The role of substance P in the progression and complications of secondary brain tumours

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Declaration

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Publications

The following articles have been published or accepted for publication during the period of my PhD candidature, and sections of these articles are included in the present thesis.

Lewis KM, Harford-Wright E, Vink R, Ghabriel MN. (2012) Targeting classical but not neurogenic inflammation reduces peritumoral oedema in secondary brain tumours. *Journal of Neuroimmunology*; 250: 59-65.

Lewis KM, Harford-Wright E, Vink R, Nimmo AJ, Ghabriel MN. (2012) Walker 256 tumour cells increase Substance P immunoreactivity locally and modify the properties of the blood-brain barrier during extravasation and brain invasion. *Clinical and Experimental Metastases.* Paper accepted on 8th May 2012, DOI: 10.1007/s10585-012-9487-z

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Abbreviations

ºC	Degrees Celsius
µL	Micro Litres
µm	Micro Metres
AQP-4	Aquaporin-4
AQP-1	Aquaporin-1
BBB	Blood-Brain Barrier
CCA	Common Carotid Artery
CNS	Central Nervous System
CPP	Cerebral Perfusion Pressure
CSF	Cerebrospinal Fluid
d	Days
DAB	3,3′-diaminobenzidine
Dex	Dexamethasone
ECA	External Carotid Artery
GFAP	Glial Fibrillary Acidic Protein
H & E	Haematoxylin and Eosin
h	Hours
IBA1	Ionized Calcium Binding Adaptor Molecule 1
ICA	Internal Carotid Artery
ICP	Intra-cranial Pressure

IFN-γ	Interferon Gamma
IL-6	Interleukin-6
IL-11	Interleukin-11
iNOS	Inducible Nitric Oxide Synthase
IU	International Units
MAP	Mean Arterial Pressure
mg	Milligrams
mL	Millilitres
mm	Millimetres
MRI	Magnetic Resonance Imaging
n	Number
NAT	n-acetyl $_{L}$-tryptophan
NHS	Normal Horse Serum
OA	Ophthalmic Artery
PPT-A	Pre Protachykinin-A
RPM	Revolutions per Minute
SEM	Standard Error of the Mean
SP	Substance P
STA	Superior Thyroid Artery
TJ	Tight Junction
TNF-α	Tumour Necrosis Factor Alpha

VEGF	Vascular Endothelial Growth Factor
wk	Weeks

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Abstract

Secondary brain tumours occur when cancer cells enter the circulation from their primary site and colonise the brain, previously shown to occur across the blood-brain barrier (BBB). Substance P (SP), a neurogenic inflammatory mediator, acting predominantly through NK1 receptors plays a role in opening the BBB and in the formation of oedema following stroke and brain trauma. It is hypothesised that SP may also promote the extravasation of tumour cells through the BBB, formation of peritumoral oedema and progression of secondary brain tumours.

Walker 256 rat breast carcinoma cells obtained from the Centre for Medical Research, Tohoku University had superior tumorigenic properties compared to cells from the American Type Culture Collection, and were therefore subsequently used in two albino Wistar rat models of tumorigenesis.

Firstly, internal carotid artery tumour cell injection was used to establish the effect of tumour cell extravasation across the BBB on brain albumin, endothelial barrier antigen (EBA) and SP immunoreactivity. I then determined if NK1 receptor antagonists could prevent tumour cell extravasation, by evaluating tumour incidence and volume.

Secondly, a stereotaxic direct inoculation model was used to investigate the effect of NK1 receptor antagonists on brain tumour growth and peritumoral oedema, compared with dexamethasone treatment. Evan's blue extravasation and albumin immunoreactivity were used to assess BBB permeability, and brain water content to evaluate cerebral oedema. Tumour volume, Ki67 immunoreactivity, caspase-3 immunoreactivity and tumour cell density were used as measures of tumour growth. Furthermore, cell viability and cell death assays determined if NK1 antagonists or dexamethasone treatment cause alterations in tumour cell growth in vitro.

In the carotid model, SP and albumin immunoreactivity increased in the brain during the extravasation of tumour cells, and in the peritumoral area of established tumours. The invaded blood vessels lacked EBA immunoreactivity, indicating loss of BBB properties. However, NK1 antagonists administered in the first three days following tumour cell injection failed to reduce tumour incidence or volume, suggesting that

extravasation may be a multifactorial process, and that NK1 receptor antagonism alone is not sufficient to prevent tumour extravasation and growth.

In the direct inoculation model, NK1 receptor antagonists did not reduce peritumoral oedema or decrease tumour growth when used to treat established brain metastases. In contrast, dexamethasone, the standard treatment for peritumoral oedema, caused a reduction in brain water content and decreased tumour volume, but not tumour growth. The decrease in tumour volume with dexamethasone reflects reduced fluid content, as there was increased tumour cell density with no change in immunoreactivity to Ki67 (marker for proliferation) or caspase-3 (marker for apoptosis). Furthermore, in vitro studies showed no effect for dexamethasone on tumour cell viability. These results suggest that peritumoral oedema is driven by classical inflammation rather than neurogenic inflammation in the direct inoculation model.

In conclusion, in these models of secondary brain tumours, SP does not appear to play a role sufficient to promote NK1 receptor antagonism as an appropriate preventative treatment for brain metastasis, as an anticancer agent, or as an alternative to dexamethasone for the management of peritumoral oedema.

1 Introduction

Metastatic brain tumours are an end stage of disease for patients suffering from breast cancer, whose incidence is steadily increasing. In part, this is due to longer survival times on account of improved methods of treatment for the causative primary tumours. However, metastatic brain tumours are particularly difficult to treat, reflecting the need for more research into the mechanisms of cancer invasion of the brain and the complications associated with tumour growth. A common complication of brain metastases is peritumoral oedema, which occurs because of increased permeability of the blood vessels that grow within the metastatic brain tumours compared to the normal blood-brain barrier (BBB) vessels. Previous studies have shown that to develop metastatic brain tumours, cancer cells arrest within the cerebral vasculature and then form cytoplasmic protrusions which penetrate the BBB during the extravasation process (Lorger 2010). The mechanisms that allow tumour cells to penetrate the BBB and metastasise to the brain are unclear. Substance P (SP) is a mediator of neurogenic inflammation and is known to increase the permeability of the BBB. It is therefore proposed that SP is secreted by tumour cells, or released by BBB endothelial cells and/or perivascular nerve terminals of primary sensory neurons through an interaction with the tumour cells. This would result in an increase in the permeability of the BBB, which may allow the tumour cells to extravasate into the brain parenchyma. Furthermore, SP release has been implicated in cerebral oedema associated with stroke and traumatic brain injury and thus may also be responsible, in part, for the formation of oedema surrounding metastatic brain tumours.

1.1 Epidemiology of brain metastases

1.1.1 Incidence

Incidence data for secondary brain tumours varies greatly within the literature. Approximately 20% of cancer patients have a secondary brain tumour found at autopsy, whereas clinical studies generally report an incidence of about 10% (Posner 1978; Cifuentes 1979; Soffietti 2002; Gavrilovic 2005; Lin 2008a). Studies of autopsy data generally report a much higher incidence because asymptomatic tumours are also included in the data. The incidence of metastatic brain tumours has increased in recent

times (Dobec-Meic 2006; Pelletier 2008), presumably because of improved treatment for the primary cancer leading to increased survival time of cancer patients, thus leaving more time for the metastatic process to take place. Furthermore, superior diagnostic techniques may have resulted in better diagnosis of secondary brain tumours.

1.1.2 Organ of origin

In many cases of brain metastases, the organ of primary tumour origin cannot be identified (Potts 1980; Clark 1989; Becher 2006). Where it has been possible, the most common primary sites responsible for metastatic brain tumours are lung, breast, renal, colorectal and skin (melanoma) (Schouten 2002; Barnholtz-Sloan 2004; Villa 2011). These cancer types are responsible for many cancer deaths and have short predicted survival times comparable to that of glioblastoma multiforme, commonly regarded as an incurable cancer type (Tran 2010). Approximately 25% of people with lung cancer will develop a metastatic brain tumour detectable at autopsy, making lung cancer patients responsible for 30-60% of all metastatic brain tumours (O'Neill 1994; Sen 1998; Tabaka 2006). Between 22-30% of patients diagnosed with breast cancer will also be diagnosed with a metastatic brain tumour (Schuette 2004; Hines 2008). Melanoma is responsible for about 1% of the total cancer burden, but 75% of these cases will result in a metastatic brain tumour (Salgado 2007).

