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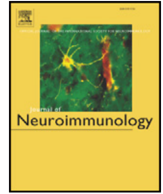

Targeting classical but not neurogenic inflammation reduces peritumoral oedema in secondary brain tumours

Kate M. Lewis, Elizabeth Harford-Wright, Robert Vink, Mounir N. Ghabriel *

Adelaide Centre for Neuroscience Research, School of Medical Sciences, The University of Adelaide, Adelaide, South Australia 5005, Australia

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ABSTRACT

Dexamethasone, the standard treatment for peritumoral brain oedema, inhibits classical inflammation. Neurogenic inflammation, which acts via substance P (SP), has been implicated in vasogenic oedema in animal models of CNS injury. SP is elevated within and outside CNS tumours. This study investigated the efficacy of NK1 receptor antagonists, which block SP, compared with dexamethasone treatment, in a rat model of tumorigenesis. Dexamethasone reverted normal brain water content and reduced Evans blue and albumin extravasation, while NK1 antagonists did not ameliorate oedema formation. We conclude that classical inflammation rather than neurogenic inflammation drives peritumoral oedema in this brain tumour model.
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1. Introduction

Cancer results in the death of more than 6 million people each year (WHO, 2002). At autopsy 20% of cancer patients will be found to have suffered from a metastatic brain tumour, while clinical studies commonly describe an incidence of approximately 10% (Posner and Chernik, 1978; Cifuentes and Pickren, 1979; Gavrilovic and Posner, 2005). It is estimated that 170,000 people per year in America suffer from at least one metastatic brain tumour, with metastatic brain tumours being 10 times more common than primary cerebral malignancies (Landis et al., 1998).

Cerebral oedema is a common complication of metastatic brain tumours, which results in an increase in the water content within the skull and subsequent increase in intracranial pressure. This may lead to localised ischaemia, brain herniation, seizures, blindness, cognitive deficit, weakness, headaches, aphasia and ultimately death if blood perfusion of the brain is critically compromised (Mukand et al., 2001; Ayata and Ropper, 2002; Marchi et al., 2007; Shinoura et al., 2010).

Angiogenic blood vessels that grow within metastatic brain tumours have the same characteristics of their tissue of origin (Cornford et al., 1992; Shuto et al., 2008). Thus, these blood vessels exhibit different properties from those of the blood–brain barrier (BBB). It is widely accepted that the angiogenic vessels, which form the blood–tumour barrier (BTB) are more permeable to serum proteins, facilitating the development of peritumoral oedema (Front et al., 1984; Zhang and Olsson, 1997). As such, peritumoral oedema is related to the size of the neoplasm and the extent of angiogenesis (Zhang et al., 1992). Both the BBB and BTB are dynamic structures with barrier properties that may vary in response to external stimuli, including vasoactive substances (Zhang and Olsson, 1997; Turner and Vink, 2007). Consequently, vasoactive substances that are able to decrease the permeability of capillaries within the brain could result in reduced peritumoral brain oedema.

Dexamethasone is the current standard treatment for brain tumour-associated cerebral oedema. Since the introduction of dexamethasone in 1962, there has been a significant decrease in deaths related to this pathology (Jelsma and Bucy, 1967). Approximately 70% of metastatic brain tumour patients receive dexamethasone treatment while they undergo radiotherapy (Hempen et al., 2002). The effects of dexamethasone on oedema have been extensively studied, but contradictory results have prevented the exact mechanism of action being fully elucidated. It is thought that dexamethasone blocks classical inflammation, acting via glucocorticoid receptors to decrease the permeability of brain and tumoral microvessels, thus leading to a reduction in cerebral oedema (Andersen et al., 1994; Heiss et al., 1996; Andersen and Jensen, 1998; Sinha et al., 2004). Unfortunately, dexamethasone treatment is associated with many harmful side-effects including immune suppression (Lesniak et al., 2004), hyperglycemia (McGirt et al., 2008), psychosis (Alpert and Seigerman, 1986) and avascular necrosis (McCluskey and Gutteridge, 1982; Fast et al., 1984).

Neurogenic inflammation is characterised by vasoactive neuropeptide release causing vasodilatation, plasma extravasation and

* Corresponding author. Tel.: +61 8 8313 5481; fax: +61 8 8313 4398.
E-mail address: mounir.ghabriel@adelaide.edu.au (M.N. Ghabriel).

