Isolating vessels from the mouse brain for gene expression analysis using laser capture microdissection

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Abstract

Studies of gene expression often examine a pool of RNA extracted from the diverse cell types making up a tissue. We have developed a method for isolating vessels from the brain in order to understand the changes occurring in the vessels during the pathogenesis of cerebral malaria. Vessels were visualised by incubating sections of mouse brain with a substrate for alkaline phosphatase. Vessels were collected by laser capture microdissection and the specificity was monitored by measuring the expression of cell-specific markers. RNA from the captured vessels was highly enriched in mRNA for genes associated with endothelial cells and pericytes. Measurement of indoleamine 2,3-dioxygenase mRNA indicated it was possible to detect changes in gene expression, due to malaria infection, occurring specifically within the vessels. Laser capture microdissection can be used to study changes in gene expression occurring at the blood–brain barrier.

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1. Type of research

Techniques for measuring gene expression have made enormous progress over the last few years with the advent of real-time PCR and microarrays. However, many studies are limited to looking at gene expression in large samples of tissue which contain many different cell types. Laser capture microdissection is a technique that allows specific cell types to be captured from a tissue section [4]. We have developed a protocol to isolate brain microvessels in order to study changes in gene expression occurring in a murine model of cerebral malaria. To develop a protocol for this procedure we had two main priorities. The first was to be able to visualise the vessels easily to aid specific capture. The second was to maximise RNA preservation.

2. Time required—11 h

1. Isolation and freezing of tissue: 30 min.
2. Cutting, staining and dehydrating sections: 90 min.
3. Capturing cells: 1 h/cap (enough for 8–10 polymerase chain reactions).
4. Extracting RNA and synthesizing cDNA: 5 h.
5. Real-time polymerase chain reaction (PCR): 3 h.

3. Materials

3.1. Animals and malaria parasite

Female CBA/T6 mice obtained from the Blackburn Animal House, University of Sydney were used at 6–8 weeks of age. Plasmodium berghei ANKA was obtained courtesy of Dr. G. Grau, Marseilles.

3.2. Equipment

Laser capture was performed on a PixCell II microscope using high sensitivity (HS) caps (Arcturus, CA, USA). Real-time PCR was performed on an ABI7700 machine (Applied Biosystems, CA, USA).
3.3. Tissue section preparation

Tissue was embedded in OCT freezing medium (Miles, IN, USA). Sections were immobilised on Starfrost uncoated slides (Germany) and stained with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate (Moss, MD, USA). The section was washed from George T. Gurr (UK) and RNAlater from Ambion (TX, USA). Ethanol was supplied by APS Chemicals (Australia) and treated with molecular sieves type 4A (BDH Laboratory Reagents, UK).

3.4. Molecular biology reagents

RNA was extracted with the Stratagene RNA microisolation kit (CA, USA). Reverse transcription was carried out using the Sensiscript reverse transcriptase kit from Qiagen (Hilden, Germany). Glycogen, RNase inhibitor and random primers were supplied by Invitrogen Lifetech (CA, USA). PCR was performed using the 2× Sybr Green master mix from Applied Biosystems and oligonucleotides from Invitrogen Lifetech.

4. Detailed protocol

4.1. Preparation of tissues

CBA mice were inoculated with 10⁶ parasitised red blood cells and were euthanised 6 days after infection, when they began to show neurological symptoms. The mice were perfused with 20 ml of cold phosphate-buffered saline immediately after death. Tissues were covered in OCT freezing medium and frozen in hexane at −120°C. Seven-μm sections were immobilised on slides. Tissue was fixed in 70% (v/v) ethanol for 1 min. After rinsing in water the section was covered with RNAlater and incubated for 4 min at room temperature. After rinsing well in water, the section was incubated for 4 min at 50°C with BCIP/NBT substrate. The section was dipped in 70% and 95% ethanol and then stained with eosin for 30 s. Excess eosin was rinsed off in 95% ethanol. The sections were dehydrated through two lots of 100% ethanol (1 min each) and dipped six times each in two lots of xylene. Sections were stored at −80°C in air-tight containers containing silica gel.

To calculate percentage of brain volume represented by vessels, 5-μm serial sections were cut through five mouse brains. After staining with BCIP/NBT substrate (no counterstain) the area of the stained vessels, as a proportion of the total area of the section, was calculated using the IPLab program (Scanalytics, VA, USA).

