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Research Article

The Application of Organotypic Brain Slice Culture to Study Microglial Differentiation by Lycopersicon esculentum and Sambucus nigra Lectin Histochemistry

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Abstract

Microglial cells are the subset of macrophages in the central nervous system (CNS). Changes in the CNS such as injury or developmental events cause morphological and physiological changes in microglial cells. In this study organotypic brain slice cultures under serum free condition were used to investigate the morphology and lectin histochemistry of microglia and macrophages in the CNS *in vitro*. Microglial cells exhibited dramatic morphological changes in the organotypic brain slice culture. Immediately after slicing microglias were seen to have the same morphology as they do in the intact brain: they had small cell bodies from which radiated several highly ramified processes. After 1 day *in vitro* (DIV) all microglia transformed into an active form with round soma and no processes. At 5 days *in vitro*, and especially at 9 days *in vitro*, many of the microglia had tended to return to the ramified phenotype. The expression of different carbohydrates was examined at the 0, 1, 5 and 9 days *in vitro* time periods by employing Lycopersicon esculentum tomato lectin (LEA lectin) and Sambucus nigra (SNA). Microglial cells with different morphology intensely stained with the LEA. SNA stained the ramified microglia only after they re-ramified at 5 DIV and 9 DIV. The results of this study confirmed that the expression of carbohydrate structures in these cells would undergo changes corresponding to the changes in morphology.

Keywords: Microglia, Macrophage, phagocyte, Lectin, in vitro

Introduction

Microglial cells are the subset of macrophages in the central nervous system (Guillemin and Brew, 2004). Changes in the CNS such as injury or developmental events cause morphological and physiological changes in microglia. Morphologically three types of microglia can be considered which are ramified, amoeboid, and intermediate (Rezaie et al., 2004). Resting or ramified microglial cells are found in normal, healthy CNS and they have an immune survival role (Czapiga and Colton, 1999). Any kinds of infection or damage can activate the microglias with change to round shapes as well as up-regulates their cell-surface antigens and contain a high proportion of hydrolytic enzymes contributing to their activated phagocytic properties (Kaur et al., 2007; Ling and Wong, 1993).

Microglial cells are labelled by many of the same antibodies as monocytes. For example, both microglia and macrophages are recognised by monoclonal antibodies against CD68, CD45,CD11c and CD 11b (Fischer and Reichmann, 2001; Rezaie et al., 1999). In addition to antibodies directed against proteins expressed by macrophages and microglia, lectins have become increasingly used to examine these cells. A variety of lectins have been shown to bind to both microglia and macrophages in the CNS (Colton et al., 1992; Czapiga and Colton, 1999). The changes of carbohydrates in cell surface have been shown to play an important role in immune defence (Boyzo et al., 2003; Mackowiak et al., 2007; Suzuki et al., 2005). Further characterization of carbohydrate structures might enable the identification of structures uniquely associated with particular macrophage and microglial phenotypes and functions.

In this study the differentiation of microglia *in vitro* and characterizing aspects of cell surface's carbohydrates has been examined by using Sambucus Nigra Agglutinin (SNA) also called Elderberry bark lectin and Lycopersicon esculentum agglutinin (LEA) lectin. SNA lectin binds preferentially to sialic acid attached to terminal galactose in α-2-6 and to a lesser degree

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α-2-3 linkage. LEA lectin is composed of single polypeptide that can bind poly N-acetyl lactosamine oligomers (Acarin et al., 1994). We have detected expression of these different carbohydrate structures in microglia and macrophages over nine days of organotypic culture of neonatal brain slices and also in intact brains of neonatal rats. It was hypothesised that the expression of carbohydrate structures in these cells would undergo changes corresponding to the changes in cell morphology.

Materials and methods

Organotypic brain slice culturing

Brain slicing

Sparge Dawley rat pups age between postnatal days (P) 0 to 8 days old were used for brain slicing as it has been described before (Lari et al., 2012). Briefly, in a laminar flow hood rat pups were sacrificed by decapitation. The brain where quickly desiccated from the head in the ice-cold slicing buffer and attached to a chuck with super glue. Warm agar (37-40°C) swirled around the brain to provide support for the brain during the cutting of 250 μm thick slices on a Leica VT 1000 vibratome.

