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Biochemical and Biophysical Research Communications 328 (2005) 281–287
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Characterization of new microglia-like cells obtained from neonatal rat brain

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Received 24 December 2004
Available online 7 January 2005

Abstract

We isolated spontaneously proliferating cells from primary astrocyte-enriched cultures prepared from neonatal rat brain. These cells proliferated and retained their characteristics for up to 50 generations. They expressed the microglial marker, OX42, but not glial fibrillary acidic protein, an astroglial marker. In addition, they possessed phagocytotic activity, and, when stimulated by lipopolysaccharide (LPS) or interferon-γ (IFN-γ), they expressed proinflammatory mediators, including cytokines (i.e., interleukin (IL)-1β and tumor necrosis factor-α) and chemokines (i.e., IL-8 and monocyte chemotactic protein-1). Protein expression of inducible nitric oxide synthase and cyclooxygenase-2, and production of NO by these cells were induced by LPS or IFN-γ. Thus, these cells possess the characteristics of microglia and can be used as a rat microglial cell line.

Keywords: New cells; Astrocyte-enriched culture; Spontaneously proliferating; Microglial cell line

Until recently, it has been thought that the brain is an immune-privileged organ, but there have been increasing numbers of reports of immune reactions in the brain [1–3]. Glial cells, which include microglia, astrocytes, and oligodendrocytes, mainly play a role in immune reactions in the central nervous system (CNS). Whereas neurons [4] and astrocytes [5] are of neuroepithelial origin, microglia are of mesenchymal origin [6] and behave so much like macrophages that they are often called brain macrophages. Besides the similarities in cellular origin, microglia, like macrophages, secrete inflammatory mediators and can carry out phagocytosis and antigen presentation [7–11]. Activated microglia secrete proinflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, chemokines, including IL-8 and monocyte chemotactic protein (MCP)-1. Activation of microglia also induces the expression of genes encoding inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), which are responsible for the production of NO and prostaglandins, respectively [12,13]. Microglia have the potential to function as important immunoregulatory cells in the brain. When the CNS is injured, microglia rapidly proliferate and migrate to the injured sites where they secrete inflammatory mediators. In this way, microglia are thought to protect neurons from external injuries. Activated microglia are observed in the brains of patients of Alzheimer's disease, stroke, and other neurodegenerative diseases [14–16]. Of course, microglial activation can occur as a result of brain injury, and there is substantial evidence that microglial activation can aggravate brain injuries, resulting in neurodegenerative disease [17–19].

Because microglia-mediated brain inflammation can participate in the initiation or progression of neurodegenerative disorders, it is important to understand not

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.12.169

only their mechanisms of activation but also their functions. In spite of the evidence that microglia play significant roles in several neurodegenerative diseases, the correlation between microglial activation and brain damage remains unclear. One of the greatest difficulties in studies of primary microglia is in obtaining sufficient cells. As a result, several murine and human microglial cell lines have been used [20–22], but cell lines differ from primary cultured microglia in several respects. Moreover, cell lines derived from rat brain microglia have not been available. In the course of experiments with primary rat astrocyte cultures, we observed rapidly proliferating cells that appeared to be similar to that of the BV2 murine microglial cell line. Here we describe the isolation and characterization of these new cells.

Materials and methods

Reagents. Lipopolysaccharide (LPS) and anti-glial fibrillary acidic protein (GFAP) antibodies were purchased from Sigma Chemical (St. Louis, MO, USA) and IFN-γ was from Calbiochem (San Diego, CA, USA). Antibodies against iNOS were purchased from Upstate Biotechnology (Lake Placid, NY, USA), COX-2 and actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and OX-42 antibodies were purchased from Boehringer–Mannheim (Indianapolis, IN, USA).