The vast majority of breast cancer brain metastases occur in the late stages of the disease (Lorger 2010; Kim 2012) and despite improved treatments for breast cancer, the survival time for patients with brain metastatic disease remains in the order of months (Sperduto 2012). Nearly 50% of patients with metastatic triple negative breast cancer will develop a metastatic brain tumour, with a median survival time of 4.9 months from diagnosis (Lin 2008b). There is a predilection for metastatic brain tumours and CNS recurrence amongst node positive, estrogen receptor-negative, young patients with high grade breast cancer, with no evidence that there is any benefit from an early diagnosis and treatment for their intracranial malignancy (Pestalozzi 2006). Furthermore, Her-2 positivity has been linked with poor prognosis

in a cohort of breast cancer patients from 1996-2010 (Berghoff 2012a). Therefore there is pressing need for improved treatment and prevention strategies for brain metastatic breast cancer.

Cancer patients who develop cerebral secondary tumours have a much poorer prognosis (Tsimberidou 2011). Brain metastases are a major cause of cancer morbidity and mortality and are often the first site of relapse in patients with systemic tumours (Atahan 2008). Brain metastatic disease progresses quickly, with one study reporting that 41% of patients undergoing gamma knife surgery for secondary brain tumours had additional lesions identified on the day of surgery (Patel 2012). Approximately 80% of patients with secondary brain tumours will have multiple intracranial lesions (Miabi 2011), substantially reducing survival time (Elaimy 2011).

1.2 Location of metastatic tumour

The most common location of metastatic brain tumour growth is in the frontal lobe, followed by the cerebellum, parietal lobe, temporal lobe, occipital lobe, deep brain nuclei and the brain stem (Potts 1980; Ghia 2007).

1.3 Clinical relevance

Metastatic brain tumours are an important area of research due to the large number of people affected and the lack of effective treatment strategies available. It is also pertinent that the mechanisms of tumour cell extravasation into the brain across the BBB be elucidated because this understanding may lead to the development of a novel prevention strategy.

Much of the current research into CNS malignancies is focused on primary tumours of the brain, despite the fact that brain metastases are ten times more common (Landis 1998). In many studies involving cancer research, CNS neoplasms are excluded,

because the BBB is a complicating factor and novel treatments are often not tested on brain metastases.

Previously, many cancer related deaths were as a result of either the primary neoplasm or systemic metastases. The isolated nature of the brain means that it is often not the preferred site of metastatic tumour formation for most tumour cell types and is generally the end stage of disease. However, with increasingly effective chemotherapeutic strategies, many patients are able to combat their primary malignancies and systemic metastases. This has unmasked a population of patients suffering and unfortunately dying from brain metastases due to inadequate treatment options. Thus, brain metastases are becoming an increasing burden on our health system and reducing the quality of life that can be expected for cancer sufferers.

1.4 Tumour associated morbidity

The presence of a metastatic tumour in the brain parenchyma can cause compression of structures within the brain and peritumoral oedema. This can result in neurological symptoms including motor weakness, aphasia, visual deficits and epilepsy (Shuto 2008). Peritumoral oedema will be considered in more detail later in this chapter after the structure of the BBB has been discussed.

1.4.1 Seizures

Often seizures are the initial symptom that leads to the diagnosis of the causative intracranial neoplasm (Shamji 2009). Furthermore, seizures often begin or return when a brain tumour is surgically excised (Beaumont 2000). The pathogenesis of brain metastases associated epilepsy is thought to be related to the tumour-associated alterations to the brain environment including oedema formation, insufficient blood supply, and release of inflammatory mediators and metabolically active substances (Shamji 2009). Changes in the pH of the peritumoral environment and loss of BBB integrity have both been linked to tumour associated epilepsy (Beaumont 2000).

When mannitol was used to compromise the function of the BBB in order to aid delivery of chemotherapy to primary CNS lymphoma, this procedure caused focal motor seizures in one quarter of the cases (Marchi 2007). In contrast, no seizures occurred when the chemotherapeutic agent was administered alone.

1.5 Current treatments for metastatic brain tumours

Functional impairment resulting from brain metastases is complex, as there are many factors that may contribute to morbidity. The tumour itself may cause neurological deficits, there may be systemic cancer symptoms as a result of the primary tumour and the prescribed treatments may impair the patient whilst combating the tumour.

The indications for different treatment options for metastatic brain tumours are complex. The most common first line treatment for metastatic brain tumours is surgery and chemotherapy, whilst whole brain radiotherapy and chemotherapy are more regularly used as second line treatment (Fabi 2011). Generally, the best results are seen when radiosurgery is combined with whole-brain radiotherapy (Rades 2012). Surgical intervention is most effective when there is only a single metastatic brain tumour, although the tumour location dictates wheather tumour excision is possible. Furthermore, more than 80% of surgically removed malignancies recur at the boundary of the excision (Veiseh 2007).

1.5.1 Chemotherapy

Treatment for the primary tumours that commonly spread to the brain has improved in recent times with the development of targeted chemotherapeutic agents. This may be the reason why the time from cancer diagnosis to brain metastases development has increased over time, alternatively primary tumours may be being diagnosed earlier (Nieder 2009). However there has not been any significant increase in survival time post-metastatic brain tumour diagnosis since 1983 (Nieder 2009), suggesting that the

improved treatment options for systemic cancer are not effective in treating the secondary brain tumours that result from these malignancies.

Chemotherapeutic agents have variable ability in crossing the BBB and reaching therapeutic concentrations within metastatic brain tumours (Lien 1991). Some lipophilic agents are able to cross the BBB in order to treat secondary brain tumours although often these agents are not best suited to target the type of tumour that has invaded the brain. Although brain metastases disrupt the BBB, increasing its permeability, this may not be sufficient to transfer targeted chemotherapeutic agents into the brain. Treatment of micrometastases in the brain with chemotherapy may be inadequate. Larger metastatic brain tumours are less protected by the BBB, as tumour angiogenesis disrupts the BBB in the tumour area (Zhang 1992). However, interstitial pressure within the tumour mass may preclude the entry of chemotherapeutic agents. In a human study, 66% of brain metastatic lung tumours showed significantly decreased uptake of a labelled chemotherapeutic agent when compared to the primary lung tumour (Front 1987).

Chemically induced increased BBB permeability, for improved delivery of chemotherapeutic agents is a popular area of investigation. The administration of hyperosmolar solutions cause dehydration of cerebral endothelial cells and cytoskeleton contraction resulting in cell shrinkage and increased TJ spacing between the cells; this can be used to increase the blood to tissue transfer in the brain (Hiesiger 1986; Kozler 2003). Furthermore, a temporary nitric oxide (NO) donor has also been used in a rat glioma model to compromise the BBB for more effective chemotherapy delivery (Weyerbrock 2003). However, the disruption of the BBB for chemotherapy delivery may cause increased toxicity in the healthy brain tissue. Therefore, agents that selectively increase the permeability of blood vessels within the tumour mass are currently under investigation. Bradykinin B2 receptor agonists are showing promise in this area, with hydrophilic agent uptake into rat implanted glioma increased after B2 agonist treatment (Cote 2010). The increased density of B2 receptors on tumour vasculature is thought to be the mechanism behind this drug selectivity (Wu 2002).

Tumoral blood flow may also influence the effectiveness of chemotherapeutic agents through delivery to the target tissue. In a study where experimental Walker 256 breast carcinoma metastatic brain tumour blood flow was measured, the range varied markedly and was correlated with tumour size and morphological features including necrosis and cyst formation (Blasberg 1984b).

1.5.2 Radiation therapy

Radiation therapy is a foundation treatment for both primary and secondary brain tumours. Indeed, patients who suffer from small cell lung carcinoma (SCLC) and are thought to be in complete remission, are often treated with cranial irradiation because of the high rate of metastatic brain tumour recurrence (Tarnawski 2011). However, acute cranial irradiation causes a breakdown of the BBB through the induction of proteolytic enzymes disrupting the basal lamina of cerebral blood vessels, such as matrix metalloproteinases (MMP) and plasminogen activators (Adair 1999). Similarly, in one study two of three patients suffering from brain metastatic lesions with breast cancer origins showed an increase in oedema in a scan following radiation therapy (Hyman 1978). This was in contrast to the overall 53% of patients with brain metastases that showed improvement following cranial irradiation (Hyman 1978).

