

Organotypic brain slice culture promotes the transformation of haemopoietic cells to the microglial like cells

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Abstract

The exact developmental origin of microglia is still under debate. In the present study we investigated which haematopoietic tissues and which features of the organotypic brain slice culture promoted microglia ramification. The potential of cells derived from embryonic yolk sac, embryonic aorta-gonad-mesonephros and adult blood monocytes was examined. These tissues were co-cultured with brain slices after the brain slices had first been maintained *in vitro* for 1 day, 5 days and 9 days. When brain slices had been maintained in culture for 1 day before the donor cells were added, the donor cells took several days to ramify. However, when donor tissues were added to brain slices that had been 5 or 9 days maintained in culture, the donor cells exhibited a ramified morphology within a day. Therefore changes in organotypic brain slices had an effect on the transformation of cells to the microglial morphology. When adult blood monocytes were added to brain slice cultures there was no evidence of any tendency to ramify over 6 days of co-culture. This study did not support the suggestion that microglia cells derive from bone-marrow (BM) cells or from circulating monocytes.

Keywords: Microglia, Macrophage, phagocyte, GFP, CSFE, *in vitro*, organotypic brain slices culture

Introduction

Microglial cells are considered to be a special form of phagocyte in central nervous system (CNS). They comprise around 10% to 15% of the total cell population of the CNS. Like macrophages in other parts of body, microglia respond to various kinds of CNS injury and, in their active state, can defend against microorganisms and remove dead cells by phagocytosis (Gehrmann et al., 1995).

The study of microglial cells, biology plays a key role in understanding of brain's fundamental tissue reaction against any kind of injuries and infections as well as cellular mechanisms of CNS development (Banati and Graeber, 1994). Although microglia have been known and studied for almost eighty years (Hortega, 1932), the origins of ramified microglia have been long-standing controversy. Several reports have described the existence of various types of microglia; resident parenchymal microglia and amoeboid microglia (Kaur et al., 2001; Monier et al., 2007). There are

many lines of evidence showing that microglia cells belong to haematopoietic system, specifically to the myeloid lineages, which give rise to monocytes and macrophages. But the origin in terms of which haematopoietic tissue (or tissues) gives rise to these cells, and relationship between microglia and macrophages are still a matter of controversy (Prinz and Mildner, 2011).

Firstly it might be postulated that microglia, like macrophages in other tissue, belong to monocyte cycle and derive from bone marrow. Support for this suggestion was obtained by several studies that, after whole body irradiation, in these studies, the labelled bone marrow were transplanted to animals to examine the long-term fate of myeloid cells in the CNS (Eglitis and Mezey, 1997; Priller et al., 2001; Zeilhofer, 2008). As a result ramified labelled microglia could be observed in brain parenchyma (Eglitis and Mezey, 1997; Priller et al., 2001; Zeilhofer, 2008). On the other hand, the first microglial precursors appear in the mouse CNS at about E9, before the brain circulation is established and before production of monocytes begin - and certainly before there is any bone marrow (Cuadros and Navascues, 1998; Ginhoux et al., 2010). There

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are experiments that indicated after bone marrow transplantation only prevascular macrophages could be detected in CNS. For example in female patients who undergo sex-mismatched BM transplantation, only prevascular macrophages could be observed, but no cells with ramified microglia characteristics in the parenchyma could be detected (Unger et al., 1993). Also in the experiment by Ajami et al. (2007), the blood-stream of GFP-positive mice was connected with a GFP-negative animals and the presence of GFP-expressing mononuclear cells in the CNS of these animals was investigated. No evidences of microglia progenitor recruitment from the circulation in CNS were found (Ajami et al., 2007). It has been suggested that the engraftment of marrow-derived myeloid cells in the CNS is affected by the experimental conditions, e.g., irradiation (Prinz and Mildner, 2011). Increasing recent evidence supported that the microglial (and macrophages) are un-replenished by constant new arrivals and turnover, and the transient haemopoietic tissues are the origin of the entire microglia population (Ginhoux et al., 2010; Monier et al., 2007; Prinz and Mildner, 2011). In the present study we investigated which haemopoietic tissues and which features of the brain slice promoted microglia ramification. The organotypic brain slice cultures were co-culture with haematopoietic tissues after the brain slices had already been in culture for periods of 0, 1, 5 or 9 days.

Materials and Methods

Organotypic brain slice culturing

Brain slicing

Sparge Dawley rat pups age between postnatal day (P) 0 to 8 days old were used for brain slicing. In a laminar flow hood rat pups were sacrificed by decapitation. The head was placed in the ice-cold (4°C) hyperosmotic (>300 mOsm) slicing buffer. The brain were 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. The chuck was placed in the slicing chamber that had been filled with ice-cold slicing buffer (4°C and pH 7.4) and the whole slicing chamber was packed in ice to keep it cold.