Cell culture. Primary mixed glial cultures were prepared from the cerebral cortices of 1- to 3-day-old Sprague–Dawley rats. Primary microglia were prepared as described previously [23]. Briefly, the cortices were triturated into single cells in minimal essential medium (MEM; Sigma Chemical) containing 10% fetal bovine serum (FBS; Hyclone, Logan, Utah) and grown in 75 cm^2 T-flasks (0.5 hemisphere/flask) for 2 to 3 weeks. Microglia were then detached from the flasks by mild shaking and applied to a nylon mesh to remove astrocytes. After 1 h, unattached cells were removed by washing, and the remaining cells were used for primary microglia. To prepare pure astrocytes, microglia were removed from the T-flasks by mild shaking. The cells remaining in the flasks after removal of the microglia were harvested with 0.1% trypsin and plated into dishes or plates. BV2 cells, a murine microglial cell line, were cultured in Dulbecco's modified Eagle's medium (Hyclone) containing 5% fetal bovine serum. New cells were cultured in MEM supplemented with 5% fetal bovine serum. All subsequent passages were performed at a split ratio of 1:4. Cells that were passaged fewer than 10 times after being detached from astrocyte bed were designated as early passage, cells passaged more than 10 times were designated as late passage. Except for comparison experiment of early passage with late one, cells between 10 and 40 passages were used for the remaining experiments.

Transmission electron microscopy (TEM). Cells were fixed in Karnovsky's fixative solution (1% paraformaldehyde, 2% glutaraldehyde, and 2 mM calcium chloride in 0.1 M cacodylate buffer, pH 7.4) for 2 h at room temperature. The cells were washed with cacodylate buffer, post-fixed for 1 h in 1% osmium tetroxide and 1.5% potassium ferrocyanide, dehydrated with a series of graded ethanol concentrations, and then embedded in Poly/Bed 812 resin (Pelco, Redding, CA, USA). Cells were cut using a Reichert Jung Ultracut S (Leica, Vienna, Austria), mounted on grids, stained with uranyl acetate and lead citrate, and examined under a Zeiss EM 902 A electron microscope (Leo, Oberkohen, Germany).

Western blot analysis. Cells were washed twice with cold phosphate-buffered saline, and then lysed in ice-cold modified RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, and 150 mM NaCl) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin, 10 μg/ml pepstatin, 1 μg/ml aprotinin, 1 mM Na_3VO_4, 1 mM NaF, and 2 mM EDTA). The lysates were centrifuged at 12,000*g* for 20 min at 4 °C, and the supernatant was collected. Proteins were separated by SDS–PAGE and then electrophoretically transferred to a nitrocellulose membrane (Schleicher and Schuell Bioscience, Keene, NH, USA). The membrane was incubated with primary antibodies, followed by peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA, USA) and then visualized using an enhanced chemiluminescence detection system (Sigma Chemical).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated using RNAzol B (Tel-Test, Friendswood, TX, USA), and cDNA was prepared using reverse transcriptase from avian myeloblastosis virus (Promega, Madison, WI, USA) according to the manufacturer's instructions. The PCR primers for iNOS, TNF-α, IL-1β, IL-8, MCP-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were as follows: iNOS, 5′-TCA CTG GGA CAG CAC AGA AT-3′ (forward) and 5′-TGT GTC TGC AGA TGT GCT GA-3′ (reverse); TNF-α, 5′-GTA GCC CAC GTC GTA GCA AA-3′ (forward) and 5′-CCC TTC TCC AGC TGG GAG AC-3′ (reverse); IL-1β, 5′-TGA TGT TCC CAT TAG ACA GC-3′ (forward) and 5′-GAG GTG CTG ATG TAC CAG TT-3′ (reverse); IL-8, 5′-GAA GAT AGA TTG CAC CGA TG-3′ (forward) and 5′-CAT AGC CTC TCA CAC ATT TC-3′ (reverse); MCP-1, 5′-ATG CAG GTC TCT GTC ACG CT-3′ (forward) and 5-CTA GTT CTC TGT CAT ACT GG-3′ (reverse); and GAPDH, 5′-TCC CTC AAG ATT GTC AGC AA-3′ (forward) and 5′-AGA TCC ACA ACG GAT ACA TT-3′ (reverse). PCR products were separated by electrophoresis on a 1.5% agarose gel and detected under UV light.