Brain culture

After cutting, the agar was gently removed from around the slices. 1 ml of cold (4°C) culture medium (see below) was placed in the wells of 6-well tray. Sterile Millicell-CM 30mm-diameter transparent culture inserts (Millipore) were used for brain slice culture. A glass Pasteur pipette was used to gently transfer the brain slices to the inserted membrane. Usually, two slices were placed in each membrane. The slices were kept in 4°C in sterile serum free Minimum Essential Medium (Sigma M-3024) (MEM) for the next two hours, and then refreshed with a change of the serum free MEM from cold 4°C C to warm 37°C. The cultures were placed in an incubator at 37°C in a 5% CO_2 95% O_2 atmosphere. The MEM was changed every two days. The cultured tissue was kept in an incubator for 1 day, 5 days or 9 days.

Cultured tissue fixation

Culture brain slices were fixed with Bouins fixative by replacing the medium inside the culture dish with Bouins fixative overnight in room temperature. To avoid over fixation the Bouins was then replaced with 70% ethanol.

Agar embedding of slice cultures

To prepare the cultured tissues for paraffin embeds warm agar in liquid form was added to the insert containing the tissues. After 30 minutes in room temperature the agar sets to solid form, thus supporting the tissue during the processing. A scalpel was used to gently cut around the membrane and agar freeing it from plastic insert. The agar with tissues inside it put in 70% ethanol and then processes for paraffin embedding. These brain slices were embedded in one of two different ways, either "flat", to enable sections of the entire face (*en face*) of the slice to be cut, or "edge on", to allow cross sections of the brain slices to be cut. Sections cut at 6 μm were floated onto 0.1% gelatin coated slides.

Perfusion-fixation the brain

Two P13 rats were deeply anesthetized (100 mg/kg Nembutal i.p.), and their hearts exposed by thoractomy. Under the fume hood and at the room temperature a cannula connected to the constant pressure perfusion apparatus was inserted into the left ventricle and the right atrium was cut to serve as an outlet. Blood and plasma proteins were removed by perfusing the animals with 0.1 M PBS for 2-3 minutes. The brains and livers were fixed by perfusion of Bouins fixative for 1-2 minutes. The tissues were removed and kept in the Bouins fixative overnight.

Lectin histochemistry

Lectin staining of paraffin sections

Paraffin sections of the cultured brain slice tissues that were 0 DIV, 1 DIV, 5 DIV and 9 DIV were de-waxed in Histolene and hydrated from absolute ethanol to 70% ethanol and then into PBS. Sections were incubated with biotinylated Lycopersicon esculentum agglutinin (LEA) and Sambucus Nigra Agglutinin (SNA) lectins (vector Laboratories, Inc.CA). Lectin was made up at a diluted of 1:3000 in fish gelatin blocker solution. After 24-48 hours they washed (4x5 times) in 0.1M PBS (0.1 M and pH 7.4). Lectin binding that remained after the washes was visualized by the avidin-biotin-HRP localization of biotinylated lectins. A 1:100 dilute solution of Avidin -Biotin Complex (ABC; vector Laboratories, CA) was made up in 0.1M PBS and applied to the sections for 12-24 hours. The sections were washed again (4x5 times in PBS) and reacted with 0.05% of diaminobenzadine (DAB) and 0.01% hydrogen peroxide (H_2O_2) in PBS.

Microscopy and imaging

Lectins stained sections were viewed with bright–field and Nomaski (DIC) microscopy (Zeiss Axioskop II). Micrographs were obtained with a Nikon digital SLR attached to the lightpath of the microscope; acquired images were adjusted for brightness and contrast using the Adobe Photoshop® software.

Results

General changes in brain slices during culture in vitro

In order to investigate microglial morphology LEA lectin was considered as a reliable type of lectin for microglial and macrophages (Velasco et al.; 1995, Acarin et al., 1994). In addition to macrophages and microglia, LEA also binds to endothelium, but it was considered that there would be no ambiguity in the identification of endothelium versus macrophages and microglia in the brain tissues.

Once cut and placed into culture (day 0), many of the microglial cells appeared with the typical morphology of resting microglia - they were highly ramified with small cell bodies. The thin cytoplasmic processes were multi-branches that tend to all directions (Fig. 1A). Many of the ramified cells located in the middle of the *en face* slices. The cross sections of the slices revealed that the branches were oriented in various directions (data not shown). At this time also some macrophages with round or oval cell body were observed, these were seen in the *en face* sections to be located mostly towards the in the edge of the slices.

After one day *in vitro* (1 DIV) some macrophage-like cells had started to migrate from the slices onto the membrane, which gave the edges of the slices an uneven appearance. These cells were apparently evenly distributed in the *en face* sections and cross sections of the brain slices. At this time dramatic changes had occurred in the LEA positive cells. All the LEA positive cells exhibited the active morphology of cells classified as ameboid microglia, with round or irregular cell body and no, or few, short cytoplasmic processes (Fig. 1C). These processes were probably due to the phagocytic action of the cells at that time.