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subsequent oedema formation (Woie et al., 1993). Substance P (SP) is a potent neurogenic inflammatory mediator, causing increased BBB permeability when it acts on NK1 receptors expressed throughout the central nervous system (Cioni et al., 1998; Paemeleire et al., 1999; Annunziata et al., 2002; Lu et al., 2008). The modulatory effect of SP on the BBB is involved in the pathogenesis of peri-lesion cerebral oedema in ischaemic stroke and traumatic brain injury. Treatment with NK1 receptor antagonists in these conditions results in reduction of water content and improved functional outcome (Nimmo et al., 2004; Vink et al., 2004; Turner et al., 2006; Turner and Vink, 2007; Donkin et al., 2009; Harford-Wright et al., 2010).

The neurally induced release of SP and its effect on the BBB and oedema formation in other brain pathologies makes it a likely candidate as a mediator of peritumoral oedema formation in metastatic brain tumours, especially given recent reports of increased SP in and around CNS tumours (Palma et al., 2000). The aim of the current study was to use an intracerebral inoculation model to produce secondary neoplasms of consistent size and location, and to elucidate the role of neurogenic inflammation in the pathogenesis and development of cerebral oedema associated with these secondary brain tumours.

2. Methods

2.1. Animals

Animal procedures were performed in accordance with the National Health and Medical Research Council (NHMRC) guidelines and were approved by the University of Adelaide and the IMVS animal ethics committees. Male albino Wistar rats weighing 250–350 g, were group housed and allowed free access to food and water. Six animals for each treatment group were randomly selected for the immunostaining study, brain water content study and Evans blue extravasation study.

2.2. Cell culture

Walker 256 rat breast carcinoma cells were obtained from the Cell Resource Centre for Medical Research at Tohoku University, Japan. Culture was performed in complete culture medium consisting of Sigma RPMI-1640 containing 10% sterile foetal bovine serum and 1% penicillin and streptomycin (Sigma, 10,000 units penicillin and 10 mg of streptomycin/mL). Cells growing in 150 cm^2 culture flasks were passaged once with >90% confluence being reached using 3.5 mL of 0.02% EDTA. The cells were spun down in a centrifuge (5 min at 1500 RPM) and resuspended in serum free medium. The cells were counted using a hemocytometer and then diluted, so that between 10^5 and 10^6 cells/8 μL of serum free RPMI-1640 medium were used for direct injection into the brain.

2.3. Tumour inoculation

For tumour cell inoculation, the animal was anaesthetised using isoflurane inhalation anaesthesia at 3% via a nose cone. The animal was placed in a stereotaxic frame, local anaesthetic injected subcutaneously in the scalp and a midline scalp incision made to expose the skull. A 0.7 mm burr hole was performed at stereotaxic coordinates, 0.5 mm anterior and 3 mm lateral to the bregma, on the right half of the skull. A 30-gauge needle was inserted and lowered 5 mm ventral to bregma using a micrometre device. Between 10^5 and 10^6 walker 256 carcinoma cells in 8 μL of sterile culture medium were injected into the striatum over 10 min. Control animals had only culture medium injected. The needle remained in place for 5 min, then withdrawn. The hole was sealed with bone wax and the wound sutured.

2.4. Treatment

All compounds were dissolved in 0.9% saline solution, which was also used as vehicle control. On days 4–6 following tumour inoculation, groups of animals were given a daily intraperitoneal injection with one of the following agents: the NK1 antagonist fosaprepitant dimeglumine (Emend^R, MERCK & CO) 3 mg/kg/day, the NK1 antagonist n-acetyl L-tryptophan (NAT) 7.5 mg/kg/day, DBL dexamethasone sodium phosphate 8 mg/kg/day, or saline vehicle (as controls). All animals were sacrificed on day 7 following tumour inoculation. The dose of 8 mg/kg/day over three days for dexamethasone was used because this had previously been effective in ameliorating cerebral oedema in a rat model of primary brain tumour (Gu et al., 2007). The concentration of NAT was determined from a previous study where a dose response was performed and 2.5 mg/kg of intravenously administered NAT caused maximal resolution of BBB permeability following traumatic brain injury (Donkin et al., 2009). This dose was tripled to allow for intraperitoneal administration, as used for dexamethasone treatment. Emend was given at three times the dose recommended clinically for IV administration that has also been used previously with central effects in animal models (Watanabe et al., 2008).