Immunohistochemistry. Cells were plated on 12-mm round coverslips (Fisher Scientific, Pittsburgh, PA, USA) that were coated with poly-D-lysine (Sigma). Cells were fixed with ice-cold 100% methanol (Merck, Whitehouse station, NJ, USA). For immunostaining, the fixed cells were washed twice with PBS and then incubated for 30 min with 1% bovine serum albumin (Sigma). The cells were then washed with PBS containing 0.01% Triton X-100 (PBS-T) and then incubated overnight at 4 °C with OX-42 and GFAP antibodies. The cells were rinsed three times for 5 min with PBS-T and incubated with fluorescein isothiocyanate (FITC)-conjugated secondary mouse antibodies and rhodamine-conjugated secondary rabbit antibodies (Cappel, Durham, NC, USA) for 2 h at room temperature. Each coverslip was rinsed with PBS-T, mounted on slides with mounting solution, and observed with the fluorescence microscope (Zeiss).

Determination of NO release. Media nitrite concentration was measured as an indication of NO release. Following the indicated incubation time, 50 μl of culture medium was removed and mixed with an equal volume of Griess reagent (0.1% naphthylethylene diamine, 1% sulfanilamide, and 2.5% H_3PO_4), and the absorbance of the mixture at 540 nm was measured.

Phagocytosis assay. The phagocytotic ability of cells was determined by following the uptake of latex beads. Latex beads (particle diameter = 1.09 μm; 1 μl beads/ml; Sigma) were added to wells containing cultured cells. After an overnight incubation, cultures were rinsed several times with PBS. The phagocytosis of latex beads by the cells was examined under a microscope.

Results

Isolation and morphological identification of new cells

While maintaining astrocyte cultures obtained from the neonatal rat brain, we unexpectedly observed a subset of cells that spontaneously and rapidly proliferated as clones on the astrocyte beds (Fig. 1A). These cells

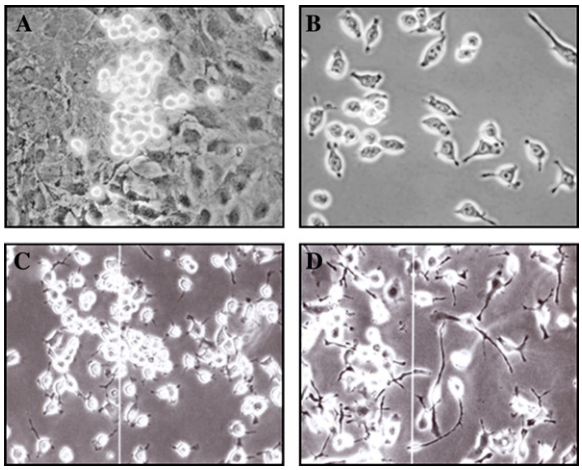

Fig. 1. Morphological characterization of the new cells. (A) Phase contrast micrograph of new cells on an astrocyte bed. The new cells appear in astrocyte-dense areas but not in astrocyte-poor areas. (B) Phase contrast micrograph of the new cells on culture dishes. New cells on an astrocyte bed were detached and reseeded on culture dishes. They appear to have a similar size and morphology as BV2 immortalized murine microglial cells. (C) Phase contrast micrograph of new cells in MEM containing 5% fetal bovine serum. Most of the cells are round without processes, but some have short processes. (D) Phase contrast micrograph of new cells in serum-free MEM. Serum-starved new cells extended long processes.

appeared in astrocyte-rich areas but not in astrocyte-poor areas. They could be detached with gentle shaking, and when they were reseeded on plates, they showed diverse morphologies, including a round shape with or without a few processes and a bipolar shape (Fig. 1B). With continued passaging, the cells had a more uniform morphology, mostly round or ovoid with a few short processes (Fig. 1C). These cells had a morphology and size similar to the immortalized murine microglial cell line, BV2. We cultured the new cells in MEM supplemented with 5% fetal bovine serum. When they were transferred to serum-free media, they extended processes and survived for up to 24 h (Figs. 1C and D).