After 5 DIV the slices adhered to the membrane and in the edge tended to flatten due to migration of cells (some clearly had a macrophage-like in morphology). At this time a large portion of microglial cells was developed towards a more ramified appearance, although round or ovaloid cells without any processes were still present (Fig. 1D and E). Labelled macrophages at 5 DIV appeared to have weaker LEA reactivity than at 0 DIV and they appeared to be smaller and had smaller nuclei than macrophage cells seen at 1DIV, but a few big macrophages still could be observed (Fig. 1E). Most of the macrophages were located around the edge of the slides and appeared less labelled than 0 DIV and 1 DIV. The ramified cells had short branches and stained less intensely than the macrophages (Fig. 1D). Most of the ramified cells were located in the middle of the slice.

At 9 DIV further development towards ramification had taken place. Few ovaloid cells could be seen around the edges (Fig. 1G). The tissues were flatter and strongly adhered to the membrane. Although adherence is believed to be due to the proliferation and growth of astrocytes that extend their presses into the insert membrane (Stoppini et al., 1991), the 9DIV specimens in this study revealed cellular processes in the insert membrane that were stained with lectins. Therefore, growth of microglial or endothelial processes into the membrane is also a possibility. Majorities of cells were highly ramified with long multi-branches processes that looked to eyes longer and thicker than 0 DIV. Microglial cell bodies were small with multi-branch cytoplasmic processes projecting in all directions (Fig. 1F). A few macrophages also reacted with LEA; these were located around the edge of the slices. Almost no blood vessels were detectable. In sections from perfusion-fixed P13 rat brain all the microglial type cells had small cell body and were highly ramified with branches in all directions (Fig. 1H). In addition to ramified microglias, there were very few highly stained macrophages in the P13 brain with round or oval cell body with no cytoplasmic processes (Fig. 1I). The macrophages mostly located around the ventricle and also in the sub-cortical white matter.

Sambucus Nigra Agglutinin (SNA)

This lectin appeared to label cytoplasmic side of macrophages evenly (Fig. 2B). At 0 DIV there was little evidence of binding to microglia (Fig. 2A), but blood vessels were strongly and evenly stained (Fig. 2B). At 1 DIV this lectin intensely stained some organelle of cells (Fig. 2C). The blood vessels were also labelled with the SNA. In contrast with 0 DIV most of the blood vessels had beaded appearance. It appeared that there was a general staining of the extra-cellular regions. After 5 DIV some ramified cells weakly reacted with SNA (Fig. 2D). Many macrophages were labelled with SNA as well and some of them had few cytoplasmic processes (Fig. 2E).