2.5. Immunostaining

Animals were transcardially perfused with 10% formalin under terminal anaesthesia induced by intraperitoneal injection of Pentobarbitone sodium (60 mg/kg). Brains were embedded in paraffin wax and 5 μm sections were cut. Slides from each treatment group were stained for substance P (Santa Cruz Biotechnology, 1:2000) and albumin (ICN Pharmaceuticals, 1:20,000). Immunohistochemistry was performed using the standard streptavidin peroxidase procedure used routinely in our laboratory with 3,3′-diaminobenzidine (DAB) for visualisation and haematoxylin counterstaining. Immunostained slides were scanned using the nanozoomer (Hammamatsu, Hammamatsu City, Japan) and objective assessment of the immunocytochemical staining was achieved through colour deconvolution techniques, to reveal the % of DAB in the scanned slides. The colour deconvolution technique has been described previously (Harford-Wright et al., 2010; Helps et al., 2012). Whole coronal sections for albumin-stained brain sections, and peritumoral areas (0.0678 mm^2) from SP immunostained sections were taken and run through the colour deconvolution software to automatically estimate the % of brown stain in the selected area.

2.6. Brain water content

The wet weight–dry weight method was used to calculate brain water content in order to quantify the effect of treatment on peritumoral oedema. Animals were anaesthetised and rapidly decapitated. The brain was quickly removed from the skull and the cerebellum and olfactory bulbs discarded. The cerebrum was placed in a quick seal jar that had been previously weighed using a fine balance, and then weighed again containing the wet brain. The lid of the jar was then removed and the jar was placed in a 100 °C oven for 24 h. The dry brain was then weighed using a fine balance. The % of brain water was calculated using the following equation:

$$((\text{wet weight} - \text{dry weight})/\text{wet weight})\text{x}100$$

2.7. Evans blue extravasation

Animals were injected intravenously with 0.8 mL of 4% Evans blue (MW 69,000; Sigma, E-2129) 30 min before they were perfused transcardially with saline under general anaesthesia induced by Pentobarbitone sodium (60 mg/kg). The brain was quickly removed from the skull and dissected to remove the cerebellum and olfactory bulbs.

The cerebrum was placed into vials, weighed and then homogenised in 7.5 mL of phosphate buffered saline. After adding 2.5 mL of trichloroacetic acid (Sigma, T-0699) the samples were vortexed for 2 min, stored overnight at 4 °C and then centrifuged at 1000 *g* over 30 min. A UV/Vis spectrophotometer was used to measure the Evans blue absorbance in the supernatant at 610 nm. The quantity of Evans blue, expressed as μg/g of brain tissue was calculated using an Evans blue standard curve that had been previously determined.

2.8. Statistical analysis

Data were expressed as mean ± SEM. To determine statistical significance, an unpaired *t* test (for two groups) or one-way analysis of variance followed by a Bonferroni post test (for more than two groups) was performed as applicable, with $p<0.05$ designated as significant.

3. Results

Tumour inoculation produced large consistent tumours by day 7. All animals were sacrificed at this time point with 100% of animals showing evidence of tumour burden upon histological analysis. Walker 256 implantation models of secondary brain tumours have been described previously in the literature (Yamada et al., 1983; Jamshidi et al., 1992; Morreale et al., 1993). However tumours in the current study grew quicker than those reported previously. No adverse effects for any treatment were seen, and tumour inoculated animals showed maximum weight loss of 19.1% of their body weight. There was no animal mortality associated with this model, 100% of animals survived until the designated euthanasia date. Treatment over 3 days was pre-determined based on the previous use of dexamethasone to treat brain tumour associated oedema in the literature (Gu et al., 2007). Days 4, 5 and 6 following tumour inoculation were chosen for treatment so that tumours were well established prior to its commencement.

3.1. SP immunoreactivity

Tumour inoculation caused a significant increase in SP immunoreactivity as evident in the peritumoral area, when compared to the same location of control brains injected with culture medium ($^{**}p<0.01$) (Fig. 1A–C). Within the tumour mass SP immunoreactivity was significantly lower than both the peritumoral area of the same animals ($^{***}p<0.001$) and the striatum of control animals ($^{*}p<0.05$) (Fig. 1A–C). This increase in SP immunoreactivity in the peritumoral area was not altered by any of the treatment regimes used in this study (data not shown).