To further confirm their morphology, we examined the cells by TEM. We found that the new cells resembled BV2 cells rather than primary microglia. The BV2 (Fig. 2B) and new cells (Figs. 2A and C) were rather small compared to primary microglia and had a relatively large nucleus, clear mitochondria, and endoplasmic reticulum. In contrast, the TEM images of primary microglia showed larger cell bodies, a smaller nucleus, and mitochondria as well as many vacuoles (Fig. 2D).

Identification of glial markers

To identify the origin of the new cells, we performed immunocytochemistry using antibodies against OX-42 and GFAP, which are markers for microglia and astrocytes, respectively. The surface of the new cells was labeled by OX-42 antibodies but not by GFAP antibodies (Fig. 3A). The BV2 and the new cells also

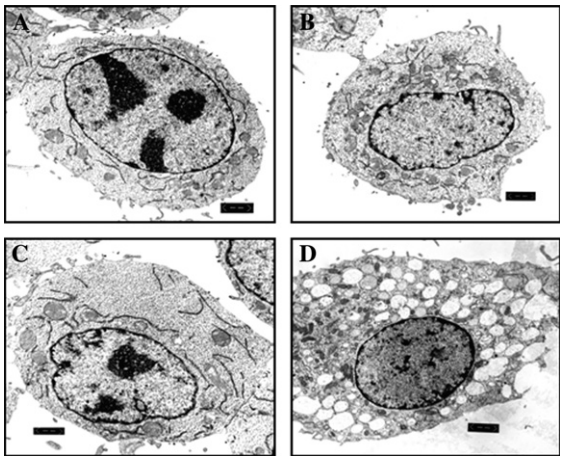

Fig. 2. TEM image of untreated new cells, BV2 cells, and primary microglia. New cells (A,B) and BV2 cells (C) are smaller in size and have relatively large nuclei and poor cytoplasm compared to primary microglia. Primary microglia (D) show larger cell bodies, smaller nuclei, and many vacuoles. Also, their mitochondria are distorted and shrunken due to the presence of enlarged vacuoles. These results indicate that the new cells resemble BV2 cells rather than primary microglia. Scale bar = 1.7 μm (A, B, and D) or 1.1 μm (C).

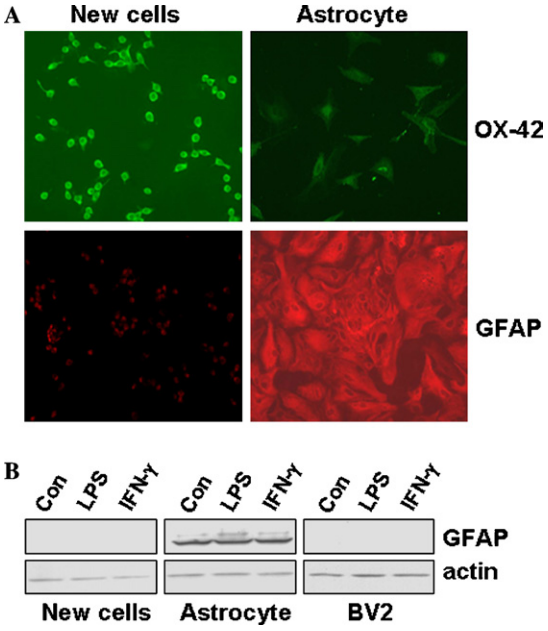

Fig. 3. Identification of the origin of the new cells. (A) Immunocytochemical characterization of new cells. New cells and primary astrocytes were labeled with antibodies against OX-42 (green) or GFAP (red). New cells were immunostained by anti-OX-42 but not by anti-GFAP (left panel). In contrast, primary astrocytes were not immunostained by anti-OX-42 but were stained by anti-GFAP (right panel). (B) New cells, primary astrocytes, and BV2 cells were treated with LPS (100 ng/ml) or IFN-γ (10 U/ml) for 24 h. Cell lysates were separated by 10% SDS–PAGE, and the Western blots were probed with anti-GFAP and anti-actin. Actin protein was used as a loading control. GFAP was expressed in primary astrocytes but not in new cells or BV2 cells. The Western blot data are representative of three separate experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