4.2. Laser capture microdissection

Loose material was blotted from the section prior to capture using a Preparation Strip (provided with the HS caps). The 7.5-μm size laser spot was always used along with a 1 ms duration of laser pulse. We used the lowest power setting possible that still enabled capture (55–70 mW). Caps containing captured cells were stored at −80°C until extraction.

4.3. RNA extraction

RNA was extracted from captured cells using a modification of the Stratagene RNA microisolation kit. After placing the Extractsure well (provided with HS caps) on the cap, 20 μl of denaturing buffer were added to the surface of the cap and incubated for at least 20 min at RT. The lysate was spun into a tube and made up to 100 μl with denaturing buffer. To this, 10 μl 2 M sodium acetate, 110 μl phenol and 30 μl chloroform were added and the mixture was vortexed. After incubation for 10 min on ice, the layers were separated by spinning in a microfuge at maximum speed for 15 min. The aqueous layer was removed to a new tube and 75 ng of glycogen and 100 μl isopropanol added. After incubation at −20°C for at least 2 h, the RNA was precipitated by centrifuging at maximum speed for 20 min. The pellet was washed in cold 70% ethanol, air-dried and resuspended in water.

4.4. cDNA synthesis and real-time PCR

cDNA was synthesised at 37°C for an hour using the Sensiscript reverse transcriptase kit in a reaction containing 1× RT buffer, 0.5 mM dNTP, 10U RNase inhibitor and 0.1 μg random primers. The cDNA was then diluted with water and used as a template in a real-time polymerase chain reaction. The reactions contained 1× Sybr green reaction mix, 200 nM of each primer and 10 μl of template in a 25-μl reaction volume. Amplification was performed with a 1-min incubation at 95°C then 40–60 cycles of a 15-s incubation at 95°C followed by 1 min at 60°C. The specificity of amplification was checked by melting curve analysis of the PCR products.

18S rRNA
5′-GCCGCTAGAGGTGAAATCTTG-3′
5′-GAAAAACATCTTGGCGAATCTTT-3′
HPRT
5′-GCTTTCCCTGTTAAGCAGTACA-3′
5′-CAAACCTTTGCTGGAATTTCAAC-3′
Indoleamine 2,3-dioxygenase (IDO)
5′-GGCTTTCTCTCTGCTCTCTATTG-3′
5′-TGACGCTCTACTGACCTGATAC-3′
Desmin
5′-TGAGGCGCTGACAACCTTGATAGA-3′
5′-CCCTCTGTAGTTGCGCTTTGAG-3′
S100 beta (S100b)
5′-TGACGCTCTACTGACCTGATAC-3′
5′-AAATACGCTGCTGGCTTGGA-3′
5. Results

5.1. Staining vessels using alkaline phosphatase activity

It has long been known that alkaline phosphatase is a marker for vascular endothelial cells, particularly those of the blood–brain barrier [11]. Alkaline phosphatase has been used as a part of a number of immunostaining and in situ hybridisation protocols. For this purpose, substrates producing coloured and insoluble end-products have been developed. Endogenous vessels in the brain, and other tissues, contain sufficient endogenous alkaline phosphatase for staining of the cells within minutes using BCIP/NBT substrate. It is possible to do the reaction at room temperature in less than 10 min, although we found that variation in ambient temperature produced variable staining intensity. For uniform staining, the reaction was carried out on a 50°C heating block for 4 min.

Counterstains, such as eosin, are not necessary to visualise the vessels but help distinguish tissue morphology and darken the vessel staining. Another stain that produced a good contrast was light green. The coloured end-product is somewhat soluble in xylene and so the section should not be left in the xylene for more than 30 s.

The alkaline phosphatase substrate produced specific staining of the brain microvasculature (Fig. 1A). We investigated how specific the alkaline phosphatase staining is in other tissues to see if it could be of more general use. The spleen, heart and liver showed specific staining of vessels (Fig. 1B and D). The lung had high background staining, although some darker coloured vessels could be distinguished (not shown). Another cell type in the kidney also stained with the BCIP/NBT substrate, although these cells could be distinguished from the vessels by the speckled appearance of the staining compared to the darker staining of the vessels (Fig. 1C).

5.2. Dissection of vessels

Adequate dehydration of the sections is vital for successful laser capture of cells. The sections were dehydrated using 100% ethanol made anhydrous with the use of molecular sieves. Slides were stored in air-tight containers containing silica gel. If frozen, they were brought to room temperature before opening the containers to prevent condensation.

