Electron microscopy and immunogold labelling of proteins involved in brain tumour growth and invasion

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Gliomas are amongst the most common tumours of the central nervous system and are derived from glial cells including astrocytes and oligodendrocytes. The most malignant of the astrocytomas is glioblastoma multiforme (GBM) which is classified as WHO grade IV [1]. The main treatment options for GBM consist of surgical resection followed by radiotherapy and chemotherapy. The characteristics of GBMs include intense cell proliferation, areas of necrosis and accentuated angiogenesis. In this study our aim was to identify by transmission electron microscopy the localization of VEGF, MMP-2 and Tenascin C using immunogold labelling techniques in the C6 rat glioma model implanted orthotopically in the brain of the Wistar rat.

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1. Introduction

The age-adjusted incidence rate of central nervous system (CNS) tumours is 6.4 per 100,000 per year in the USA, with astrocytomas being the most frequent primary brain tumours (40%) [2,3]. In the case of glioblastoma multiforme (GBM), the most malignant astrocytoma, the main treatments consist of surgical resection followed by radiotherapy and chemotherapy. However, in spite of multimodal therapies, the average survival of patients with GBM is between 9-12 months after surgical resection, due to post-surgical re-incidence of the tumour of almost 100% [4,5]. The main characteristics of GBMs are: high rates of cell proliferation; ability to migrate and to invade the surrounding cerebral structures (mainly along vessels and myelinated fibres); areas of necrosis and accentuated angiogenesis (which is reflected by an increased endothelial cell proliferation). Indeed, GBMs are one of the most densely vascularized human tumours [6]. Some reports have suggested that this neo-angiogenesis is more closely related to GBM migration than to tumour nutrition [7]. Angiogenesis consists of new vessels sprouting from pre-existing vessels and its regulation depends on inducers and inhibitors released by both tumour and endothelial cells. In GBMs, the best characterized pro-angiogenic factor is vascular endothelial growth factor (VEGF), whose overexpression is correlated with increasingly malignant phenotypes [8,9]. This growth factor induces endothelial cells to proliferate, degrade the extracellular matrix surrounding the vessel and also promotes endothelial migration to increase the new blood vessel. Some studies show that angiogenesis facilitates the invasiveness of GBM cells. Another characteristic of GBM cells is the expression of large amounts of metalloproteinases (MMPs) which are proteolytic enzymes that degrade different extracellular matrix elements including collagens, laminin and some proteoglycans [10]. Several reports have shown, in gliomas, a strong correlation between MMP-2 expression and the malignant phenotype [11]. Moreover, a strong correlation between MMPs expression (mainly MMP-2 and MMP-9) and angiogenesis has been shown in GBMs in vivo [12]. Indeed, the expression of MMP-2 and MMP-9, both in tumour and endothelial cells, shows a pivotal role of these enzymes in invasion and in matrix remodelling induced by angiogenesis [13,14].

Tenascin-C (TNC) is an extracellular matrix glycoprotein which is expressed in the central nervous system (CNS) during embryogenesis but is not normally present in the adult CNS (except in areas of cellular migration and secondary neurogenesis). TNC can be expressed by astrocytes, neurons and endothelial cells. In GBM, TNC is expressed at the tumour border, stimulating proliferative and invasive characteristics in these tumour cells. Some studies have also related TNC expression with metalloproteinase and VEGF synthesis [15].

There are various methods to detect these proteins at the level of light microscopy but the exact cellular location cannot be reliably confirmed without using electron microscopy methods. Since each antigen and each tissue has its own peculiarities for immunogold labelling, methods must be individually developed for each model system. Therefore the aim of the present study was develop a suitable method to identify the exact location of MMP-2, VEGF and TNC in brain tumour tissue, in order to complement our previously established methods using light microscopy.
2. Immunogold Labelling Techniques

2.1 Surgical procedures
Male and female Wistar rats were anaesthetised with an intramuscular injection of ketamine:xylazine 10mg:1.5mg/100g body weight to provide deep anaesthesia and analgesia. The rats were placed on a stereotaxic surgical table, a midline incision was made and a burrhole was drilled 0.4mm anterior and 3 mm lateral to bregma. The C6 cell suspension was slowly injected into the striatum using a Hamilton syringe at a depth of 5.4 mm to the bone surface and the needle left in situ for 3 minutes before its removal. The rats received 5x10⁵ cells in a total of 4-5μl of sterile saline. After 28 days the animals were anaesthetised and perfused transcardiatically with ice cold saline, pH7.2, followed by ice cold fixative solution. The fixative solution used was 4% formaldehyde in 0.1M potassium phosphate buffer (KPB) pH7.2, containing 0-0.5% glutaraldehyde. This procedure was approved by the Ethical Commission for Animal Experimentation of the Biomedical Institute (University of São Paulo) – protocol number 190/02. The use of perfusion fixation greatly improves the quality of fixation and allows for the use of lower concentrations of glutaraldehyde. When necessary, for example in the case of human brain tumour samples, immersion fixation can be used on small tissue fragments of less than 1mm³.