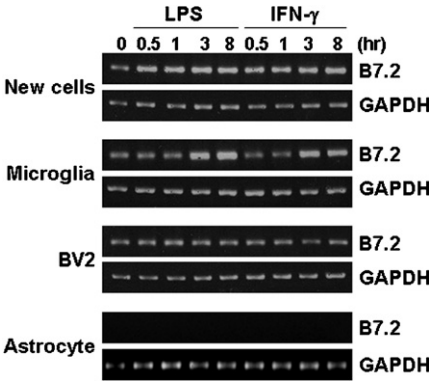

Fig. 4. B7.2 expression in new cells, primary microglia, BV2 cells, and primary astrocytes. Cells were treated with LPS (100 ng/ml) or IFN-γ (10 U/ml) for the indicated times. Total RNA was isolated and analyzed for the level of B7.2 transcript by RT-PCR. The B7.2 transcript was expressed in unstimulated new cells, BV2, and primary microglia, and its level was further increased by stimulation with LPS or IFN-γ. In contrast, B7.2 was not expressed in unstimulated or stimulated astrocytes. The level of GAPDH transcript was used for normalization. RT-PCR data are representative of three separate experiments.

showed no immunoreactivity with GFAP antibodies in Western blots, regardless of whether or not they were stimulated with LPS or IFN-γ (Fig. 3B). Moreover, we found that unstimulated and LPS- or IFN-γ-stimulated new cells expressed mRNA for B7.2 (Fig. 4), a co-stimulatory molecule expressed in primary microglia and BV2 cells but not in primary astrocytes [24]. Together, these results indicate that the new cells are derived from primary astrocyte-enriched cultures and are of microglial lineage.

Expression of proinflammatory mediators in new cells

Several studies have found that activated microglia produce inflammatory mediators, including iNOS, TNF-α. Therefore, we compared the activation profiles of new cells with those of primary microglia and BV2 cells. First, we investigated the mRNA expression level for the proinflammatory mediators using a RT-PCR-based assay (Fig. 5A). As in primary microglia and BV2 cells, in the new cells, LPS or IFN-γ induced the transcription of iNOS, the proinflammatory cytokines TNF-α and IL-1β, and the chemokines, IL-8 and MCP-1. Second, we examined the protein expression level of iNOS and COX-2, enzymes that are responsible for the production the inflammatory mediators NO and prostaglandins, respectively. Western blot analysis (Fig. 5B) showed that, as in primary microglia and BV2 cells, the expression of iNOS and COX-2 protein in the new cells was induced by LPS or IFN-γ. Lastly, we determined whether the new cells produced NO. After 2 days of treatment with LPS or IFN-γ, NO production increased 3- to 5-fold compared to untreated