Brain culture

After cutting, the agar was gently removed from around the slices. 1 ml of cold (4°C) culture medium 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 pasture pipette was used to gently transfer the brain slices to the insert membrane. Usually, two slices were placed in each insert. The slices were kept at 4°C for next two hours, and then refreshed with a change of the MEM from cold 4°C to warm 37°C. The cultures were placed in an incubator at 37°C in a 5% CO₂ 95% O₂ atmosphere. The MEM was changed every two days. The cultured tissue was kept in an incubator for 1 day, 5 days or 9 days before co-culturing with haematopoietic tissues.

Lectin staining for paraffin section

Paraffin sections of the cultured brain slice tissues that were 0 day in vitro (DIV), 1 DIV, 5 DIV and 9 DIV were de-waxed in Histolene and hydrated from absolute ethanol to 70% ethanol and then into phosphate buffer solution (PBS). Sections were incubated with biotinylated Lycopersicon esculentum agglutinin (LEA) lectin (vector Laboratories, Inc.CA). Lectin was made up at a diluted of 1:3000 in fish gelatin blocker. After 24-48 hours they washed (4x5 times) in 0.1 M PBS (0.1 M and pH 7.4). Lectin binding that remained after the washes was visualised by the avidin-biotin-HRP localisation of biotinylated lectins. A 1:100 dilute solution of Avidin Biotin Complex (ABC; vector Laboratories, CA) was made up in 0.1 M PBS and applied to the sections for 12-24 hours. The sections were washed again (4-5 times in PBS) and reacted with 0.05% of diaminobenzadine (DAB) and 0.01% hydrogen peroxide (H₂O₂) in PBS.

Haematopoietic tissues preparations

Haematopoietic tissues were dissected from embryos, taken by Caesarean section from deeply anaesthetised (100 mg/kg Nembutal i.p.) time-mated mothers. Time mating involved housing two female rats with a male for one hour in the morning and subsequently examined for vaginal plugs. The day of the appearance of vaginal plugs was designated as day 0. Embryos from 11 to 13 day of gestation (E11–E13) were obtained within sections of uterus and placed in a Petrie dish containing cold (4°C) sterile PBS, this dish was transferred to a laminar flow hood where subsequent dissection and washing took place. In warm MEM the embryos (in their yolk sacs) were dissected from the uterine tissues. Then, by using a plastic transfer pipette, the embryos were transferred to a separate Petrie dish containing warm MEM. After dissecting the yolk sac or AGM from embryo, scissors were carefully

cleaned to avoid transferring cell between haematopoietic tissues.

Vital labelling of embryonic haematopoietic tissues

To label the haematopoietic tissues, they were infected with recombinant adenoviruses that encoded the gene for green fluorescent protein (adeno- GFP) (Kindly provided by Dr Steve Petrou from the department of Physiology University of Melbourne). The viral lysate was diluted 1:100 in MEM and added to haematopoietic tissue cultures. Tissues were left in incubator at 37°C in 5% CO₂ / 95% O₂ overnight to allow for infection.

Co-culturing the tissues

After 24 hours the infected tissue was washed 2-4 times with MEM to prevent transferring free virus to cultures. Then the infected haematopoietic tissues co-cultured with brain slices that have been 1, 5 or 9 DIV. These co-culture slices were harvested after 1 day, 3 day and 6 days (MEM was changed every two days).

In order to control for endogenous infection with free viral particles, the culture medium from last wash of adeno-GFP infected haematopoietic tissues was added to one well of 1DIV organotypic brain slice culture. No infections were observed.

CFSE labelling the monocyte cells

Monocyte cells were resuspended in sterile PBS to 2 ml. A 2 ml stock solution of CFSE (5-(and -6)-carboxyfluorescein diacetate succinimidyl ester, Molecular probes, Eugene, OR) in DMSO (stored desiccated at -20 C) was added for 1 hour at room temperature. Then the monocytes were washed twice before added to brain slice culture.