1.5.3 Dexamethasone

Dexamethasone is currently used clinically to treat peritumoral oedema, with significant improvements to patient outcomes since it began being used in 1962 (Jelsma 1967). It is recommended that patients suffering from metastatic brain tumours, with symptoms related to the mass effect of the neoplasm, be treated with 4-16mg/day of dexamethasone depending on the severity of symptoms and not the size of the tumour (Ryken 2010). 75% of patients report neurological benefits within 48-72 hours after treatment initiation (Soffietti 2006). Approximately 70% of metastatic brain tumour patients receive dexamethasone treatment concomitantly with radiotherapy (Hempen 2002). Limitations of dexamethasone use include many harmful side-effects including insomnia, immune suppression (Lesniak 2004), hyperglycemia (McGirt 2008) and occasional psychosis (Alpert 1986).

In humans, several magnetic resonance imaging (MRI) studies have shown that dexamethasone reduced peritumoral oedema resulting from primary glial tumours (Armitage 2007) and metastatic brain tumours, but not meningiomas (Andersen 1994a; b). However a variable response to dexamethasone treatment has been reported between different brain metastases with the same organ of origin, meaning that tumours of the same type may have an inconsistent oedema response (Wolfson 1994).

The exact mechanism of dexamethasone action to reduce cerebral oedema remains controversial, although it is thought to be through suppression of classical inflammation and actions on glucocorticoid receptors, resulting in reduction of brain microvessel permeability (Andersen 1994a; Heiss 1996; Andersen 1998; Sinha 2004). Classical inflammation is characterised by accumulation and proliferation of microglia along with perivascular macrophages (Graeber 2011). Animal models of peritumoral oedema have been used in an attempt to elucidate the precise molecular actions of dexamethasone on BBB permeability. Models used are predominantly intracerebral implantation of rat C6 or 9L glioma cells, with dexamethasone treatment causing a decrease in Evans blue or radiolabelled serum albumin extravasation into the neuropil (Nakagawa 1987; Guerin 1992; Gu 2009a; Gu 2009b) and decreased MRI measures of oedema (Nakagawa 1987; Ewing 2008).

Dexamethasone induced decrease in oedema and BBB permeability was also associated with decreased vascular endothelial growth factor (VEGF) (Heiss 1996), elevated GLUT1 (Guerin 1992), increased occludin and increased calcium activated potassium channel expression (Gu 2009a) by cerebral vasculature. These studies indicate possible pathways by which dexamethasone counteracts tumour-initiated increased BBB permeability. Furthermore, actions of dexamethasone on cerebral vasculature include decreased plasma vascular volume and decreased total vascular density (Nakagawa 1987; Badruddoja 2003). However conflicting results exist for the density and volume of brain microvessels in response to dexamethasone treatment, although different methods of detection were used for these experiments (Wolff 1993;

Badruddoja 2003). These results suggest a possible angiogenic modulation effect of dexamethasone along with its established effects on BBB permeability and oedema.

Despite the positive effects of dexamethasone on peritumoral oedema, some debate exists about its use in conjunction with chemotherapeutic agents. In vitro studies have shown inhibition of TRAIL, taxol, doxorubicine, gemcitabinetemozolomide, cisplatin and 5-fluorouracil chemotherapy induced tumour cell apoptosis with dexamethasone treatment (Kim 2004; Sur 2005; Zhang 2006). These experiments were fairly consistent showing the protective effect of dexamethasone on chemotherapy induced bone (MG63, HOS, SAOS, HT1080), brain (HS683, H4, A172, U-373-MG, TE671, T98G), breast (BT-474, BT-20, Colo-824, MCF7, MDAMB-436), cervix (P5, CASKI, MRH-215, MRH-186, MRH-196), melanoma (HS695T, WM1341, WM98.1), lung (A549) and neuroblastoma (IMR32, KELLY, SKN-SH, SHEP, IMR5) cancer cell line apoptosis, although the opposite effect was seen in primary lymphoid cells (Kim 2004; Sur 2005; Zhang 2006). Dexamethasone treatment enhances chemotherapy induced T cell leukaemia cell apoptosis (Zhang 2006). However, interaction of dexamethasone with chemotherapeutic agents in vitro is a phenomenon that has not been confirmed in animal models of brain tumours.

Dexamethasone is well known to cause significant toxicity to human patients and to animals used in oedema studies, as evidenced by animal weight loss and necrosis at the site of administration (Villeneuve 2008; Moroz 2011). When administered alone, several studies have found that dexamethasone has an anti-proliferative, pro-apoptotic effect on cancer cells in vitro (Bavaresco 2007; Piette 2009; Tazik 2009). Furthermore, dexamethasone caused a decrease in tumour volume in numerous animal studies (Guerin 1992; Wolff 1993; Badruddoja 2003; Villeneuve 2008), although this is likelydue to reduced oedematous fluid within the tumour mass. Furthermore, dexamethasone treatment has not extended animal survival with cerebral tumour implantation (Moroz 2011).

1.6 Blood brain barrier

The BBB is the interface between the blood and the brain, separating the brain parenchyma from the blood within cerebral capillaries and involves the interactions between endothelial cells, astrocytes, pericytes and the capillary basement membrane. Often cells, but not solutes, are able to move through a supposedly non-permeable and undamaged BBB (Perry 1997). An example of this is when no leakage of Evan's blue was evident surrounding capillaries within a metastatic brain tumour, indicating an intact BBB, although macrophages were able to infiltrate the brain parenchyma (Schackert 1988b).

1.6.1 Cerebral capillary endothelial cells

Cerebral capillary endothelial cells are simple squamous cells of uniform thickness, with few pinocytotic vesicles (Schackert 1988b). These cells rest on the basal lamina and lack fenestrations (Freed 2002; Petty 2002). There is an increase in the number of mitochondria in the endothelial cells of the BBB when compared to the capillary endothelial cells found systemically, as there is increased demand for active transport across those in the brain. This is because cerebral capillary endothelial cells have tight junctions (TJ) between them, which prevent many substances from entering the brain parenchyma via the intercellular space. These blood vessels enter the brain parenchyma through the peri-vascular spaces that are lined by the pia mater. Thus there is also a pia-glial membrane present between the brain parenchyma and the endothelial cells of the BBB (Saito 2008).

1.6.2 Tight junctions

TJ are located on the most apical region of the cleft between cerebral capillary endothelial cells and form a seal to prevent substances from passing between them (Petty 2002). Claudins are involved in the primary makeup or backbone of TJs, forming dimers which interact with opposing claudin molecules and form the primary seal of the TJ (Petty 2002; Persidsky 2006). In the brain, claudin 5 is predominant (Petty 2002). Junctional adhesion molecule (JAM) is an immunoglobulin with a

single transmembrane segment, which mediates the initial cell to cell attachment and is able to mediate permeability through this avenue (Persidsky 2006). Occludin has four transmembrane segments and is present in higher concentractions in endothelial cells of the BBB than in those in systemic capillary endothelial cells. It induces high membrane resistance, which is indicative of low ion permeability (Joo 1996; Persidsky 2006). Occludin interacts with the cytoskeleton of BBB endothelial cells through zona occludens-1 (ZO1), ZO2 and ZO3 molecules, which form the sub-membranous plaque of the TJ (Petty 2002; Persidsky 2006).

1.6.3 Basement membrane

The basement membrane of the BBB provides another obstacle to prevent the entry of unwanted substances into the brain. It is made up of proteins found within the extracellular matrix including collagens, vitronectin, fibronectin, tenascin and proteoglycans (Baumann 2009). These components provide stability to the structure of the blood vessels and a surface upon which cerebral capillary endothelial cells can rest.