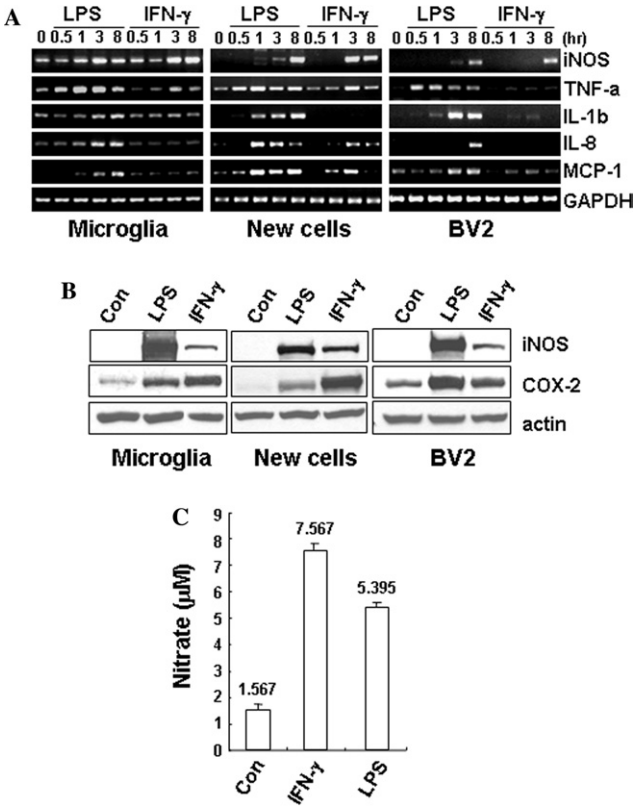

Fig. 5. Expression of inflammatory mediators in new cells. (A) Primary microglia, new cells, and BV2 cells were treated with LPS (100 ng/ml) or IFN-γ (10 U/ml) for the indicated times. Total RNA was isolated and analyzed for level of iNOS, TNF-α, IL-1β, IL-8, and MCP-1 transcripts by RT-PCR. LPS or IFN-γ treatment of new cells enhanced the expression of transcripts for the inflammatory mediators. Very similar patterns were found in primary microglia and BV2 cells. The level of GAPDH transcript was used for normalization. RT-PCR data are representative of three separate experiments. (B) LPS or IFN-γ-treated new cells induced proteins of proinflammatory mediators. Primary microglia, new cells, and BV2 cells were treated with LPS or IFN-γ for 24 h. Cell lysates were separated by 10% SDS–PAGE, and Western blots were performed using anti-iNOS, anti-COX-2, and anti-actin antibodies. Actin protein was used as a loading control. As in primary microglia and BV2 cells, LPS or IFN-γ treatment enhanced the level of iNOS and COX-2 protein in new cells. Western blot data are representative of three independent experiments. (C) Determination of NO release. Cells were treated with LPS (100 ng/ml) or IFN-γ (10 U/ml) for 48 h. The amount of NO was determined by measuring the amount of nitrite in the media. Data represent means ± SEM of three independent experiments.

cells (Fig. 5C). Collectively, these results indicate that the new cells possess the characteristics of microglia.

Phagocytotic activity of the new cells

As the resident macrophage-type cells of the CNS, one of the physiological functions of microglia is the phagocytosis of large particles. Microglia can be elevated to cytocidal status by stimulation with agents such as LPS or IFN-γ [25]. To examine phagocytotic activity of the new cells, we performed a phagocytosis assay