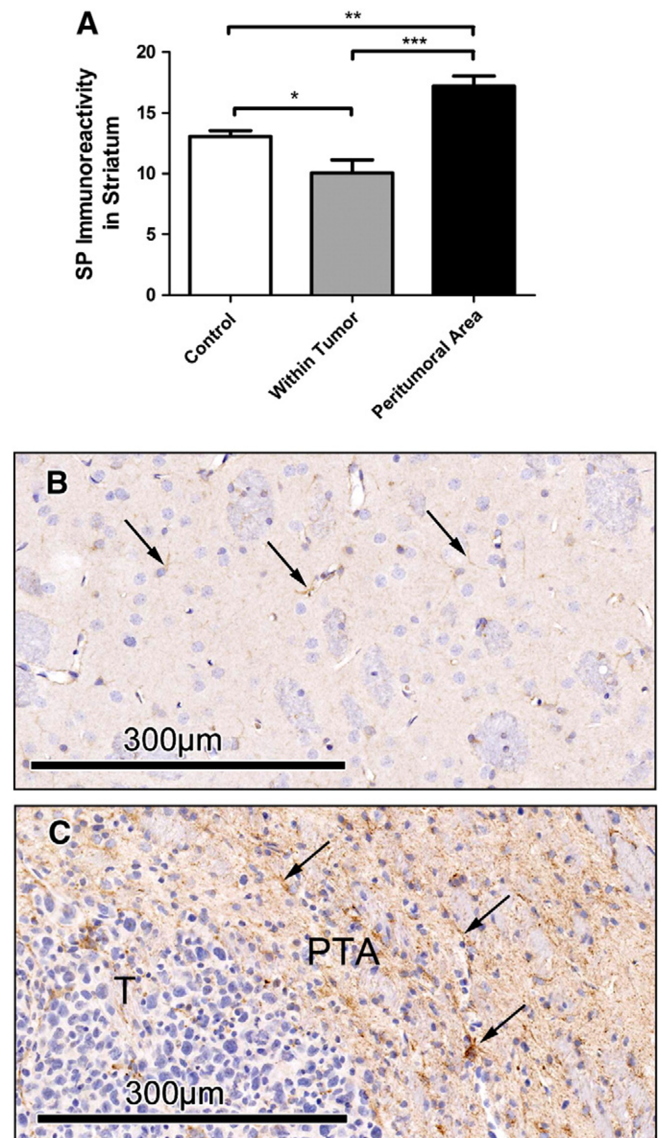

Fig. 1. (A) Graph showing substance P (SP) immunoreactivity within the tumour mass and in the peritumoral area in walker 256 inoculated animals compared with the striatum of control animals; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$. The values were obtained using a colour deconvolution software that measures the % of brown stain representing DAB and presented as the mean +/− SEM. (B) Brain from a control animal injected with culture medium showing faint SP immunoreactivity (arrows) appearing as fine network of brown stain in the striatum. (C) Peritumoral area (PTA) stained for SP showing increased immunoreactivity (arrows) surrounding the tumour mass (T).

3.2. Brain water content

Tumour inoculation caused a significant increase in brain water content when compared to injection of culture medium alone ($p<0.05$, Fig. 2). This was not resolved following treatment with Emend or NAT, but dexamethasone treatment reduced brain water content to the level of the culture medium control group (Fig. 2).

3.3. Blood–brain barrier permeability

Evans blue was used as an indicator of BBB permeability along with albumin immunoreactivity. Albumin is located within the blood vessels and not in the neuropil of the brain under normal conditions. However, pathological conditions that increase the permeability of the BBB allow albumin to leak out of the cerebral vasculature and into the brain tissue. Evans blue binds to serum albumin and is thus used as an exogenous indicator of BBB permeability at the time of tracer application. Albumin immunoreactivity was used as an endogenous indicator of BBB permeability over a longer period of time. Evans blue extravasation was increased in all tumour-injected and treated groups when compared to the culture medium control group (Fig. 3). However, there was a small non-significant decrease in Evans blue extravasation in the dexamethasone treated group compared to other treatments (Fig. 3). Albumin immunoreactivity was significantly elevated in all tumour-inoculated groups injected with the vehicle ($p<0.001$), Emend ($p<0.001$), NAT ($p<0.001$) and dexamethasone ($p<0.05$), when compared with the control animals injected with culture medium alone (Fig. 4A–F). Similar to Evans blue, there was a slight decrease in albumin immunoreactivity in the dexamethasone treated brains compared to other treatments (Fig. 4A). Fig. 4E and D shows more extensive peritumoral albumin immunoreactivity in the NAT treated brain when compared to the Emend treated brain, likely due to the variation in tumour size.