1.6.4 Astrocytes

Astrocyte end feet surround 99% of BBB endothelial cells and act to support and enhance the TJs between them (Persidsky 2006; Bundgaard 2008). Gap junctions between astrocytes allows for quick transfer of substances and information (Escartin 2008). Astrocytes also mediate the connection between neurones and endothelial cells (Kim 2006). Astrocytes become activated in response to pathological stimuli which results in the hypertrophy of the astrocytic processes and over-expression of intermediate filaments, namely glial fibrillary acidic protein (GFAP) (Escartin 2008).

1.6.5 Pericytes

Pericytes have a stellate appearance and cytoplasmic processes. They cover 22-32% of the capillary cell surfaces (Fisher 2009). Gap Junctions (GJ) between pericytes and

cerebral capillary endothelial cells allow communication to occur (Persidsky 2006). The main function of pericytes seems to be in blood flow regulation particularly in the pre-capillary arterioles that supply the brain with blood (Joo 1993). The structure of pericytes makes them ideal for this function, as they are contractile and express the smooth muscle actin isoform (Fisher 2009). Collagen type IV glycosaminoglycans and laminin are also synthesised in pericytes to be used in formation of the basement membrane (Fisher 2009). They have the ability to regulate endothelial cell proliferation, survival, migration and differentiation (Persidsky 2006).

1.7 Formation of brain metastases

In order for metastatic tumour cells to reach the brain through the blood stream, they must first have the ability to leave the site of the primary systemic tumour. By migrating through the basement membrane of vessels in the primary tumour, some cells are able to reach the systemic circulation. This process is called intravasation and will involve the tumour cells either passing between or through the endothelial cells of the blood vessels. The tumour cells must be able to resist programmed cell death, due to the loss of cellular contact with the primary tumour, in order to form viable metastases (Khanna 2005). Similarly, the host immune system must be evaded.

Mechanical forces associated with narrowing blood vessels allow tumour cell attachment to endothelial cells at a distant site (Marchetti 2003). Firstly, weak carbohydrate-carbohydrate locking reactions allow tumour cells to interact with endothelial cells of the microcirculation of the brain (Petty 2002). Following this, the bond between tumour cells and endothelial cells of the BBB are strengthened with the interaction of adhesion molecules (Petty 2002).

The metastatic pathway continues with extravasation, where the tumour cells must pass through the BBB (Marchetti 2003). This means that the tumour cells must pass through both the endothelial barrier and the basement membrane (Petty 2002). The mechanism for this is yet to be elucidated.

Many tumour cells that are able to break away from the primary tumour will not survive in the circulation to form metastatic tumours at distant sites. For example, in a study using radiolabeled B16 melanoma cells, less than 1% of the tumour cells survived the first 24 hours in the circulation and less than 0.1% of the cells went on to form metastatic tumours (Fidler 1970). In addition, when an in vivo MRI was performed on a nude mouse that had human breast cancer cells injected into its left ventricle, 81% of the tumour cells evident at day 0 were no longer detectable at day 3 (Heyn 2006).

Tumour cells have variable propensity for metastases to the brain, based on the properties of the tumour cell that aid its progression through the metastatic process. In a study where eight different melanoma tumour cell lines were injected both intracerebrally and into the carotid artery of nude mice, all eight cell lines were able to grow tumours in the brain as a result of their intracerebral inoculation (Schackert 1990). However, only seven of the cell lines resulted in any metastatic melanomas in the brain through intra-carotid delivery (Schackert 1990). The TXM-31-3 cell line that was derived from a lymph node metastatic melanoma in a human patient did not enter the brain after intra-carotid inoculation, whereas other cell lines derived from brain metastatic melanoma tumours had already acquired the characteristics needed to pass through the BBB into the brain parenchyma (Schackert 1990). It has been shown that cancer cells round up inside the cerebral capillaries before cytoplasmic processes extend through the BBB (Lorger 2010).

Specific qualities are required of tumour cells in order to metastasise to the brain. It has been established that melanoma cells derived from central nervous system metastases are more likely to form experimental metastases in the brain, than tumour cells derived from other areas of the body (Schackert 1988a; Fidler 1990; Kusters 2001). For example, tumour cell lines that were derived from human CNS metastatic melanoma tumours (cell lines TXM-13 and TXM-40) were more likely to form brain parenchyma lesions when injected into the carotid artery of nude mice, whereas

tumour cells that were derived from human melanoma lymph node metastases (cell lines A375-SM, DM-4, TXM-1 and TXM-31-4) and subcutaneous metastases (DX-3) preferentially formed meningeal or ventricle metastases when inoculated in the same way (Schackert 1990).

1.7.1 Immune system interactions

Microglia are the macrophage like cells of the CNS (He 2006). The population of microglia in the brain is maintained by local replication, although some infiltration of macrophages originating from outside the CNS is evident when the brain is injured (Perry 1997). When microglia are activated, usually by neuronal degeneration or decreased interactions with neuronal terminals, they become scavenger cells that repair neurons and promote regeneration through alerting the wider immune system through signalling cascades (Kreutzberg 1996). Surrounding metastatic brain tumours, microglia have been found to be enlarged with thicker processes, which are thought to be involved in the activation of astrocytes (Zhang 1995). The activated microglia also proliferate, forming a barrier like pattern surrounding brain metastatic lung cancer (He 2006). Some, but not all of these microglia showed expression of inducible nitric oxide synthase (iNOS) and Tumour necrosis factor alpha (TNF-α) (He 2006). Injection of SP into the brainstem induces microglial activation as indicated by an increase in the expression of class II MHC proteins (McCluskey 2001).

Leukocytes may migrate into the brain from the blood through the choroid plexus and into the CSF, as choroidal endothelium is fenestrated with no tight junctions between the cells (Ransohoff 2003). There are approximately 3,000 leukocytes present in 1ml of CSF from a normal healthy individual (Ransohoff 2003). Leukocytes may also migrate through the vasculature into the subarachnoid or Virchow-Robin spaces, although it is thought that during pathological events leukocytes travel directly through the BBB and into the perivascular spaces, although the mechanism for this is unclear (Ransohoff 2003). It is possible that inflammatory mediators increase the permeability of the BBB to allow leukocyte migration into the brain.

Metastatic brain tumours may also be infiltrated by macrophages that are of blood monocyte origin, even with an intact BBB (Schackert 1988b). This has been shown to occur even in the absence of a major T-lymphocyte response (Schackert 1988b). One study showed that all six metastatic brain tumours from various primary tumours were positive for macrophage infiltration using anti-Leu-M3 monoclonal antibody, in the brain tumour itself and in the surrounding oedematous tissue (Shinonaga 1988).

Activated T-lymphocytes have also been known to infiltrate the brain across an intact BBB in the absence of inflammation as part of immune surveillance (Perry 1997). Increased number of regulatory T cells (CD4+CD25+) in glioma infiltrates, are thought to play an important role in suppressing the immune response of CD8+ T-cell infiltration of tumour cells expressing self antigens in the CNS (El Andaloussi 2006; Grauer 2007). This was shown when animals with glioma cells implanted in their brains had increased survival times when regulatory T cells were depleted (El Andaloussi 2006).

Tumour xenografts implanted into the brains of non-immunologically compromised rodents show a delayed rejection when compared to those same tumours implanted into the skin. This may be because in contrast to the skin, the brain responds to noxious stimuli with microglial activation and then delayed monocyte infiltration and no neutrophil response (Ransohoff 2003). Biphasic tumour growth at the primary site for the 4T1 mammary tumour, which occurred at week three to four in animals with a normally functioning immune system, did not occur in immune compromised mice (Tao 2008). Therefore it is likely that the immune system was responsible for this tumour regression, which was associated with tumour necrosis and leukocyte infiltration (Tao 2008).

TNF-α and interferon gamma (IFN-γ) are cytokines that are released by immune cells and have the ability to destroy tumour cells directly (Lampson 2003). However, toxicity can become a problem if doses sufficient to kill tumour cells are administered. Lower doses are able to be used to initiate an indirect pathway of tumour cell death through the immune enhancing effects of these cytokines (Lampson 2003). Mice that were immunised with peptide pulsed dendritic cells and systemic application of interleukin 2 (IL-2) caused a significant reduction in the size of metastatic brain tumours and increased the survival time of the animals (Prins 2008).