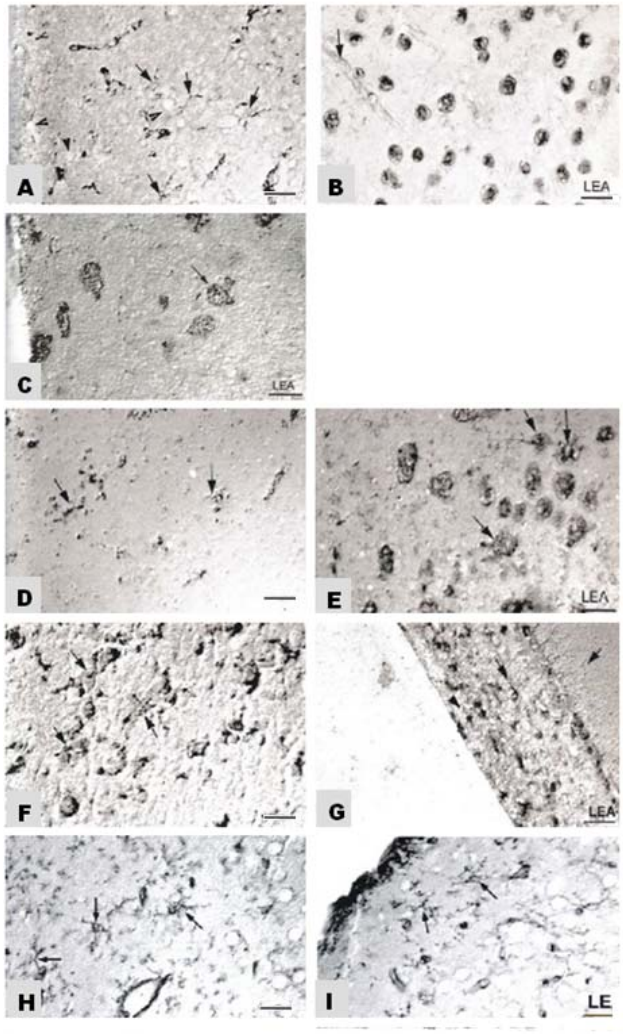

Fig. 1 Microglial changes in brain slices culture in vitro: brain slices fixed at different time points in and stained for LEA.
Slices at 0 DIV (A) and (B); (A) Ramified microglia stained with LEA exhibiting long thin processes (long arrows). Neuronal cell bodies were unstained (arrowheads). (B) LEA positive macrophages: reactivity was observed in the cytoplasm and plasm membrane. LEA also labels blood vessels (arrow). (C) At 1 DIV LEA stained activated microglial.
Slices at 5 DIV (D) and (E); (D) LEA stained ramified cells with short-branched processes. (E) LEA positive macrophages, some with a few cytoplasmic processes (arrow) and some without any processes. Slices at 9 DIV (F) and (G): (F) LEA stained highly ramified microglia with long multi-branched processes (arrows) (G) a cross section of LEA stained ramified microglia. These cells were distributed throughout the depth of the slice extended and cytoplasmic process in different directions -some eventually growing into the insert membrane (arrow). Perfused-fixed P13 brain (H) and (I): (H) LEA positive microglial cells in the sub cortical grey matter (arrows) (I) Macrophages with round or oval cell bodies with no or a few cytoplasm process and darkly stained with LEA(big arrow head). Blood vessels also stained. Scale bar: 25 µm in all panels.

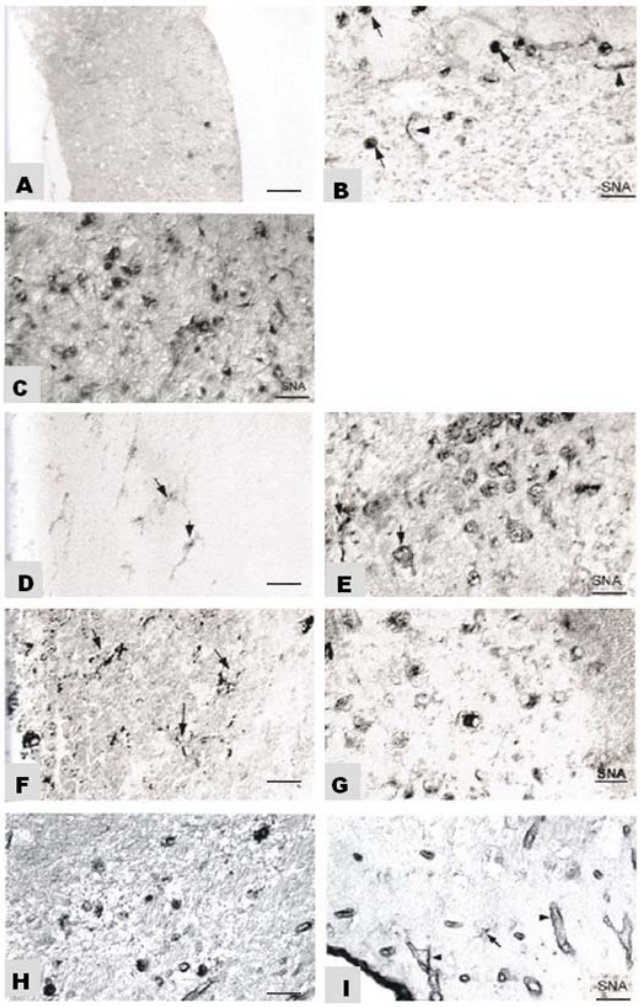

Fig. 2. SNA staining of microglial and macrophages in organotypic brain slice cultures**.**
Slices at 0 DIV (A) and (B); (A) Cross-section of the brain slice revealing some ramified cells that were faintly stained with SNA. (B) SNA appeared to label macrophages (long arrow) and strongly stained blood vessels (arrowhead) as well as some staining of entities in most or all cells giving the impressioof a general background staining. (C) At 1 DIV SNA strongly stained some parts of cells. Slices at 5 DIV (D) and (E); ramified cells weekly reacted with SNA (arrows). Many macrophages were labelled with SNA as well. Slices at 9 DIV (F) and (G): (F) 9 DIV SNA had a weak reaction with a few ramified cells (arrows) (G) some macrophages stained with SNA. In perfused-fixed P13 brain (H) and (I) SNA stained some macrophages and blood vessels but no ramified cells. Scale bar: 50 µm in panel A, 25 µm in all other panels.