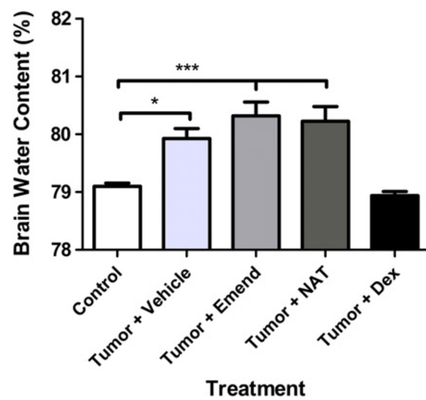

Fig. 2. Graph showing brain water content as a percentage (%) of brain weight in tumour-inoculates and control animals. NAT = n-acetyl L-tryptophan; Dex = dexamethasone. The values represent the mean +/− SEM; $^{*}p<0.05$; $^{***}p<0.001$.

However, there was no significant difference among these groups using colour deconvolution of albumin immunoreactivity.

4. Discussion

This study showed that inoculated tumour cells produced large, consistent tumour masses that increased brain water content and barrier permeability. For this reason, the model used in this study was suited to the investigation of potential therapeutic benefits of NK1 antagonists on peritumoral cerebral oedema. Direct injection models of secondary brain tumours have been frequently used previously to test treatments for tumour associated cerebral oedema (Yamada et al., 1983; Jamshidi et al., 1992; Engelhorn et al., 2009). The requirement of the model was that enough oedema be produced by the tumour growth, such that a therapeutic intervention could be effective and that the results be measurable. This is evident in the current study by the significant decrease in brain water content seen in the dexamethasone treated group when compared to the vehicle treated group. Direct injection of tumour cells into the brain bypasses the usual route of tumour cell invasion through the BBB in human metastases. Models of internal carotid artery or intra-cardiac injection of tumour cells to produce metastatic brain tumours allow for the study of extravasation through the BBB, although these models

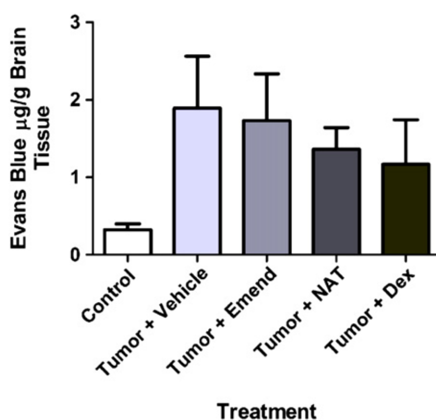

Fig. 3. Graph showing Evans blue concentration in brain tissue (μg/g) of controls injected with culture medium only and tumour-inoculated animals injected with saline vehicle, Emend, n-acetyl L-tryptophan (NAT) and dexamethasone (Dex). The values represent the mean +/− SEM.

often produce multiple secondary brain tumours without predictable location (Ushio et al., 1977; Hasegawa et al., 1983; Song et al., 2011; Budde et al., 2012).

BBB permeability was measured by Evans blue extravasation and albumin immunoreactivity in the brain parenchyma. By seven days following tumour inoculation, brain water content was 0.8% above the control level of 79.1%. While significant, this elevation in brain water content is less than that seen in some other CNS pathologies reporting vasogenic oedema. For example, water content increases of 3.6%, 3.2% and 2.2% have been reported for rat models of ischaemic reperfusion stroke, intracerebral haemorrhage and traumatic brain injury respectively (Nimmo et al., 2004; Turner et al., 2006; Li et al., 2009). However, the brain water content increase seen in the current study is comparable to the 0.87% elevation observed in a rat model of subarachnoid haemorrhage (Barry et al., 2011). The substantially larger percentage change in brain water content for models of trauma and reperfusion ischaemic stroke may be indicative of a different pathogenesis associated with oedema formation compared to that seen in the current study and in models of subarachnoid haemorrhage. Thus, the extent of increase in brain water content may be linked to the degree to which neurogenic inflammation contributes to breakdown of the BBB in different models of neurological diseases.