The immune system plays an important role in fighting metastatic brain tumours, but the location of the tumour within the brain may dictate how effective this system of defence is. For example, when tumour cells were implanted directly into the cerebral ventricles, the survival time was increased when compared to the same volume of cells injected into the brain parenchyma (Thomas 2008). Similarly, when a larger volume of cells was infused into the same intra-parenchymal site, the cells leaked into the ventricles, and thus caused an immune reaction similar to that seen for the direct implantation of tumour cells into the ventricles (Thomas 2008). This also resulted in an increase in animal survival time when compared to the smaller intra-parenchymal inoculation (Thomas 2008). Activated T-cells will cross the BBB and accumulate within an intra-parenchymal tumour mass, although the environment in the brain parenchyma is more hostile to inflammatory cells than that in the ventricles (Thomas 2008).

1.8 Experimental models of brain metastases

1.8.1 Tumour injection into the internal carotid artery

Injection of tumour cells into the internal carotid artery accurately simulates haematogenous spread of metastatic tumour cells to the brain, allowing the extravasation process to be investigated. Injection of tumour cells into the internal carotid artery of the animal is very effective in producing brain metastases, as the brain contains the first capillary bed that the tumour cells encounter following their injection (Khanna 2005). In addition, when tumour cells are injected into the right

internal carotid artery, metastases preferentially form in the right hemisphere of the brain (Zhang 1992). The pattern of tumour growth as a result of carotid artery injection has been shown to include both angiocentric collars of neoplastic cells where the pia-glial membrane was intact, along with invasive proliferations of neoplastic cells with fragments of collagen fibers from the breakdown of the pia-glial membrane (Saito 2008). When tumour cells are injected into the internal carotid artery, only the last steps of the metastatic process are able to be examined (Fidler 1990). However, this model is suitable for investigation of tumour cell extravasation.

1.8.2 Syngeneic model

Syngeneic brain metastases models, where the animals have the same genetic background as the tumour cell line used, allow the normal interactions between the animal and the tumour cell of the metastatic pathway to take place (Khanna 2005). However, the cells used in these models may lack the complexity seen in human tumour cells, both genetically and in the formation of mutations. This is because tumour cells used in syngeneic models are usually derived from inbred mice or rats (Rangarajan 2004; Khanna 2005). An example of a syngeneic murine model of brain metastases is the Walker 256 carcinoma cells, which have previously been injected into the internal carotid artery of Wistar rats (Hasegawa 1979).

1.8.3 Walker 256 breast carcinoma

The Walker 256 carcinoma cell line is a rat tumour that originally occurred spontaneously in the mammary gland of a pregnant albino rat and grew with the morphology of a carcinosarcoma (Buffon 2007). The Walker 256 cell line has been used previously to establish experimental brain metastases through an internal carotid artery injection and direct implantation into the cerebral cortex (Hasegawa 1979; Hasegawa 1983; Blasberg 1984a; b; Blasberg 1986; Hiesiger 1986; Jamshidi 1992).

Walker 256 cells are highly immunogenic (Ferreira 2004), with a regression in tumour growth seen following oxidative burst, involving the release of reactive oxygen species and hydrolytic enzymes, from granulocytes that infiltrate tumours implanted into the hind leg of rats (Jaganjac 2008). The infiltration of an implanted Walker 256 tumour with granulocytes occurred in just five hours (Jaganjac 2008). The Walker 256 carcinoma is also sensitive to cisplatin and chlorambucil (Simpkins 1991). An angiogenic substance has been purified from the Walker 256 carcinoma (Fenselau 1981). This substance causes proliferation of fetal bovine aortic endothelial cells in vitro and similarly acts to stimulate blood vessel growth when applied in vivo (Fenselau 1981). It is possible for this angiogenic factor to be detected in the serum of a Walker 256 bearing animal and therefore to determine if animals are in a neurovascular diseased state through detection of this factor (Fenselau 1981).

Walker 256 tumours have the ability to cause changes in oxidative metabolism in tumour-free tissues (Freitas 2001). For instance, when a Walker 256 tumour cell suspension was injected into the right flank of male Wistar rats, there was evidence of oxidative stress in the brain tissue (Freitas 2001). This is possible because of an increase in the production of compounds that favour oxidation and decrease the activities of antioxidants, which ordinarily would combat oxidative stress (Freitas 2001). The possible results of this oxidative stress placed on the brain in Walker 256 tumour bearing rats is that the hypothalamic responses to starving are impaired through the increase in compounds such as vasopressin, IL-1, prostaglandin E2 and TNF-α. This may be the mechanism by which cachexia is induced in Walker 256 bearing rats, which is one of the characteristic symptoms of this tumour cell type.

When 10^7 Walker 256 cells were injected sub-cutaneously into the left flank of male albino wistar rats, the first apparent signs of cachexia were seen 7 days after tumour cell inoculation (Ferreira 2004). Furthermore, these animals died as a result 15-16 days post tumour cell inoculation (Ferreira 2004). In contrast to this, when 10^6 cells were injected into the right common carotid artery of female Wistar rats, the cancer related weight loss began at approximately day 28 post inoculation (Hasegawa 1979).

1.9 Features of metastatic brain tumours

1.9.1 Tumour morphology

Metastatic brain tumours vary greatly in morphology, as a result of their wide range of cell types associated with their organs of origin. Their features can range from yellow and soft to abundantly necrotic, with dark coloured tumours metastasising to the brain from melanomas (Potts 1980; Subramanian 2002).

1.9.2 Tumour border

Many CNS metastases invade the brain parenchyma via tumour expansion, compressing the surrounding structures, rather than tumour cells infiltrating the surrounding tissues as is the case for primary brain tumours like gliomas (Hasegawa 1983). However, there is variation in method of expansion between different organs of origin, for example SCLC brain metastases have a less defined border than seen in other metastatic brain tumours, although this type does not grow particularly quickly (Tabaka 2006).

1.9.3 Blood-tumour barrier

The capillaries within metastatic brain tumours are characteristic of the organ of the primary tumour (Cornford 1992; Shuto 2008), as indicated by the expression of aquaporin 1 (AQP-1). AQP-1 is not normally present in brain parenchyma, but is expressed in the endothelial cells of the blood vessels within many metastatic brain tumours (Papadopoulos 2004). Endothelial cells are also able to be recruited from the brain tissue surrounding the tumour due to the highly vascular nature of the brain (Strugar 1994). This process is known as vessel co-option, where the cerebral capillary endothelial cells may de-differentiate in response to lack of interaction with astrocytes and other signalling from the tumour cells themselves (Leenders 2003; Papadopoulos 2004). Therefore the co-opted vessels do not retain the properties of the

BBB and are often dilated, irregular and permeable to proteins normally found in the blood plasma (Leenders 2003).

If a metastatic brain tumour exceeds approximately 2mm in diameter, then angiogenesis must occur to ensure adequate blood supply (Blasberg 1984a; Fidler 1990; Zhang 1992). These newly formed blood vessels make up the blood tumour barrier, which may have variable barrier properties (Front 1984). Some common features of the blood tumour barrier include short, elongated or open endothelial junctions, a non-continuous basement membrane of varying width that has lost its 3 layered appearance and fenestrated membranes that lead to pores involving a fused basal and luminal plasma membrane when endothelial cells become thinner (Hirano 1975; Shibata 1989; Strugar 1994). Furthermore, there are increased pinocytotic vesicles and vacuoles when compared to the brain endothelial cells, along with increased perivascular space between the basement membrane and the tumour cells (Hirano 1975; Shibata 1989). Interestingly, one study showed that pertechnetate uptake was correlated to the absence of TJ, but not to the extent of endothelium fenestration (Bar-Sella 1979). Brain metastases from five bronchiogenic carcinomas studied showed significantly elevated extravasation of tracer and capillary transit times when compared to normal brain tissue (Koh 2006).

The structural integrity of blood vessels surrounding metastatic brain tumours is controversial. Only the blood vessels within a metastatic brain tumour mass are angiogenic and thus distinct from the normal BBB. However many believe that the mass effect of tumour growth and associated oedema may result in disruption of the BBB in the brain parenchyma surrounding metastatic brain tumours. Ultrastructural studies report that the features of the blood-tumour barrier are not shared by their counterparts outside of the tumour mass in oedematous tissue (Long 1979). Similarly the pathological changes in GLUT1 expression on blood vessels within metastatic brain tumours are not evident in the peritumoral environment (Zhang 1996b). However, the peritumoral area of some metastatic adenocarcinomas have been shown to be devoid of the tight junctional protein occluden, meaning that tight junctions both

outside and inside the metastatic brain tumour mass are disrupted (Papadopoulos 2001) . Therefore further investigaton is reqired to determine the extent to which the BBB is disrupted in the peritumoral area.