A flat tissue section aids with the capture process. Very wrinkled sections of tissue were removed with a scalpel blade before LCM. Capturing the longitudinally sectioned vessels provided a large amount of vessel material but also was more likely to pull away adjoining tissue (Fig. 1E–G). Circular cross-sections of vessels, although smaller and therefore yielding less material, were more likely to come away cleanly (Fig. 1H–J).

5.3. RNA yields

According to the manufacturer the RNA stabilising agent RNAlater is only taken up by living cells and should not be effective in this step of the protocol. However, incubating the section before staining did increase the RNA yield 2–4-fold (data not shown). We tried placing tissues in the solution before freezing but found it extremely difficult to obtain good sections afterwards.

To minimise the number of steps in our RNA extraction protocol and maximise the amount of RNA obtained, we omitted any DNase treatment. HPRT, IDO, desmin and S100b primers were all designed to be cDNA specific (with primers situated on different exons or spanning introns). Amplifying 18S rRNA from reactions with or without reverse transcriptase indicated that genomic 18S rRNA made an insignificant contribution to the total 18S rRNA detected (data not shown). We also measured IDO, desmin, neurogranin and S100b expression relative to the housekeeping gene HPRT as well as 18S rRNA to check that the results were comparable.

5.4. IDO expression in vessels

IDO expression is induced in the endothelium of many tissues during malaria. To see whether it is possible to detect differential expression of the gene in the vessels, we measured IDO mRNA levels by real time PCR in captured vessels from uninfected and infected mice (Fig. 2). We also compared the IDO expression in captured material that did not appear to contain any vessels (‘non-vessels’). After normalising to 18S rRNA levels the induction of IDO was expressed relative to uninfected mice. Even after extending the PCR run to 60 cycles, no IDO expression could be detected in vessels from uninfected animals or in the ‘non-vessel’ material from either uninfected or infected mice. Using a cycle threshold of 60, the average induction of IDO mRNA in PbA infected mice was calculated to be over 200,000-fold.

5.5. Enrichment of vessels

We determined the volume occupied by the alkaline phosphatase positive vessels through serial sections of five murine brains. Vessels represented 0.99±0.008% (mean±S.D.) of the total volume of the brain. By comparing IDO expression relative to 18S rRNA in captured brains to that in a lystate from a whole brain section, the average enrichment of endothelial cells was calculated to be over 90-fold (Fig. 3A). If the proportion of endothelial cells in the brain is similar to the area they cover (~1%) then the captured material is highly enriched for endothelial cells. PbA-infected mice were used for this experiment as there is very little expression of IDO in uninfected mice.

Pericytes are a relatively rare cell type in the brain and are found associated with vessels. The enrichment of pericytes after LCM was assessed by measuring the amount of desmin mRNA, a marker for pericytes, in the captured cells relative to the whole brain (Fig. 3A). The ratio of desmin mRNA to 18S rRNA was ~240 times.
Fig. 1. (A) Brain incubated with BCIP/NBT substrate (20× magnification). (B) Spleen incubated with BCIP/NBT substrate (40× magnification). (C) Kidney incubated with BCIP/NBT substrate (40× magnification). (D) Heart incubated with BCIP/NBT substrate (20× magnification). (E) Longitudinal brain vessel before and after (F) capture (40× magnification). (G) Captured vessel from (E) on the surface of the cap. (H) Cross-sectional vessel before and after (I) capture (40× magnification). (J) Captured vessel on the surface of the cap.
higher in the captured cells compared to whole brain, indicating the captured material was highly enriched in pericytes.

5.6. Presence of other cell types

The expression of two genes was used to assess the purity of the captured vessels (Fig. 3B). Astrocytes are adjacent to endothelial cells lining the vessels and specifically express S100b. The proportion of S100b mRNA to 18S RNA did not change significantly in the captured vessel mRNA compared to whole brain mRNA suggesting that there was some contamination with this cell type. Neurogranin is a calmodulin-binding protein that is expressed in nerve cell bodies and dendrites. The neuronal marker was only sometimes detectable, suggesting that neuronal contamination was insignificant.