NK1 receptor antagonist treatment did not change the brain water content, albumin immunoreactivity or Evans blue extravasation when compared to vehicle treated controls. These results were seen despite the increase in SP immunoreactivity evident in the peritumoral area. Therefore, it is possible that the increase in SP expression was not of sufficient magnitude to be the primary mediator of cerebral oedema formation in this model. These results contrast with other studies using NK1 receptor antagonists to block neurogenic inflammation and treat vasogenic oedema. NAT has previously been used to decrease brain oedema and improve functional motor outcome after experimental traumatic brain injury and ischaemic reperfusion stroke (Donkin et al., 2009, 2011; Turner et al., 2011). In contrast, NK1 antagonists have not been shown to be effective in reducing vasogenic oedema associated with subarachnoid haemorrhage (Barry et al., 2011). This suggests that the mechanisms of peritumoral vasogenic oedema formation in the current study and that observed in subarachnoid haemorrhage are likely to be non-neurogenic, and distinctly different from the vasogenic oedema following stroke and traumatic brain injury, in both of which NK1 receptor antagonists appear to be more effective as treatment.

Both classical inflammation and neurogenic inflammation involve increased permeability of the BBB, thus both have the potential to mediate cerebral oedema in many neurological pathologies. Neurogenic inflammation typically involves the release of SP and calcitonin gene related peptide from primary sensory nerve endings resulting in vasodilatation and plasma extravasation (Nimmo and Vink, 2009). Thus in this study, the failure of NK1 receptor antagonist treatment to ameliorate peritumoral oedema indicates that its formation is not mediated by SP-driven neurogenic inflammation.

Dexamethasone is a classical anti-inflammatory agent, having previously been shown to decrease bradykinin and prostaglandin E2 production by white blood cells in cattle (Myers et al., 2010). In the CNS, classical inflammation is characterised by accumulation and proliferation of microglia along with perivascular macrophages (Graeber et al., 2011). This leads to blood vessel alterations driven by classical inflammatory mediators like bradykinin (Donkin and Vink, 2010). The well-documented effects of dexamethasone in treating peritumoral oedema, also seen in the current study, suggest that classical inflammation is the mechanism behind peritumoral oedema formation.

In the current study, treatment with dexamethasone was used as a positive control to determine if the model of brain metastases used here produces enough peritumoral oedema for a treatment intervention to have an effect. Treatment of animals with dexamethasone has