Co- culture of organotypic brain slices with adult monocytes

In order to compare of adult monocytes with embryonic haematopoietic tissues in capacity of producing microglial like cells. Monocytes were

separated from adult blood by using Boyum method (Boyum, 1968) and co-cultured with organotypic brain slices. Female adult rats were anesthetized by (1 ml) Nembutal. Via the cardiac 4-ml blood were drawn into the heparinized syringe. 3 ml of lymphocyte separation medium (LSM) were transferred to a 15- ml centrifuge tube and 4-ml blood diluted with 4 ml sterile PBS (0.1 M and PH 7.4). Supplemented the blood layered carefully over the LSM. The tube was centrifuged at 1200 H x g at room temperature for 20 minutes resulting a band of mononuclear lymphocytes observed between LSM and plasma. Top layer of clear plasma aspirated to 2-3 mm above lymphocytes layer aspirated the lymphocytes layer and half of the LSM below it transferred to centrifuge tube. The lymphocytes were washed several times by pelleting and resuspended in sterile PBS (0.1 M and PH 7.4).

Results

Endogenous microglia ramification in brain slice cultures

Endogenous microglial cells exhibited dramatic changes during the 9-days *in vitro*. At 0 DIV of the most of the LEA labelled cells had ramified processes typical of resting microglia (figure 1a), and some were macrophages. The great majority of macrophages were found in the white matter tracts and near the pial and ventricular surfaces, whereas microglia were seemingly evenly distributed throughout the parenchyma. After 1 DIV all of the Lectin labelled cells were rounded, consistent with a change of microglial which is the active phenotype (figure 1b). At 5 DIV some of the LEA labelled cells were ramified, some cells in intermediate phase, and macrophages were also observed (figure 1c). After 9 DIV most of the cells were highly ramified or had the intermediate phenotype, suggesting that the cells were still ramifying (figure 1d).

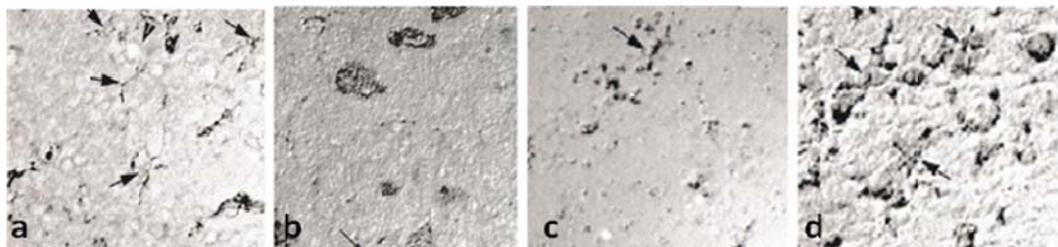


Figure 1. Endogenous microglial changes in brain slices culture *In vitro* (a) at 0 DIV ramified microglia stained with LEA (b) after 1 DIV all of the LEA labelled cells were rounded. (c) at 5 DIV some of the LEA labelled cells were ramified with short-branched processes. (d) At 9 DIV LEA stained highly ramified microglia with long multi-branched processes (arrows). Scale bar: 25 µm in all panel.

Brain slices co-cultured with yolk sac and AGM after one DIV

In this experiment five brain slices from a rat co-cultured with yolk sac and one slice co-cultured with AGM after one DIV. At day 1 of co-culture no ramified cells were observed. After six days of co-culturing the cultures were fixed. Fluorescent green protein (GFP) positive cells were found in both AGM and yolk sac co-cultured slices, mainly around the pial surface of the slices. In most of the cells the nuclei were brighter than the cytoplasm. Many of the GFP labelled cells were highly ramified. Also, some intermediate and round cells were observed, a few of which had one very long process along with some stout branches (figure 2 a, b).

Brain co-cultured with yolk sac and AGM after 5 DIV

In total eight brain slices were cultured for 5 days and then co-cultured with yolk sac or AGM. After one day co-culturing a great number of GFP labelled cells could be observed which distributed around the edges of the brain slices. Both AGM and yolk sac produced the fluorescent cells without any obvious distinction between them. Some of these cells were ramified with multi-branched cytoplasmic processes and strongly fluorescent green (figures 2 c, d). There were also some ramified and round cells with faint green fluorescent.

Brain co-cultured with yolk sac and AGM after 9 DIV

From a rat ten brain slices prepared and cultured. After 9 DIV the slices co-cultured with AGM or yolk sac. The co-cultured tissues were fixed in the following day. There were some haematopoietic-derived cells with highly green fluorescent in brain slices, many of which had a ramified morphology while others had a round appearance (figures 2 e, f). Some of the labelled cells had only weak green fluorescent. There was not any obvious difference between yolk sac and AGM in term of producing ramified cells. Density and morphology of the GFP labelled cells in this experiment resembled the GFP labelled cells of the 5 DIV co-cultures.