2.2 Free-floating pre-embedding technique
After transcardiac perfusion with 4% formaldehyde in 0.1M KPB pH7.2, containing 0-0.5% glutaraldehyde the brain tumours were dissected and post-fixed in the same solution for 3 hours at 4°C. After post-fixation 50μm sections were cut on a vibrating microtome (Vibratome series 1000) in 0.02M phosphate buffered saline (PBS). The individual sections were placed in 24-well culture plates in 0.02M PBS pH7.2.

In all immunocytochemical methods it is important to block aldehyde groups to avoid non-specific background labelling. For this step the sections were incubated for 10 minutes in a solution of 0.1 M glycine in PBS. This is a relatively gentle blocking procedure which does not usually adversely affect tissue morphology. The sections were then washed three times in PBS for 5 minutes. From this point onwards most of the washing steps used PBS together with 0.2% Triton X-100 detergent solution (PBST) to improve tissue permeability to antibodies. The non-specific binding sites were blocked with PBST containing 5% pre-immune serum of the species in which the secondary antibody was raised for 60 minutes. Primary antibody was diluted 1:200 in PBST and incubated overnight at room temperature or at 4°C. The sections were rinsed in PBST and incubated with colloidal gold-conjugated secondary antibody (1:50) in PBST for 90 minutes at room temperature. This basic immunocytochemistry protocol was modified according to the different techniques used during the study.

The primary antibodies used were anti-MMP-2, VEGF and TNC from Santa Cruz, USA. The secondary antibodies conjugated with 6, 10 or 15nm colloidal gold particles were purchased from Electron Microscopy Sciences (EMS), USA.

After incubation the sections were rinsed, fixed in 2% glutaraldehyde in PBS and incubated with 1% OsO₄ for 60 minutes. Sections were then polymerized at 60°C for 16 hours. During resin polymerization it is important to maintain the tissue slice as flat as possible for further ultrathin sectioning. After resin polymerization the sections were removed from the Thermax coverslips with liquid N₂. The included brain slice was trimmed to select the tumour area of interest and mounted in silicones with Epon 812 resin. These blocks were polymerized at 60°C for a further 16 hours. Ultrathin sections of approximately 80 nm were placed on 200 mesh copper grids and contrasted with lead citrate and uranyl acetate before viewing at 80 kV on a JEOL transmission electron microscope (JEM 1010). While the free-floating technique improves interaction between the tissue antigen and primary antibodies, a much larger volume of antibody is required than post-embedding methods. This may be a problem when only small quantities of a primary antibody are available for research.

2.3 Freeze-cracking pre-embedding technique
When trying to improve tissue permeability to antibodies the freeze-cracking technique may also be useful. After perfusion and post-fixation as described in 2.2, 50μm sections were cut on a vibrating microtome in 0.02M phosphate buffered saline (PBS). The individual sections were placed in 24-well culture plates in 0.02M PBS pH7.2. The sections were then placed in a solution containing 25% sucrose and 6%glycerol in 0.05 M KPB for 30 minutes. The sections were then placed in 1% osmium tetroxide solution (OsO₄) for 1 hour. After dehydration in a series of ethanol solutions, the sections were then placed in a solution of 100% Epon 812 resin and polymerized at 60°C for 24 hours. Ultrathin sections were then prepared for viewing as described above.

2.4 Post-embedding technique
After transcardiac perfusion with 4% formaldehyde in 0.1M KPB pH7.2, containing 0-0.5% glutaraldehyde the brain tumours were dissected and post-fixed in the same solution for 3 hours at 4°C.
The brain tumours were serially dehydrated and after dehydration the samples were gradually embedded in LR White resin in three increasing concentrations of resin. The first solution contained 100% ethanol: LR White (1:1) and the samples were incubated overnight. The second 3 hour incubation was in 100% ethanol: LR White (1:3). The final step involved two incubations in pure LR White, the first for 2 hours and the second overnight. All of the LR White incubation steps were performed at 4°C. After embedding in LR White the tissue blocks were polymerized in silicone moulds at 37°C overnight.