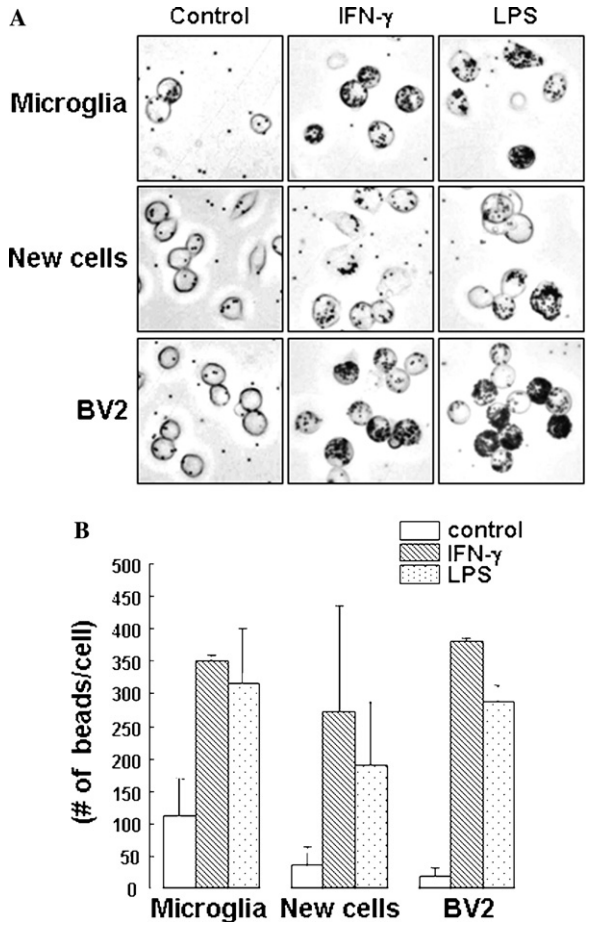

Fig. 6. Phagocytic activity of new cells. Primary microglia, new cells, and BV2 cells were stimulated with LPS (100 ng/ml) or IFN-γ (10 U/ml) for 6 h. The cells were then incubated for 12 h with latex beads. Phagocytosed beads were observed by phase contrast microscopy (A) and counted (B). All of the cells absorbed beads in response to stimulators. Data represent means ± SEM of three independent experiments.

using latex beads. Phagocytosed beads were counted using phase contrast microscopy. The new cells, like primary microglia and BV2 cells, showed an increase in the number of phagocytosed bead particles in response to LPS or IFN-γ (Fig. 6).

Comparison of early passage cells with late ones

We investigated whether the microglial characteristics of new cells were maintained across the passages. First, we compared the OX42 immunoreactivity of early and late passage new cells. As shown in Fig. 3A, the surface of cells was labeled by OX-42 antibodies but not GFAP antibodies and OX-42 immunoreactivity was similarly detected in both early and late passage cells (data not shown). Second, we tested LPS-induced gene expression in two different passages of cells. In both of the early and late passage cells, LPS induced the transcripts of iNOS, pro-inflammatory cytokines and the chemokines,

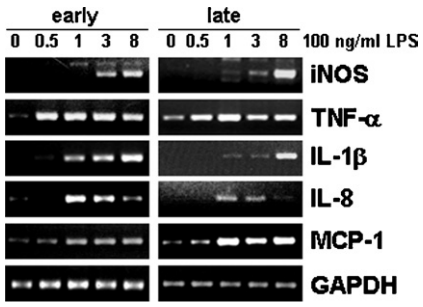

Fig. 7. Comparison of early passage cell with late ones. Early passage and late passage cells were treated with LPS (100 ng/ml) for the indicated times. Total RNA was isolated and analyzed for level of iNOS, TNF-α, IL-1β, IL-8, and MCP-1 transcripts by RT-PCR. The level of GAPDH transcript was used for normalization. RT-PCR data are representative of three separate experiments.

including TNF-α, IL-1β, IL-8, and MCP-1 (Fig. 7). Together, these results indicate that the major microglia phenotypes of the new cells are maintained with continued passaging.

Discussion

In the current study, we isolated spontaneously proliferating cells from primary astrocyte-enriched cultures that had been prepared from neonatal rat brain. These cells appeared in astrocyte-rich areas, and, when detached and reseeded, they continued to proliferate for up to 50 generations. With continued passaging, their morphology became more uniform, resembling the BV2 murine microglial cell line. Furthermore, they had morphological and functional characteristics of microglia [20,26,27].

In early generations, the new cells extended processes when serum deprived, whereas those from later generations did not. This suggests that the new cells have the potential to differentiate. There are several reports that fetal and adult microglia can differentiate into neurons and astrocytes under the appropriate conditions. Specifically, when cultured rat brain microglia are incubated in 70% serum for 2 days, they differentiate into neurons, astrocytes, and oligodendrocytes [28]. In addition, the majority of microglia within the neonatal brain remain in an undifferentiated state [29]. Even adult microglia remain in a relatively immature and unactivated state compared to macrophages in other tissues [30]. However, in MEM supplemented with 5% fetal bovine serum, we obtained only one cell type, which was similar to BV2 cells.