6. Discussion

Cerebral malaria is an example of a disease where a particular cell type (endothelial cells) in a complex tissue (the brain) plays a crucial role in the disease process. This complication of malaria occurs in a minority of Plasmodium falciparum infections and has a high mortality rate. Examining brains of patients who died of CM reveals adherence of parasitised red blood cells in the brain microvasculature, haemorrhages, glial proliferation, oedema and possibly hypoxia–ischaemia [10]. The pathogenesis of this condition is not fully understood, although changes in the microvasculature play a crucial role. During murine cerebral malaria, endothelial cells upregulate their expression of adhesion molecules which results in adherence of leukocytes within the microvasculature [5]. Breakdown of the blood–brain barrier is also necessary for the development of the disease [9]. Formerly, to investigate changes in gene expression, we have looked at RNA from homogenates of brain. Differentially regulated genes were then further investigated by immunohistochemistry. To focus our search on pathways differentially regulated in the vessels during cerebral malaria we wished to specifically study RNA from the vessels. As the vessels represent a small proportion of the area of the brain it would be difficult to detect changes in gene expression in the vessels using RNA from a homogenate. For this reason we have employed laser capture microdissection to isolate vessels from the murine brain and look for changes in gene expression in the selected cell populations.

Laser capture microdissection (LCM), using the system developed by Arcturus, involves placing a plastic cap onto a tissue section. The tissue can be viewed through the top of the cap with an inverted microscope [4]. A low-power, infra red laser is fired at cells of interest, melting the thermoplastic film on the surface of the cap. The cells fuse to the plastic and are lifted away from the tissue section when the cap is lifted. RNA, DNA or protein can be extracted from the captured cells. In comparison with other cell isolation techniques, LCM has several advantages. The tissue can be frozen, along with its gene expression patterns, immediately after removal. In contrast, disrupting the tissue into a single cell suspension and separating cells using antibodies is a lengthy procedure during which gene expression could alter further. Tissue fixation can often make it difficult to extract RNA afterwards. In situ
hybridisation can specifically locate mRNA to a cell type but can be difficult to quantitate and only allows the study of one gene at a time.

Specific capture requires good visualization of the vessels. A skilled operator may be able to detect vessels with a non-specific stain, however utilising the endogenous alkaline phosphatase activity of endothelial cells offers a quick, specific staining method for distinguishing vessels in the brain and still allows for subsequent RNA isolation. Alkaline phosphatase is a particularly good marker for endothelial cells in the blood–brain barrier [11]. This staining method may be useful for other tissues, for example, spleen and heart. However there is a high level of background staining in the lung, and in the kidney another cell type has a speckled staining pattern. These cells probably represent the proximal convoluted tubules which are known to express high levels of alkaline phosphatase [2].

Electron microscopy has been used to localise alkaline phosphatase activity within the endothelial cell during cerebral malaria [8]. Infecting C57 Bl/6 mice with a low dose of *Plasmodium berghei* K173 results in cerebral malaria. As the course of the disease progresses, changes in the localisation of alkaline phosphatase along the endothelial cell membranes occur. These changes correlate with the formation of pinocytic vesicles in some endothelial cells and may be a sign of disruptions to the blood–brain barrier. The lower level of magnification used with
LCM reveals that the alkaline phosphatase appears a little more diffuse in mice with cerebral malaria compared to controls. The oedema in the sick mice resulted in better separation of the cells and enhanced specific capture.

IDO expression is induced in the endothelium during malaria infection as detected by immunostaining [7]. These results were substantiated in our study using laser captured vessels. IDO mRNA was undetectable in the material that did not contain any vessels, confirming the specificity of the induction. This demonstrates that we can detect changes in gene expression in captured vessels that correlate with immunohistochemistry results. The inability to detect changes in IDO expression in captured tissue without any visible vessels (non-vessels) demonstrates the specificity of the technique.

Measuring the levels of cell specific markers is a useful way of determining the purity of the captured population. The levels of desmin, relative to 18S rRNA, in captured vessels versus whole brain indicates that the captured cells are highly enriched for pericytes. This is not surprising as pericytes are contained within the basal lamina of blood vessels and are therefore a component of the vasculature. Astrocytes are adjacent to the vessels and signals from these cells are responsible for many of the unique properties of the endothelial cells that comprise the blood–brain barrier. Assessing the levels of S100, an astrocyte marker, suggested that astrocytes represented a similar proportion of cells in the captured vessels to that observed in the whole brain. As S100b-positive astrocytes represent a minority of cells in the total cell population, the contaminating astrocytes in the captured material are similarly outnumbered by the vessel cell types. Only traces of a neuronal cell marker, neurogranin, were detectable in the captured vessels, indicating there was insignificant contamination with this cell type. It is possible that an additional source of contamination may arise from adherent leukocytes in the vasculature of mice with cerebral malaria. We sought to minimise this by intravascular perfusion of the mice after death and by avoiding capturing vessels where the rounder, adherent leukocytes were visible.