Co-culture the organotypic brain slices with adult monocytes for 6 days

In order to detect the potential of the precursor of definitive macrophages (monocytes) to produce the ramified type cells, the brain slices co-cultured with monocytes isolated from adult blood. The monocytes labelled by CFSE produced an intense green fluorescence not unlike that produced by

GFP. Six brain slices of P6 rats co-cultured with adult monocytes after one DIV. The co-cultured tissues were fixed after 3 days or 6 days of co-culture. Fluorescent microscopy revealed the huge number of green fluorescent cells on the brain slices in both 3 and 6 days cultures. They had typical morphology of the monocytes, with round cell body and no branches. The density of the green cells after 6 days appeared to be higher than at 3 days in culture (figures 2 g, h).

Discussion

This and previous studies have demonstrated that the organotypic culture of neonatal brain slices provides an environment that maintains microglial cells and permits their reversible transformation from resting to active phenotypes (Coltman and Ide, 1996; Czapiga and Colton, 1999; Strassburger et al., 2008). This allows the brain slice to be employed in an assay for the microglial generating potential of candidate haemopoietic tissues. In this study, it was shown that both yolk sac and AGM from E12 rat embryos can produce vast numbers of microglia-like cells after being co-cultured for several days with brain slices. The observation that both yolk sac and AGM can produce these microglial-like cells does not address which one, or both, tissues was the ultimate origin of these cell, as they are connected by the circulation from E9, thus have the opportunity to mutually seed one another.

Although this study confirmed the previous report that embryonic tissue can give rise to microglial-like cells in brain slice cultures (Alliot et al., 1999; Prinz and Mildner, 2011), the positive characterisation of the ramified cells could not be extended beyond morphological features, as lectin labelling of these cells could not be achieved. It is important to note that while labelling with LEA did not label the "microglial-like" embryonic cells, this does not mean they did not express the carbohydrates that allow microglial labelling in paraffin sections. This is because the lectin labelling of the whole mount cultured slices (using fluorescent streptavidin to localise the biotinylated lectins) also failed to label the endogenous microglia of the host brain slice, which are readily labelled with lectin using the ABC technique on paraffin sections. Thus using the fluorescent methods to localise LEA ligands is not sufficiently sensitive to stain microglia in these whole-mount preparations. The option of paraffin processing was not available for this particular study, as it would result in the loss of the GFP fluorescence which identified the cells as being of donor origin.