Immunohistochemical and Western blot studies also showed that the new cells have microglial characteristics (OX-42-positive and GFAP-negative) rather than those of astrocytes. We additionally found that, like primary microglia and BV2 cells, the new cells expressed mRNA

for B7.2 when unstimulated or when stimulated by LPS or IFN-γ. In contrast, we could not detect B7.2 mRNA expression in primary astrocytes when they were unstimulated or when they were stimulated by LPS or IFN-γ. Because B7.2 is expressed in microglia but not in astrocytes, these results further confirm that the new cells have characteristics of microglia rather than those of astrocytes. Microglia can be elevated to cytocidal status by treatment with agents such as LPS or IFN-γ. When activated by these agents, they produce proinflammatory mediators, including cytokines (e.g., TNF-α and IL-1β), chemokines (e.g., IL-8 and MCP-1). They also express iNOS and COX-2, and release NO. As in primary microglia and BV2 cell lines, we detected the expression of these proinflammatory mediators in the new cells following exposure to LPS or IFN-γ. Microglia are known to have not only immune competent but also phagocytotic capacities; thus, they are often called brain macrophages. Indeed, like primary microglia and BV2 cells, the new cells had phagocytotic activity. Therefore, our proliferating new cells have the characteristics of microglia and can be used as a rat microglial cell line.

A previous report by Cheepsunthorn et al. [26] described very similar findings. They also characterized a novel microglial cell line derived from neonatal rat brain. These cells expressed markers of microglia rather than astrocytes, and upon exposure to LPS, they secreted proinflammatory cytokines, such as TNF-α, and released NO. However, the new cells described here are derived from an astrocyte-enriched culture, whereas their cells were derived from microglia-enriched cultures.

That the new cells are derived from astrocyte-enriched cultures suggests two possible origins. The first possibility is that new cells are derived from astrocytes. Laywell et al. [31] reported a multipotent astrocytic stem cell in the immature and adult brain. Thus, certain in vitro conditions transiently confer neural stem cell-like attributes to astrocytes during a critical period of CNS development. In addition, subventricular zone astrocytes, and not ependymal cells, are reported to give rise to cells that grow into multipotent neurospheres in vitro [32]. These reports support the possibility that not only microglia but also astrocytes act as pluripotent neural stem cells. However, it is unlikely that microglial cell lines are generated from neural stem cells that originate from astrocytic stem cells because, unlike neurons and astrocytes, microglia appear to derive from mesenchymal cells rather than neural stem cells.

The second possibility for the origin of the new cells is that astrocytes induce remnant microglial precursor cells to differentiate into spontaneously proliferating microglial cells. Based on total cell numbers, astrocytes are the major glial cells in the brain. In addition to the classical supporting roles, they actively participate in the regulation of embryonal and adult neurogenesis [33], inducing and stabilizing CNS synapses [34,35]. Wagner et al. [36] described that coordinated induction of ventral mesencephalic dopaminergic phenotype in an immortalized multipotent neural stem cell line required both the overexpression of Nurrl and factors derived from type 1 astrocytes. Also, Nakayama et al. [37] reported the effect of soluble factors from astrocytes that instruct embryonic stem (ES) cells to differentiate into neural stem cells. Although the role of astrocytes in the generation and differentiation of the new cells is currently uncertain, the cells appear only from astrocyte-enriched culture systems and not from pure microglia culture conditions. Therefore, astrocytes may play a critical role in the generation of immortalized microglial cells. The questions of how and under what conditions the spontaneously proliferating cell lines are generated remain to be determined. Although we have performed many experiments, we have not yet found conditions that consistently produce these cells. Despite this, it is clear that the microglial cell lines are spontaneously generated from astrocyte-dense glial mixed cultures and that astrocytes may play a critical role in the process. In summary, our experimental results demonstrate that new cells from astrocyte-enriched primary cultures of rat brain possess most of the immunological and functional properties of primary rat microglia. Like microglia, these new cells could be activated by LPS or IFN-γ. Thus, the immortalized microglial cells described in the present study should be a useful tool for the investigation of microglial function and for the study of the pathophysiology of microglia in Alzheimer's disease, stroke, and other neurodegenerative diseases.

Acknowledgment

This work was supported by Korea Science and Engineering Foundation (KOSEF) through Chronic Inflammatory Disease Research Center (CIDRC) at Ajou University (Grant R13-2003-019) to I. Jou.

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