Isolation of two distinct subpopulations of microglia

REAGENTS & EQUIPMENT

Reagents

PKH26 (Zynaxis, Malvern, PA)

Diluent B (a phagocytic cell-labeling solution, Zynaxis, Malvern, PA)

FITC-labeled IgG (Cappel, West Chester, PA)

anti-MacI antibody (1:100 dilution, BMA Biomedical, Switzerland)

anti-ER-MP12 antibody (1:50 dilution, BMA Biomedical, Switzerland)

FITC-labeled anti-rat IgG (1:100 dilution, Cappel, West Chester, PA)

phosphate-buffered saline (pH 7.2) containing 0.02% EDTA

Microglial Cell Culture medium: Eagle's MEM supplemented with 10% fetal calf serum, 5 mg/ml bovine insulin, and 0.2 % glucose

Animals: neonatal C57BL/6 mice (Jackson Laboratory, Bar Habor, MA)

EQUIPMENT

Culture Flask: F75 culture Flask (Falcon 3024, Becton-Dickinson Japan, Tokyo, Japan)

Purification plate: non-coated plastic dishes (Falcon 1001, Becton-Dickinson Japan, Tokyo, Japan)

EPICS Elite fluorescence activated cell sorter (Courter, Hialeah, FL)

PROTOCOL

Preparation of Mixed Brain Cell Culture

Primary mixed glial cultures were prepared as a previous report (Sawada, et al., 1990).

1. a brain is isolated from a pup carefully
2. the meninges are removed carefully in chilled Hanks' balanced salt solution
3. each brain is dissociated by nylon mesh (75 mesh) in chilled Hanks' balanced salt solution
4. dissociated cells are washed with Hanks' balanced salt solution
5. the cell suspension is triturated with a fire-stretched glass pipette and plated in a F75 culture Flask in 10 ml of microglial cell culture medium
6. medium was changed every 3 days.

Isolation of two types of microglia

type I microglia

1. one fraction of microglia is liberated by mechanical agitation at 150 rpm for 120 min in an orbital shaker at 37^{o}C on the 14th day
2. liberated cell suspension is plated on a non-coated plastic dish (Falcon 1001 plate) for 30 min
3. remove non attached cells with culture medium and wash gently with pre warmed medium twice.
4. attached cells (type I microglia) are liberated by a rubber policeman in 1 ml of microglial cell culture medium
5. an aliquot (7 ul) of cell suspension is counted; an average yield of the cells was 1.7 x 10^{6} cells/brain in a typical experiment.
6. The purity of type I microglia is determined by immunostaining with FITC-labeled IgG (used in 1:100 dilution); about 3000 cells in 100 ul medium is placed and attached on a glass cover slip briefly, then stained with diluted FITC-IgG for 10

min. In a typical experiment, the purity of type I microglia is more than 99 %.

type II microglia

Another fraction of microglia is liberated by trypsinization following mechanical agitation;

1. mixed brain cell culture in a F75 flask is washed with 10 ml of phosphate-buffered saline (pH 7.2) twice
2. digest by 2 ml of trypsin-EDTA solution for 15min at 37^{o}C
3. reaction is terminated by adding 10 ml of microglial cell culture medium and flash by pipetting
4. the cell suspension is triturated with a fire-stretched glass pipette and plated on a non-coated plastic dish (Falcon 1001 plate) for 30 min
5. remove non attached cells with culture medium and wash gently with pre warmed medium five times.
6. attached cells (type II microglia) are liberated by a rubber policeman in 1 ml of microglial cell culture medium
7. an aliquot (7 ul) of cell suspension is counted; an average yield of the cells was 1-2 x 10^{6} cells/brain in a typical experiment.
8. The purity of type I microglia is determined by immunostaining with FITC-labeled IgG (used in 1:100 dilution); about 3000 cells in 100 ul medium is placed and attached on a glass cover slip briefly, then stained with diluted FITC-IgG for 10 min. In a typical experiment, the purity of type II microglia was about 90-95%.