Semi-thin sections of 1µm were cut and stained with 1% toluidine blue to localize the tumour for trimming of the blocks. Ultrathin sections of approximately 80 nm were placed on 200 mesh gold grids. Gold grids were used to reduce possible non-specific background labelling which is more likely with copper grids. The immunocytochemical protocol began with section hydration in water for 5 minutes, followed by a gentle aldehyde block with glycine 0.02M in Tris buffered saline (TBS) pH 7.2 for 15 minutes. The sections were then incubated with 3% bovine serum albumin (BSA) and pre-immune serum (1:1) in TBS Tween 20 (TBST) 0.1% pH7.2 for 30 minutes. Primary antibody (1:50-1:200) incubation was overnight at 4°C and the primary antibody was prepared in TBST pH 7.2 containing 0.6% BSA. Sections were rinsed 5 times in TBST for 5 minutes. This was followed by incubation with secondary antibody (1:10) in TBST pH 8.0 with 0.1% BSA for 2 hours at room temperature. After incubation the sections were washed in TBST 5 times for 5 minutes. The reaction was fixed with 2.5 % glutaraldehyde in TBS pH7.2. The sections were finally contrasted with lead citrate and uranyl acetate before viewing as described in 2.2. This method allows for the use of much smaller volumes of reagents but may have reduced sensitivity for certain antigens which will be more readily detected using the free-floating technique.

3. Results

The invasive phenotype of glioma cells is directly related to MMP-2 activity. Using immunogold labelling techniques MMP-2 was localized throughout the extracellular matrix (ECM) (Fig.1) and in some cases very close to the cell membrane (Fig.2). This correlates with the light microscopy findings where MMP-2 is largely present in the ECM, where it is responsible for the degradation of ECM components that surround the tumour cells. MMP-2 was also observed in close proximity to both tumour cells and endothelial cells at the basement membrane of blood vessels, showing the preference of tumour cells to migrate along blood vessels in the brain (Fig.2).

![Fig. 1 Transmission electron microscopy of immunogold labelled matrix metalloproteinase 2 (MMP-2). MMP-2 is distributed throughout the extracellular matrix. The scale bar represents 200nm.](image)
VEGF was strongly immunogold labelled close to blood vessels in the tumour tissue (Fig. 3). VEGF was also detected in the ECM where it is known that both fibrillary and amorphous matrix can act as a VEGF reservoir, which liberates the growth factor when the ECM is degraded by enzymes including MMP-2 (Fig. 4). Both VEGF and MMP-2 were successfully detected using each of the immunogold labelling methods described in this chapter. The upper limit for glutaraldehyde concentration before labelling was lost was 0.2% but the best labelling was obtained using the lower concentration of 0.1% glutaraldehyde.
VEGF was present in the extracellular matrix surrounding a tumour cell. The scale bar represents 200nm.

TNC was detected close to the tumour cell surface and in the pericellular ECM (Fig. 5). In the ECM, TNC was observed in the fibrillary and amorphous matrix, as seen in previous light microscopy studies (Fig. 6). While all of the labelling methods detected TNC, this protein required lower concentrations of glutaraldehyde to preserve immunogold labelling with the most success at <0.1% glutaraldehyde.
Fig. 6 Transmission electron microscopy of immunogold labelled Tenascin C (TNC). TNC is present in the extracellular matrix surrounding a tumour cell. The scale bar represents 200nm.

4. Conclusions

When testing the different methods used in this study we observed that glutaraldehyde concentration influences the immunogold labelling of all three proteins MMP-2, VEGF and TNC in brain tumours. All of the methods permitted detection of the antigens of interest to a greater or lesser degree. Although we observed labelling with all the techniques described in this chapter, when antibody penetration is important and morphology is less important to the study freeze-cracking may be the best method to choose. However, the tumour tissue is easier to manipulate using the post-embedding method, as free-floating sections often lose the tumour tissue during the many washing stages of the protocol. Individual modifications of this basic methodology have been used to detect cell surface and intracellular antigens including the receptor for hyaluronic acid-mediated motility (RHAMM) and cyclooxygenase-2 (COX-2).

References