1.9.4 Angiogenesis

Malignant brain tumours cause more angiogenesis than systemic malignancies (Ito 1993). Vasoactive substances secreted by metastatic brain tumours may induce angiogenesis. Tumour cell expression of VEGF is directly correlated with rate of angiogenesis (Yano 2000). Furthermore, there is evidence to suggest that SP may be involved with metastatic brain tumour associated angiogenesis. When brain metastases from malignant melanomas, lung carcinomas and breast carcinomas were compared, the mean number of blood vessels in brain metastatic breast carcinomas was significantly elevated when compared to the CNS metastases of melanomas (Maniotis 1999).

Angiogenesis is not the only way in which metastatic brain tumours supply themselves with sufficient blood to be able to grow. Light microscopy has revealed that 45% of the brain metastases from malignant melanomas showed evidence of hollow channels containing blood that had a basement membrane and were not lined by endothelial cells, but tumour cells instead, without signs of necrosis or inflammation (Maniotis 1999; Zhang 2007). This phenomenon of tumours forming their own blood supply without the need for angiogenesis is known as vascular mimicry. This process is not unique to melanoma tumours and has been identified in many other tumour types including inflammatory breast cancer and astrocytomas (Shirakawa 2002; Yue 2005). In one study the presence of vascular mimicry in primary invasive ductal carcinomas was linked to a more invasive and metastatic phenotype (Shirakawa 2001). In an oxygen-deprived environment, B16 melanoma cells showed increased incidence of vascular mimicry and expression of MMP-2, MMP-9 and VEGF when compared to the same tumour in non-ischemic limbs (Sun 2007). However, it seems that vascular mimicry is more prevalent in the early stages of tumour growth, progressing to an intermediate stage where a combination of

tumour and endothelial lining is present lining the vessels within the tumour, called mosaic blood vessels (Zhang 2007).

1.9.5 Peri-tumoral environment

In the area around metastatic brain tumours there is often white matter tract disruptions indicated by β-amyloid precursor protein accumulation (Zhang 1996a), neuronal degradation and cytoskeleton breakdown (Toh 2007). The reasons for this could include cytotoxic products secreted by the metastatic brain tumour or disturbances in the microcirculation (Toh 2007). When compared to meningiomas, brain metastases were found to contain more extracellular water and white matter tract disorganisation in the peri-tumoral environment (Toh 2007).

A rim of reactive glial cells is often evident surrounding metastatic brain tumours, one function of which may be to act as a barrier to the flow of oedematous fluid (Zhang 1997). There is often an increase in reactive astrocytes in the peri-tumoral area, which may proliferate and become activated in response to contact with serum proteins, such as albumin, present in oedematous fluid that accumulates around the tumour (Jamshidi 1992). The reactive astrocytes surrounding metastatic brain tumour samples from 73 autopsy cases that originated from many different organs showed endothelin expression, which may be partially responsible for peri-tumoral gliosis (Zhang 1995).

1.10 Peritumoral oedema

Oedema in the brain is most commonly vasogenic in nature and results from a compromised BBB (Zhang 1997). Vasogenic oedema is characterised by the abnormal increase in the volume of interstitial fluid and may be caused by increased blood pressure in the capillaries, increased capillary permeability and decreased plasma protein concentration within the capillaries (Zhang 1997). Due to oncotic forces, plasma protein leakage into the tissue is followed by fluid, which results in oedema. It is thought that this inflammatory vasogenic oedema may provide a

favourable environment for the proliferation of malignant cells (Van Den Brenk 1974a; Van Den Brenk 1974b; Zhang 1997). It is thought that the secretion of substances that act on vascular permeability, like neurogenic inflammatory mediators, may play a role in the development of oedema surrounding metastatic brain tumours (Zhang 1997).

1.10.1 Tumour size

Tumour size and subsequent angiogenesis is thought to determine the extent of peritumoral oedema formation through effects on overall vascular permeability. Metastases larger than 0.2mm^2 have the ability to decrease the integrity of the BBB as indicated by the leakage of sodium fluorescein out of the cerebral blood vessels and into the brain parenchyma (Zhang 1992). The leakage of sodium fluorescein was directly correlated with the size of the tumour, but not with the anatomical location of the tumour in the brain or the origin of the primary tumour (Zhang 1992). Paradoxically, no leakage of sodium fluorescein was seen in another study using a similar protocol, although the size of the tumours was not reported (Schackert 1988b). Therefore it is possible that the experimental metastases in that study were too small to disrupt the BBB. However, the capillaries within the tumours were enlarged and irregular with regions of astrocytosis and oedema, although the BBB was intact (Schackert 1988b). The plasma transfer constant, which takes into account the capillary blood flow, surface area and permeability, for small tumours in the brain were very similar to that of the normal brain tissue, but in the same study the value was increased 5-20 fold for tumours larger than 0.6mm^2 (Blasberg 1984a).

1.10.2 Complications of cerebral oedema

Approximately 80% of the brain is made up of water (Tait 2008). Inside the skull, brain tissue is made up of approximately 1.1 L of water in the intracellular compartment and 100ml in the interstitium. Furthermore, the intravascular contents and CSF each make up approximately 100ml of the intracerebral volume (Lukaszewicz 2011). However, when the BBB is disrupted, then fluid is able to move from the intravascular compartment into the interstitium, thus increasing the volume

taken up by the brain tissue within the skull. There is limited capacity for blood or cerebrospinal fluid (CSF) to counteract the chages in intracranial pressure (ICP). This compensation is responsible for the initial plateau the ICP / volume curve, which becomes exponential once compensatory mechanisms are exhausted (Smith 2008) Raised ICP results in alterations to cerebral blood flow, because when the ICP is raised, the cerebral perfusion pressure (CPP) is reduced. With a reduced CPP, the brain may become deprived of oxygen, resulting in vasodilation of cerebral blood vessels. This further exacerbates the problem as it results in further increase in ICP. Complications of raised ICP can include compression of cerebral vasculature leading to localized ischaemia (Ayata 2002). Since the brain is divided into discrete compartments by membranous structures, local oedema may result in herniation of the brain tissue from one compartment into the other (Zhang 1997).

1.10.3 Clearance of oedema

Oedematous fluid spreads via bulk flow, as particles of different diffusion coefficients move at the same rate through the brain in the oedematous fluid (Reulen 1977; Ito 1988; Stummer 2007). Oedema may be absorbed into the CSF or astrocytes may resorb proteins in order to reduce the oncotic pressure that drives fluid out of the capillaries (Stummer 2007). Furthermore, oedema may be cleared through the trancellular pathway, for example through AQP-4 water channels. AQP-4 is expressed by activated astrocytes that surround metastatic brain tumours, which is postulated to aid in the removal of peritumoral oedema (Saadoun 2002).

1.11 Substance P

Substance P (SP) is an excitatory tachykinin that is predominantly released from primary sensory nerve endings in the central nervous system, but is also localized in brain endothelial cells (Cioni 1998; Ribeiro-da-Silva 2000; Lazarczyk 2007). SP preferentially binds to NK1 receptors, although there is some cross reactivity with the other tachykinin receptors, NK2 and NK3 (Harrison 2001). The pre-protackykinin A (PPT-A) gene encodes for SP (Bruno 2003; Chappa 2007). The transcription of this gene is influenced by nerve growth factor, interleukin-11 (IL-11) and interleukin-6

(IL-6) and is increased in response to noxious chemical stimulation of tissue (Noguchi 1988; Bruno 2003). Throughout the body, SP acts to increase microvascular leakage (Nimmo 2004).

When SP acts on NK1 receptors, which normally reside in the cell membrane of primary sensory neurons, it is internalized (Hokfelt 2001). NK1 is a G-protein coupled receptor (Lazarczyk 2007). SP inserts itself in the hydrophobic ligand binding pocket located between the transmembrane domain of the NK1 receptor and the extracellular surface (Harrison 2001).