The amount of RNA obtained after laser capture microdissection is very small. It is not possible to study changes in gene expression without some kind of amplification of the nucleic acids. Real-time PCR is useful for known genes and representative difference analysis may be feasible for discovering differentially expressed genes. Microarray analysis requires much larger amounts of material. We are currently optimising a linear amplification procedure based on the Eberwine T7 RNA polymerase method [3] to enable the material from captured vessels to be analysed using microarrays. Current microarray studies on gene expression during cerebral malaria have used RNA from whole brain homogenates. Laser capture microdissection will allow us to target our search for differentially regulated genes to cell types in the vessels which have an important role in the development of the disease.

7. Troubleshooting

To obtain the maximum amount of RNA from the captured cells, section preparation should be performed in RNase-free conditions. RNase inhibitors can be added to the BCIP/NBT substrate if RNA degradation is thought to be a problem. The incubation lengths should also be kept to a minimum. In our experience longer staining protocols (e.g. lectin-staining and immunostaining) resulted in the degradation of all the RNA. The use of an RNA stabilising agent, RNAlater, increased RNA yields although according to advice from the manufacturer the product should only be active in living cells. It is obvious that some cellular activities are at work even after fixation, e.g. RNases and alkaline phosphatase, so it is possible that the agent can still be taken up by the cells after light fixation. Without knowing the composition of the commercial reagent it is difficult to speculate.

Efficient capture depends upon the dehydration of the sections. Therefore using anhydrous alcohols (treated with molecular sieves) is important. We also found the ambient humidity can effect the dissection and the use of a dehumidifier in the LCM room prevents this becoming a problem.

8. Alternative and support protocols

There are other histochemical techniques available for detecting alkaline phosphatase including the Gomori method [6] and the azo dye technique [1]. Another recently described technique utilises a substrate, ELF (Enzyme-Labeled Fluorescence)-97 phosphate [2]. A comparison of the four histochemical techniques found that ELF-97 phosphate localised alkaline phosphatase activity in seconds rather than the minutes or hours required by the other methods. This suggests that the ELF-97 phosphate method would be optimal for preserving RNA. Unfortunately many laser capture microscopes (including our own) do not have the modifications required for fluorescence detection.

We have used other molecular biology reagents with success (e.g. Qiagen RNAeasy kit, MMLV-RT from Invitrogen Lifetech and Brilliant-QPCR kit from Stratagene) although it is not possible to use phenol-based reagents to lyse cells on the caps as they react with the plastic.

9. Essential literature cited

The laser capture microdissection method is first described in the paper by Emmert-Buck et al. [4].
10. Quick procedure

1. Euthanise mice and perfuse with cold PBS. Remove tissue, cover in O.C.T. freezing medium and freeze in hexane. Store at −80 °C.
2. Cut 7-μm sections onto glass slides. Fix in 70% ethanol for 1 min.
3. Rinse in water and cover section with RNA later for 4 min.
4. Rinse well in water and cover section in BCIP/NBT substrate. Incubate for 4 min at 50 °C.
5. Slides were dipped in 70% and 95% ethanol followed by a 30-s incubation in eosin.
6. Excess eosin was rinsed off in 95% ethanol and the sections dehydrated by 1 min in 100% ethanol (twice).
7. Several hundred cells were captured using a 7.5 micron spot size, 1 ms duration of pulse and a power setting of 55–70 mW.
8. Denaturing solution (20 μl) was added to the surface of the cap and incubated for 20 min. The lysate was spun into a tube and 80 μl denaturing solution, 10 μl 2 M sodium acetate, 110 μl phenol and 30 μl chloroform were added. After vortexing the tube was placed in ice for 10 min.
9. After centrifuging for 15 min, the aqueous layer was removed to a new tube and 75 ng glycogen and 100 μl isopropanol were added. The RNA was precipitated by incubation at −20 °C for 2 h followed by a 20-min centrifugation. The pellet was washed with cold 70% ethanol and resuspended in water.
10. Reverse transcription was carried out according to the manufacturer’s instructions (Qiagen Sensiscript reverse transcriptase kit). The cDNA was diluted with water.
11. Real time PCR was performed in a 25-μl reaction containing 1× Sybr Green master mix and 200 nM each primer. Amplification was performed with a 1-min incubation at 95 °C then 40–60 cycles of a 15-s incubation at 95 °C followed by 1 min at 60 °C.

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References