SNA also barely labelled the blood vessels and some background cells. At 9 DIV although the background was highly stained, SNA had a weak reaction with some ramified type cells and also some macrophages (Fig. 2G). The macrophages were the same size as macrophages in 0 DIV. This lectin in P13 brain only labeled a few macrophages around the ventricle and in the cortex (Fig. 2H). No ramified microglia reacted with SNA. These results are summarised in table 1. Some macrophages and

blood vessels labeled with SNA lectin at P13 (Fig. 2H and I).

Table 1. Summary of lectins staining in normal brain and culture

Lectins		P13 brain	0 DIV	1 DIV	5 DIV	9 DIV
LEA	R.microglia	++	++	_	++	++
	macrophages	+++	+++	+++	+++	_
SNA	R. microglia	_	_	_	++	++
	macrophages	+++	+++	+++	+++	++

Discussion

The organotypic brain slice culture used for investigating microglial function change was first described by (Hailer et al., 1996), but the few subsequent studies that have employed this method have not significantly extended this initial description of microglial activation and re-ramification. Most of the histochemical changes in microglia and macrophages of the CNS have been on the fixed intact brain or mixed glial primary cultures (Ananth et al., 2003; Song et al., 2006).

In the current study, the brains of rats age between 0 to 8 were used because at this age, there is a peak in the density and the ramification of microglia (Navascues et al., 2000), thus this age brain can support (or is at least permissive to) the development of microglia. During the slicing procedure, the brain was dissected out quickly in the 4°C hyperosmotic buffer to minimize cell swelling glutamate-mediated toxicity. Also, calcium free condition Mg^{2+} was added to minimize mediated toxicity and in result, reduces the cell death in culture. The serum free conditions were used to prevent serum stimulation on activating microglia and also possibly to limit the proliferation of astrocytes (Czapiga and Colton, 1999). The common use of fetal serum on microglial cultures appears to maintain these cells in an activated state (Czapiga and Colton, 1999).

The detection of poly N-acetyl lactosamine residues by LEA lectin resulted in staining of both macrophages and ramified microglia in the intact and cultured brain (Billiards et al., 2006; Rezaie et al., 2005). Previous studies have demonstrated N-acetyl lactosamine associated with glycoproteins in the cell surface and lysosomal membrane (Carlsson and Fukuda, 1990). The cytoplasm of macrophages would be expected to contain numerous secretory granules and lysosomes with high level of N-acetyl lactosamine due to their phagocytic action in removing dead cells (Acarin et al., 1994). It can be the reason that macrophages and the activated microglia invariably exhibited more intense staining that the ramified microglia. Endothelial cells in blood vessels also stained with LEA lectin, in sections from intact and from cultured brains. However, the blood vessel labelling appeared to change over time in the cultures: at 0 DIV blood vessels similar to normal brains evenly stained with LEA but after 1 DIV and at 5 DIV they had lumpy appearance. After 9 DIV only a few blood vessels labelled with the LEA. One possible explanation for these changes could be the blood vessels were degenerating, or reorganising, in the organotypic brain slice culture after a few days - presumably due to the loss of blood flow within.

SNA has been reported to label microglia in meningoencephalitis (Lutsik et al., 1991) and in Alzheimer's disease (Zambenedetti et al., 1998). Sialic acid has also been reported to play an important role in the activation of macrophages by facilitating attachment of bacteria to macrophages (Maganti et al., 1998). It has been reported that the level of sialoglucoconjugated was changed in several diseases such as cancer, diabetes and rheumatoid arthritis (Alturfan et al., 2007; de Castro et al., 2008; Holzhauser and Faillard, 1988; Maruhama et al., 1983). As SNA did not label microglia at day 0 and also in the P13 brain, but it strongly interacted with re ramified microglia in day 5 and day 9. These results indicated that re-ramification increased the expression the level of sialic acid attached to terminal galactose in α-2-6 or α-2-3. Therefore, SNA could be a modulator for stress on ramified microglia cells.

Sialic acid is known to involve in neural plasticity in the adult CNS. It changes in hormonal patterns, adaptations to pain and stress, and aspects of learning and memory (Rutishauser, 2008) and also it has role in microglia-neuron interactions (Wielgat and Braszko, 2012). Therefore, increasing the level of sialic acid after re-ramification of microglia may play a key role in repair of adult CNS tissue.

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