1.11.1 Immunoreactivity in the brain

SP is the most abundant neuropeptide in the central nervous system (Black 2002). Human CSF is positive for SP and therefore SP can come into contact with the brain tissue surface (Zubrzycka 2002).

The areas of the brain where SP is present include the rhinencephalon, telencephalon, basal ganglia, hippocampus, amygdala, septal areas, diencephalon, hypothalamus, mesencephalon, pons, myelencephalon and spinal cord (Harrison 2001). NK1 receptors are expressed in the highest concentration in the caudate, putamen and superior colliculus, which are areas of the brain that modulate behaviour and affect stress responses (Harrison 2001; Black 2002). In the rat brain, SP is predominantly expressed in the olfactory tubercle, amygdala, nucleus caudatus, septum, hypothalamus, habenula, posterior pituitary, thalamic nuclei, globus pallidus, substantia nigra, peri-aqueductal central gray, locus coeruleus, nuclei parabranchiales, medulla oblongata, dorsal and ventral horns of the spinal cord (Severini 2002).

1.11.2 Substance P effects on the blood-brain barrier

Virtually all blood vessels are surrounded by primary sensory nerve fibres that secrete SP and the cerebral blood vessels are particularly well innervated. In brain endothelial cells the normal resting level of free Ca2+ is 100nM (Revest 1991). SP causes calcium responses in the endothelial cells of the BBB of approximately 1000nM and hence increased Ca2+ levels lead to increased BBB permeability through cell contraction (Revest 1991; Paemeleire 1999). In conjunction with this, SP treatment of cerebral capillary endothelial cells co-cultured with astrocytes, has been shown to decrease the concentration of ZO-1 and claudin-5 tight junctional proteins, resulting in increased permeability of the simulated BBB (Lu 2008).

SP is present in cerebral capillary endothelial cells and its secretion by these cells can be increased through treatment with high doses of cytokines, including IL-1β and TNFα (Cioni 1998; Annunziata 2002). This increase in SP released from brain endothelial cells was found to be associated with an increase in the expression of b-preprotachykinin mRNA, a precursor for SP, inside the cells (Cioni 1998). Spantide, a NK1 antagonist, reversed this increase in SP release from endothelial cells and the subsequent increased permeability of the BBB in a dose-dependent fashion (Annunziata 2002). Through the use of electron microscopy, it was discovered that the morphological changes associated with SP interactions with endothelial cells were also neutralized (Annunziata 2002).

1.11.3 Substance P and oedema formation

Substance P is involved in neurogenic inflammation and subsequent cerebral oedema formation. Arterial infusion with SP causes blood vessels to dilate along with extravasation of plasma proteins and fluid (Severini 2002). Similarly, inoculation of the skin with SP causes a dose dependant oedema as a result of the increase in vascular permeability and vasodilation (Black 2002; Severini 2002).

Traumatic brain injury is associated with significant oedema formation, proposed to be mediated by the SP and thus neurogenic inflammation. In the human condition, SP immunoreactivity is increased following traumatic brain injury (Zacest 2010). Similarly, perivascular SP immunoreactivity was increased in a rat model of brain trauma, which was closely associated with increased Evans blue leakage into the neuropil (Donkin 2009). Supporting this, capsaicin depletion of SP resulted in decreased BBB disruption and vasogenic oedema formation in a rodent model of diffuse traumatic brain injury (Nimmo 2004). Likewise, NK1 antagonist treatment has been shown to reduce BBB permeability and cerebral oedema, and to improve functional outcome in this model (Vink 2004; Donkin 2009). Therefore, there may be a link between the increased perilesional neurogenic inflammatory mediators and increased BBB permeability leading to cerebral oedema formation following both experimental and clinical traumatic brain injury.

Ischemic stroke is also the cause of substantial vasogenic cerebral oedema, with SP thought to play a similar role in its pathogenesis to that seen in traumatic brain injury. The expression of SP is increased by cerebral ischemia and may exacerbate the damage caused (Bruno 2003). This can be partially reversed by NK1 antagonist administration, preventing the increase in vascular permeability normally associated with SP release (Souza 2002). Similarly, when SP was significantly elevated in a model of cerebral ischaemia, treatment with the NK1 antagonist SR-14033 resulted in a reduction in the area of the infarct (Yu 1997). Another model of brain ischemia, using a middle cerebral artery occlusion, showed increased SP expression and associated oedema at 24 hours after the reperfusion of the ischaemic site (Turner 2006). In this case, treatment with an NK1 antagonist 4 hours after the occlusion and reperfusion of the middle cerebral artery was able to reduce the amount of oedema evident (Turner 2011). It is proposed that SP may play a similar role in the pathogenesis of oedema formation surrounding metastatic brain tumours.

1.11.4 Substance P and NK1 expression in cancer cells

It is well documented that NK1 receptors are expressed in many human cancer cell types, particularly breast carcinomas, melanoma and astrocytomas (Table 1). In many of these studies, NK1 receptor levels in cancerous tissue have been compared to the equivalent non-cancerous tissue, with the non-neoplastic tissues exhibiting significantly reduced NK1 receptor expression (Khare 1998; Singh 2000; Friess 2003). NK1 receptors have also been identified on peritumoral blood vessels and adjacent epithelial tissue (Hennig 1995; Gonzalez-Moles 2009), although there are few studies that have investigated NK1 receptos in this location, but have rather been focused on the tumour cell expression. Some human tumours have also been found to be positive for SP expression (Table 1), although this has been studied less frequently than NK1 receptors. Furthermore, SP has been found to be upregulated in the peritumoral area of several human tumours (Tarkkanen 1983; Gonzalez-Moles 2009).

SP and NK1 receptor expression has been studied extensively in cell culture conditions along with many human tumour cell lines. Similar to the experiments on human surgical specimens, many human tumour cell lines express NK1 receptors, although less studies were performed regarding SP secretion (Table 2). There were few studies that identified human cell lines where NK1 receptors were not expressed (Palma 1999). Furthermore, it was rare that murine cell lines were investigated, with only one showing an absence of NK1 receptor mRNA using RNA blots (Fukuhara 1996). One study showed that with increased invasiveness of breast cancer cell lines, there was an associated increase in both SP and NK1 using ELISA and RT-PCR respectively (Castro 2005). Similar to the studies done in human tissue, when cancerous cell lines were compared to normal epithelial cell lines, there was increased SP and NK1 receptors in the neoplastic cells (Singh 2000; Ramkissoon 2007).

Table 1.1 Expresssion of substance P (SP) and NK1 receptors within cancer cells in human cancer specimens

Reference	Cancer Type	SP	NK1
(Singh 2000)	Breast carcinoma	Yes	yes
(Hennig 1995)	Breast carcinoma	-	yes
(Huang 2010)	Breast carcinoma	-	yes
(Schulz 2006)	Breast carcinoma	-	yes
(Khare 1998)	Melanoma	Yes	-
(Allen 1985)	Astrocytoma	Yes	-
(Hennig 1995)	Astrocytoma	-	yes
(Schulz 2006)	Glioblastoma	-	yes
(Hennig 1995)	Glioblastoma	-	yes
(Schulz 2006)	Carcinoid meningioma	-	no
(Allen 1985)	Meningioma	Yes	-
(Schulz 2006)	Insulinoma meningioma	-	no
(Hennig 1995)	Ganglioneuroblastomas	-	yes
(Nowicki 2002)	Neuroblastoma bone marrow metastases	Yes	-
(Hennig 1995)	Medullary thyroid carcinomas	-	yes
(Rosso 2008)	Gastric adenocarcinoma	-	yes
(Rosso 2008)	Colon adenocarcinoma	-	yes
(Schulz 2006)	Colorectal carcinoma	-	no
(Schulz 2006)	Ovarian carcinoma	-	yes
(Schulz 2006)	Prostate carcinoma	-	no
(Friess 2003)	Pancreatic carcinoma	-	yes
(Schulz 2006)	Pancreatic carcinoma	-	yes
(Zhu 1995)	Pituitary adenoma	Yes	-
(Schulz 2006)	Pituitary adenomas	-	yes
(Schulz 2006)	Pheochromocytoma	-	no
(Schulz 2006)	Thyroid carcinoma	-	yes
(Gonzalez Moles 2008)	Odontogenic tumours	Yes	yes
(Gonzalez-Moles 2009)	Oral carcinomas	Yes	yes
(Brener 2009)	Oral squamous cell carcinoma	Yes	yes
(Esteban 2009)	Human laryngeal cancer	Yes	yes