Fluorescent Dye Staining

Astrocytes, microglia and mixed glial cultures were stained with the fluorescent dye PKH26 (Zynaxis, Malvern, PA) as follows;

1. cells in 10-cm id. plastic culture dishes were incubated with PKH26 staining solution containing 10 mM of PKH26, 50% Diluent B (a phagocytic cell-labeling

solution, Zynaxis, Malvern, PA), and 50% culture medium for 15 min

2. washed with 10 ml serum-containing medium for three times
3. PKH26-stained cells were harvested using a rubber policeman in 2ml of ice-cold phosphate-buffered saline (pH 7.2) containing 0.02% EDTA
4. washed with 5ml ice-cold phosphate-buffered saline (pH 7.2) by centrifugation three times
5. analyzed with an EPICS Elite fluorescence activated cell sorter (Courter, Hialeah, FL).

Immunostaining

Type I and type II microglia were labeled with anti-MacI antibody (1:100 dilution, BMA Biomedical, Switzerland) or anti-ER-MP12 antibody (1:50 dilution, BMA Biomedical, Switzerland) at 0^{o}C for 30 min, stained with FITC-labeled anti-rat IgG (1:100 dilution, Cappel, West Chester, PA), then analyzed with an EPICS Elite fluorescence activated cell sorter (FACS) (Courter, Hialeah, FL).

RNA preparation and RT-PCR

PKH26-stained microglia were sorted into two fractions; one with a high forward scattering (FS) intensity (type I microglia) and the other with a low FS intensity (type II microglia). Total RNA was extracted from approximately 1x10^{5} microglia of both fractions by a modified acid phenol-guanidine-chloroform method (Sawada, et al., 1992a). PCR was performed as described previously (Sawada, et al., 1993a) with the following primers; IL-1β sense, 5'-atggcaactgttcctgaactcaact, antisense,

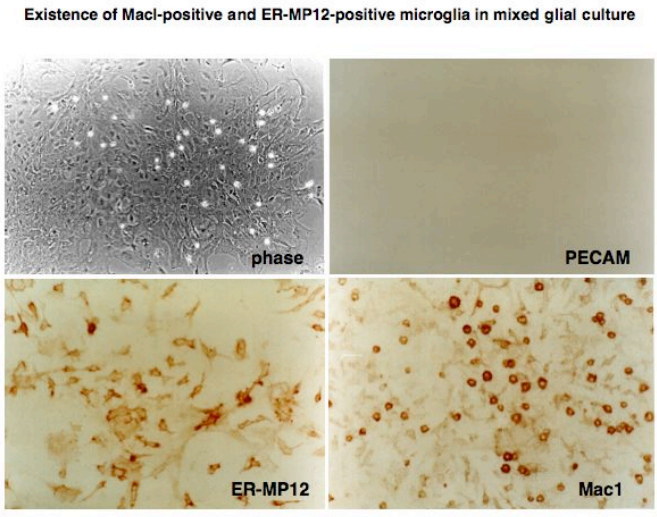
5'-caggacaggtatagattctttccttt; class I MHC sense, 5'-acatggagcttgtggagacc, antisense, 5'-agtcggagagacatttcagagc; CD14 sense, 5'-ccttagtcacaattcactgcgg, antisense, 5'-atcaggggtcaagtttgc; and β-actin sense, 5'-.gtgggccgctctaggcaccaa, antisense 5'-ctctttgatgtcacgcacgatttc. Aliquots of PCR product were subjected to electrophoresis in 2% agarose gels in TBE buffer. The gels were then stained with ethidium bromide and photographed.

TYPICAL PROTOCOL RESULTS

mixed glial culture

Mixed glial culture from the neonatal mouse brain is a model system for glial cell differentiation; the three major types of glial cells, astrocytes, oligodendrocytes and microglia, sequentially differentiate and increase in number as they do *in vivo* (Suzumura et al., 1987). At 10 to 14 days *in vitro* (DIV) in normal mouse mixed glial cultures, many phase-bright round cells appeared on top of the astrocyte monolayer and these cells show a burst-forming proliferation (Fig. 1 A) which paralleled microglial growth *in vivo* (Suzumura, et al., 1987). There are two types of

Fig. 1 Sawada & Suzuki



microglia in the 14 DIV mixed glial culture; one is positive for Mac I antibody staining and shows round shape, and another is positive for ER-MP12 antibody staining and shows ramified shape.

type I microglia

Since the phase-bright round cells could be easily liberated from the astrocyte monolayer by mechanical agitation (Sawada, et al., 1990), we isolated these cells from mixed glial culture, stained them with rhodamine-labeled IgG to examine Fc receptor binding which is a hallmark of microglia, and counted rhodamine-positive cells. More than 99% of the liberated cells from normal mouse mixed glial culture were Fc receptor-positive microglia.