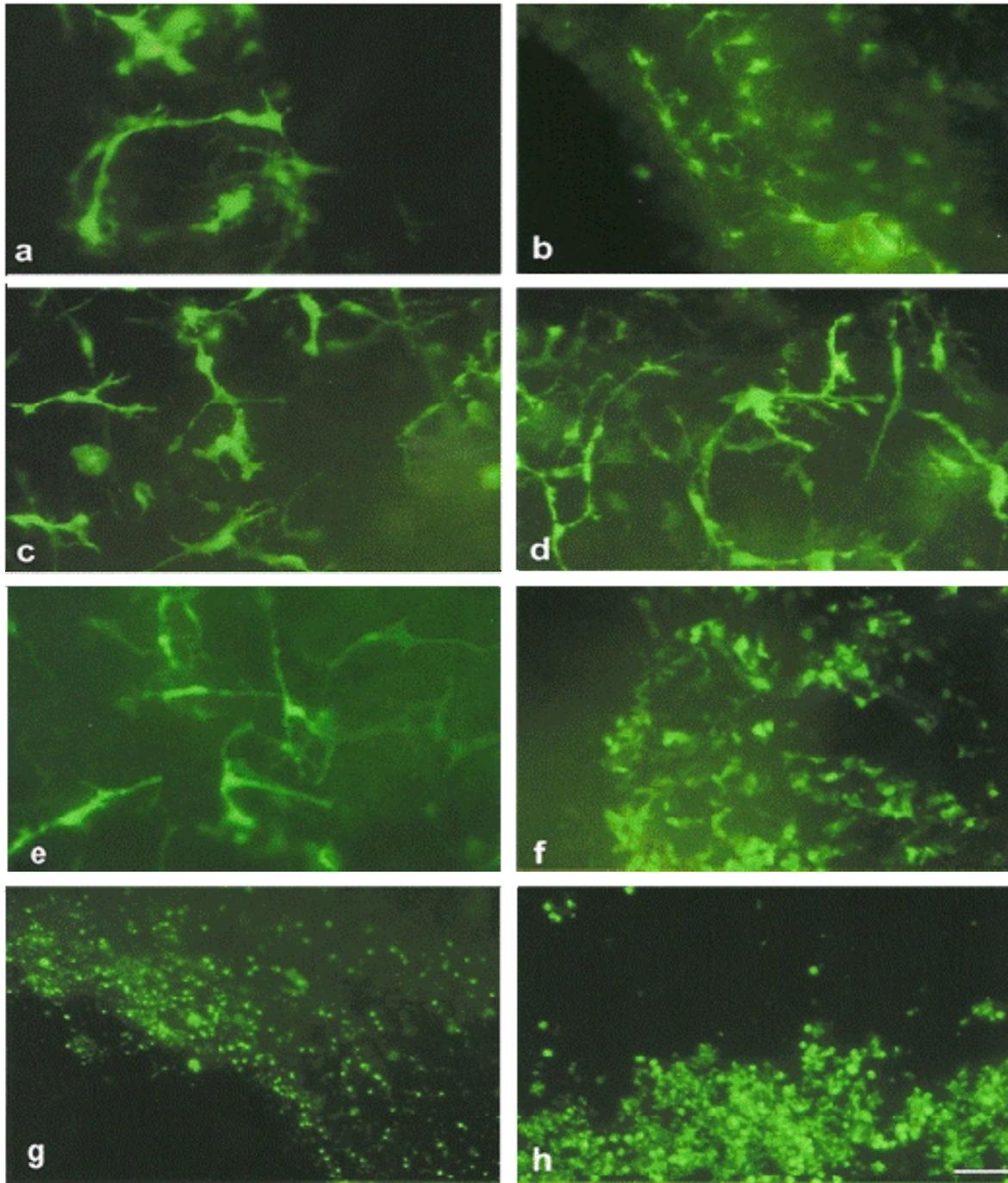


Figure 1. (a-b) brain slices co-cultured after one DIV with adeno-GFP-labelled E11 yolk sac (a) or AGM (b) and fixed after 6 days. Some ramified microglia like cells could be observed in both tissues. (c-d) brain slices co-cultured after 5 DIV with adeno-GFP-labelled yolk sac (c) or AGM (d) and fixed after one day. Some ramified microglia like cells could be observed in both tissues. Also some macrophages and some cells with fewer ramifications could be observed. (e-f) brain slices co-cultured after 9 DIV with adeno-GFP-labelled yolk sac and fixed after 1 day. Some ramified microglia like cells (e) and some macrophages (f) could be observed. (g-h) Organotypic brain slices co-cultured with CFSE labelled adult monocytes 3 days (g) or 6 days (h). No ramified microglia like cells could be observed. Scale bar 50 μ m in panel g and h, 25 μ m in other panels.

While failing to provide cytological evidence of the microglial phenotype, other aspects of these cells were characterised in the present study. Khan's results showed that highly ramified donor derived cells were present after 6 days of co-culture (un-

published); this time *in vitro* could be effected by the state of the slice, or it may be that the expression of the ramified phenotype takes many days to emerge. As discussed above, the brain slice culture is an environment that induces activation of

phagocytes after cutting but subsequently this environment changes to be one which permits the re-ramification of microglia. The role of the state of the brain slice in the adoption of the ramified morphology was investigated by co-culturing the embryonic tissues with brain slices that had spent various times in culture before the donor tissues were added. It was clear that the ramified phenotype of donor cells could be observed within a day of co-culture if the brain slice was 5 or 9 days *in vitro* before the donor tissue was added.

This result strongly suggests that the status of the brain slice has a major influence on the adoption of the ramified phenotype. While the elimination of the dying and dead cells (caused by the slicing) might be why the older slices are more conducive to ramification, another possibility is that astrocyte proliferation may be the crucial factor facilitating the acquisition of the ramified phenotype. It has been reported that astrocyte monolayers induce ramification of isolated microglia, monocytes and macrophages (Wilms et al., 1997). The findings of Wilms et al. (1997) that not only microglia, but also macrophages and monocytes transform into a ramified phenotype suggest that the monocytes in our experiments should have ramified on the brain slices. However, monocytes isolated from the blood of adult rats did not exhibit the transformation into the ramified phenotype at any stage of co-culture (up to 6 days). A reason for this discrepancy might be that other factors are needed by monocytes that are not needed by the embryonic tissues. For example, monocytes and macrophages do not ramify if serum is present, unless astrocytes are included in the culture, but serum may in fact be required by monocytes for reasons unrelated to ramification, and thus the culture conditions employed in the present studies did not allow monocytes to differentiate to a stage where they could exhibit a ramified morphology.

The methods developed and characterised by this and other studies- such as brain slice cultures, serum free culture systems and the isolation of embryonic and adult haemopoietic tissues - will permit further elucidation of the identity, origin, nature and behavior of the brains immune cells.

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