partially inhibited both LDH release (our unpublished results) and production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-8 and GM-CSF, thereby proving paracrine and autocrine inhibitory effect of TGF-β [6]. We also observed that when LPS-stimulated microglia phagocytized opsonized-bead, microglial production of TNF-α was up-regulated, but production of TGF-β was down-regulated (Fig. 2, C and D). From this result, we speculate that phagocytosis of opsonized-bead influences microglial state in a favor of pro-inflammatory state.

TGF-β plays a role in resolution of inflammation not only by acting as an anti-inflammatory cytokine as afore-mentioned, but also by promoting healing and repairing of damaged tissue/cell during inflammation [28]. The abilities of TGF-β for promotion of healing [29] make TGF-β ligands as potential therapeutic agents in several disease such as cutaneous wounds [30], mucositis [31, 32], bone defect [25] and stroke [33, 34]. Especially in stroke, administration of TGF-β into the brain reduced infarct size in experimental animal models of ischemia, while antagonizing the endogenous actions of TGF-β with an injection of a soluble TGF-β type II receptor resulted in a drastic increase in infarct area [35]. Considering anti-inflammatory/cytoprotective activities against TNF-α and anti-oxidant activities, TGF-β would be of great therapeutic benefit to inflammation-related diseases such as cerebral stroke, Parkinson's disease and others.

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