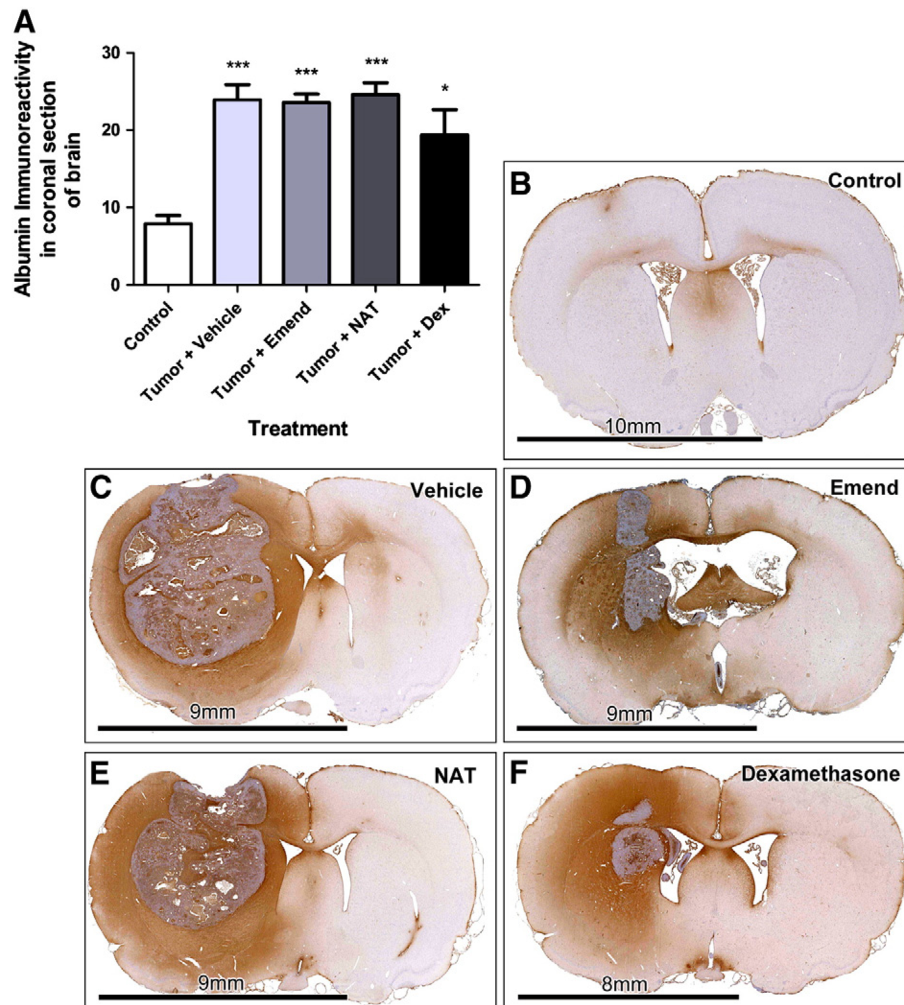

Fig. 4. (A) Graph showing comparison of albumin immunoreactivity, in controls injected with culture medium only, and in tumour-inoculated animals injected with vehicle, Emend, n-acetyl L-tryptophan (NAT), dexamethasone (Dex). The values were obtained using a colour deconvolution software that measures the % of brown stain representing DAB and represented as the mean +/− SEM; $^{*}p<0.05$; $^{***}p<0.001$. (B) Coronal brain section of culture medium control animal stained for albumin showing minimal albumin immunoreactivity in brain parenchyma appearing as a brown reaction product. (C) Albumin immunostaining in the brain of a vehicle-treated animal showing a large tumour mass surrounded by extensive albumin immunoreactivity in the right hemisphere. (D) Brain coronal section from Emend-treated animal stained for albumin showing peri-tumoral immunoreactivity predominantly within the right hemisphere. (E) Albumin immunostaining in a brain form NAT-treated animal showing extensive albumin immunoreactivity throughout the right hemisphere. (F) Coronal brain section from a dexamethasone-treated animal stained for albumin showing a small tumour with widespread immunoreactivity in the peritumoral area.

previously been used effectively in models of vasogenic oedema to reduce brain water content, blood–brain barrier permeability and other measures of cerebral oedema (Betz and Coester, 1990; Guerin et al., 1992). The outcomes associated with dexamethasone treatment are thought to be through its actions on glucocorticoid receptors, with modulation of VEGF (Heiss et al., 1996; Kim et al., 2008), and occludin is proposed to play a role in its activity (Forster et al., 2006; Gu et al., 2009). Despite the undefined mechanism of action, dexamethasone has been shown to decrease transendothelial fluid movement and extravascular fluid volume (Nakagawa et al., 1987; Andersen and Jensen, 1998). However, the benefits of improved fluid homeostasis were not sufficient to improve survival when animals bearing U87 or C6 intracranial gliomas were treated with dexamethasone (Moroz et al., 2011).

The decrease in brain water content in the dexamethasone-treated group was comparable to that seen in the control group injected with culture medium only. In contrast, the decrease in Evans blue and albumin immunoreactivity with dexamethasone treatment was non-significant and was elevated above that seen in the control groups. These data suggest that the mechanisms of extravasation of water and protein components of oedema are different, and that the mechanism of dexamethasone-induced resolution of peritumoral oedema is only partially mediated by decreasing the permeability of the BBB. Therefore, further investigation is needed to elucidate the specific mechanistic effects of dexamethasone on peritumoral oedema.

In conclusion, the results of this study demonstrate that dexamethasone is more effective in treating peritumoral oedema than the NK1 receptor antagonists in this model of brain secondaries. This suggests that in this model the pathogenesis of peritumoral oedema may be mediated by classical inflammation, rather than neurogenic inflammation driven by substance P.

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