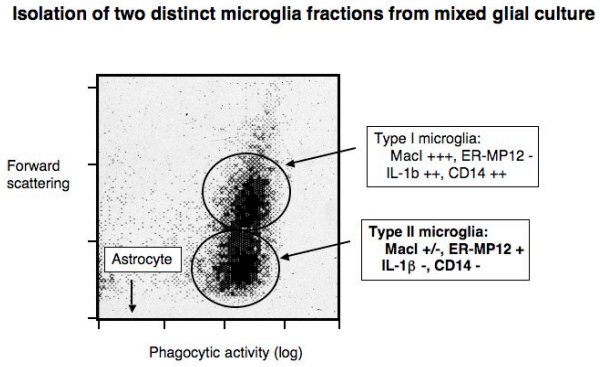
type II microglia

Fc receptor-positive microglia were also observed in an astrocyte monolayer, and these cells could be discriminated and purified from astrocytes and other remaining cells by trypsinization followed by allowing the cells to attach to non-coated plastic dishes. We found similar numbers of Fc receptor-positive microglia in the collected adherent cells.

FACS

We analyzed characteristics of microglia in whole cells of the mixed glial cultures from mouse brains with a FACS with a fluorescent dye specific for

Fig. 2 Sawada & Suzuki



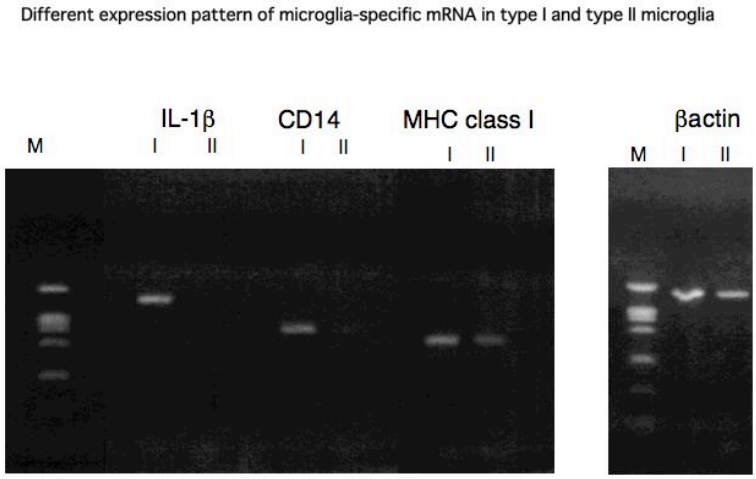
phagocytic cells, PKH26 (Horan and Slezak, 1989, Melnicoff, et al., 1988). PKH26 stained microglia efficiently; purified microglia were stained with intensity at least two-orders higher than purified astrocytes which is the major cell population in mixed glial cultures (indicated by an arrow in Fig. 2). PKH26-stained microglia from normal mouse brains were divided into two populations by intensity of FS (Fig. 2). The type I microglia liberated from astrocyte layer by mechanical agitation were enriched in high-FS intensity cells, while those which were not liberated by agitation but separated from astrocytes by trypsinization and non-coated plastic dish treatment (type II) were enriched in low-FS intensity cells. When mixed glial cultures from normal mice were treated with human recombinant M-CSF, the high-FS population increased in number while the low-FS population decreased. On the other hand, these two sub-populations of microglia had similar phagocytic capacities because both populations had the same fluorescent intensity following PKH26 incorporation (Fig. 2).

To characterize the differences between the two subpopulations of microglia further, we compared surface antigen expression on both types of cells. Type I microglia were stained with an anti-Mac1 antibody which recognizes complement C3b receptor on the surface of cells of the monocyte lineage (Griffin, et al., 1975). Type II microglia were stained faintly with this antibody. On the other hand, staining with ER-MP12 and ER-MP20 antibodies gave the opposite results.

Distinct phenotypes of the two microglial sub-populations were also observed at the level of mRNA expression. We fractionated the high- and low-FS microglial subpopulations by sorting with a FACS and analyzed these cells by RT-PCR

(Sawada, et al., 1993a). The sorted type I microglia showed comparable levels of interleukin-1 β, class I MHC, and CD14 mRNA expression. The sorted type II microglia, however, did not express interleukin-1 β or CD14 mRNAs, and expressed small amounts of class I MHC mRNA (Fig. 3). Contamination with other types of glial cells was negligible because both types of microglia showed phagocytic activity indicated by PKH26 fluorescent dye staining (data not shown).

Fig. 3 Sawada & Suzuki



summary/future directions

From this experiment, it is clear that there are two major phenotypically different populations of microglia (type I and type II) in mixed brain culture. Type I microglia have surface markers for mature monocytic cells and produce microglia-specific cytokines. Type II microglia have cell-surface characteristics exclusively observed in immature bone marrow cells but not in mature monocytic cells, and do not express the hallmarks of mature microglia such as C3b receptors, CD14 or cytokine mRNAs.

Type II microglia did not express CD14 mRNA, which is a receptor for LPS-LPS-binding protein complex and one of the major signal transduction molecules involved in LPS stimulation (Lee, et al., 1992), suggesting they might be less responsive to LPS stimulation than type I microglia. Furthermore, type II microglia express less class I MHC mRNA than type I. From these observations, the two distinct types of microglia seemed to be functionally different. Further studies should be performed to elucidate the *in vivo* functions of both types of microglia. We have found that type II, but not type I, microglia were observed in the M-CSF-deficent *op/op* mice. A previous study showed that post-traumatic microglial proliferation was not observed in *op/op* mouse brain (Raivich, et al., 1994), suggesting type I microglia may have a role in degeneration/repair of brain injury.

The type II microglia have a novel surface characteristic in that they are immunoreactive for ER-MP 12 and 20, antibodies to which react with immature bone marrow cells but not mature monocytes, macrophages, or other tissue-resident macrophages (Leenen, et al., 1990a, Leenen, et al., 1990b). We previously reported that ER-MP12 and 20 positive microglia exist in the brain of young-adult mice (Tanaka et al. 1997). These findings do not support the current hypothesis (Ling and Wong, 1993) that microglia arise from blood monocytes which are thought to be a source of brain macrophages observed in blood-brain-barrier rupture.

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the developing and adult mouse brain. Neurosci Lett. 239:17-20.

Figure Legends

Fig. 1 Mixed glial cultures at 14 days *in vitro* (DIV) from normal brains had many phase-bright cells on top of the astrocyte layer (A). Mixed glial cultures were stained with PE-CAM (B), ER-MP12 (C) and Mac I (D).

Fig. 2 Fluorescence flow cytometric analysis of PKH26-labeled microglia. Two-dimensional histograms of PKH26-stained mixed glial cultures from normal brain indicating the existence of two populations of microglia; high-FS and low-FS populations. Histogram of microglia purified by mechanical agitation (type I) indicating the similar FS intensity of high-FS population of microglia in mixed glial cultures from normal brain. That of microglia purified by trypsinization (type II) indicating the similar FS intensity of low-FS population of microglia in mixed glial cultures from normal brain.

Fig. 3 Distinct mRNA expression in microglia subpopulations. mRNA expression of Interleukin 1β (IL-1β, lane 2 and 3), CD14 (lane 4 and 5), class I MHC (lane 6 and 7), and β-actin (lane 9 and 10) were examined. Lane 2, 4, 6, and 9 indicate RT-PCR products from type I microglia. Lane 3, 5, 7, and 10 indicate those from type II microglia. lane 1 and 8, Hae III digested pUC119 as a molecular weight marker.