(Munoz 2007)	Retinoblastoma	Yes	yes
(Tarkkanen 1983)	Retinoblastoma	No	-

Table 1.2 Expression of substance P (SP) and NK1 receptors in cancer cell lines

Reference	Cancer Type	Cell line	SP	NK1
(Singh 2000)	Human breast cancer	ZR-75–30, BT-474, T-47D, MDA-MB-330, 184B5, CP-96 345-1, DU4475, BT 483	yes	yes
(Aalto 1998)	Human breast cancer	MDA-MB-231, MDA-MB-231 Dox	yes	-
(Castro 2005)	Breast cancer	MCF7, LCC1, LCC2	yes	yes
(Ramkissoon 2007)	Human breast cancer	ZR-75-30, BT-474, T47D, DU4475, BT 483, MDA-MB-330, SK-BR-3	yes	yes
(Bigioni 2005)	Human breast carcinoma	MDA-MB-231	-	yes
(Huang 2010)	Human breast carcinoma	T47D	-	yes
(Munoz 2010b)	Human melanoma	COLO 858, MEL HO, COLO 679	-	yes
(Palma 1999)	Human glioma	SNB-19, DBTRG-05 MG, U373 MG	-	yes
(Palma 1999)	Human glioma	U138 MG, MOG-G-GCM	-	no
(Munoz 2005a)	Human neuroblastoma	SKN-BE(2)	-	yes
(Munoz 2005a)	Human glioma	GAMG	-	yes
(Fowler 1994)	Human astrocytoma	UC11MG	-	yes
(Johnson 1992)	Human astrocytoma	UC11	-	yes
(Eistetter 1992)	Human astrocytoma	U373MG	-	yes
(Akazawa 2009)	Human glioblastoma	U373	-	yes
(Fukuhara 1996)	Murine	C1300	-	no

	neuroblastoma			
(Munoz 2007)	Human retinoblastoma	WERI-Rb-1, Y-79	-	yes
(Munoz 2008)	Human laryngeal cancer	HEp-2	-	yes
(Rosso 2008)	Human gastric adenocarcinoma	23132/87	-	yes
(Rosso 2008)	Human colon adenocarcinoma	SW-403	-	yes
(Friess 2003)	Human pancreatic cancer	PANC-1, MIA-PaCa-2, ASPC-1, CAPAN-1, T3M4	-	yes
(Munoz 2012)	Human acute lymphoblastic leukaemia	T-ALL BE-13, B-ALL SD-1	-	yes

1.11.5 Role of substance P and NK1 receptors on cancer growth

In vitro, the predominant action of SP on cancer cells is to cause replication and often induce tumour cell migration (Table 3). Similarly, NK1 antagonist treatments has been fairly consistently shown to inhibit tumour cell growth and cause apoptosis (Table 3). However, several studies showed opposing effects (Ogasawara 1997; Nagakawa 1998; Korcum 2009) or were not able to detect any effect of SP on tumour cells in vitro (Palma 1999; Nagakawa 2001). Interestingly two of the cell lines in which SP was not beneficial for cancer growth, were the only two murine cell lines described in this literature (Ogasawara 1997; Korcum 2009). This may be indicative of differential treatment responsive properties between species, as it is generally accepted that human and murine tumour development is fundamentally different (Rangarajan 2004). In contrast, studies that used NK1 antagonist treatment in vitro consistently showed inhibition of tumour cell growth or induction of apoptosis (Table 3).

The effects of NK1 antagonists are proposed to be cancer specific, with one study demonstrating no effect on non-cancerous epithelial cell lines when compared to the growth inhibition seen in human breast cancer cell lines (Singh 2000). NK1 antagonists reported to be effective in inhibition of tumour growth were effective at concentrations on the order of micromoles and acted in a dose dependant manner, whereas SP was reported to cause tumour cell mitogenesis at nanomolar concentrations (Munoz 2004b; Munoz 2005a; Munoz 2005b; Munoz 2007); (Palma 1999; Friess 2003; Munoz 2004a; Munoz 2010a; Munoz 2010b). The most common agents investigated in cancer in vitro studies were L-733,060, L-732,138, MEN 11467, SR140333 and aprepitant (Bigioni 2005; Munoz 2007; Rosso 2008; Huang 2010). Similar to these studies are experiments performed on cancer cells in vitro where SP was inhibited using antibodies, also resulting in breast, prostate and colon carcinoma apoptosis using multiple cell lines (Mayordomo 2011).

Far fewer animal studies have investigated the role of SP in cancer (Table 3); these need to be performed to confirm the results seen in the numerous in vitro studies (Table 4). In contrast to the cell culture studies, which are fairly consistent in the conclusions of the stimulatory effect of SP on cancer, experiments in vivo are much more inconclusive. The marginally predominant finding of the in vivo studies is that SP is beneficial for the treatment of cancer, rather than stimulating the growth of cancer cells as seen in cell culture studies (Harris 2003; Erin 2004; Manske 2005; Erin 2006). Interestingly, all animal models that showed a positive effect of SP on tumour inhibition were syngenic or carcinogen induced tumour models and therefore did not use immune compromised animals. A possible explanation of this is that the stimulatory effect of SP on the immune system aids the natural response to the presence of neoplastic cells. Manske and colleagues showed that in a subcutaneous inoculation model of melanoma, SP delivered by an osmotic pump caused delay in tumour growth, but only in the presence of natural killer and T cells (Manske 2005). Similarly, Harris et al (2003) showed that the incidence of side stream cigarette exposure induced lung carcinomas were reduced along with increased survival and immune activation when treated with aerosolised SP. The possible role of T lymphocytes in SP mediated inhibition of cancer makes nude mice, lacking functional T cells, an imperfect host for models evaluating the role of SP or NK1 receptors in cancer progression.

In contrast, treatment with the NK1 antagonists MEN 11467 and MEN 11149 has been shown to reduce tumour volume when U373 MG astrocytoma grade III cells were inoculated subcutaneously into the right flank of female nude mice. The fact that nude mice were used in the study may explain why blocking the actions of SP were beneficial in this model. Similar studies, where A2780 ovarian carcinoma and MDA-MB-231 breast carcinoma cells were also subcutaneously injected into the right flank of female nude mice showed no effect of MEN 11467 on tumour volume (Palma 2000; Bigioni 2005). Therefore, SP does not play a sufficient stimulatory role on tumour growth in these models for NK1 antagonist treatment to be effective in decreasing tumour burden. Furthermore, it has been reported that subcutaneous xenografted tumours are poor predictors of human response to anticancer treatments

because they do not reflect normal tumour host interactions and rarely metastasise (Cespedes 2006). Therefore, the role of SP on the growth of cancer in vivo remains controversial.

Table 1.3 Effect of exogenous substance P (SP) application and NK1 antagonist treatment on cancer cells in vitro

Reference	Cancer type	Cell line	Exogenous SP	NK1 antagonist
(Munoz 2005b)	Human retinoblastoma	WERI-Rb-1, Y-79	Mitogenesis	Inhibited growth
(Munoz 2007)	Human retinoblastoma	WERI-Rb-1, Y-79	Mitogenesis	Apoptosis
(Munoz 2004b)	Human melanoma	COLO 858, MEL H0, COLO 679	Mitogenesis	Cytotoxic
(Munoz 2005a)	Human neuroblastoma	SKN-BE(2)	Mitogenesis	Apoptosis
(Munoz 2005a)	Human glioma	GAMG	Mitogenesis	Apoptosis
(Palma 1999)	Human glioma	SNB-19, DBTRG-05 MG and U373 MG	IL-6 secretion, mitogenesis	Inhibited growth
(Palma 1999)	Human glioma	U138 MG and MOG-G-GCM	No effect	-
(Friess 2003)	Human pancreatic cancer	ASPC-1, CAPAN-1	Mitogenesis	Inhibited growth
(Luo 1996)	Human astrocytoma	U-373MG	Mitogenesis	-
(Munoz 2008)	Human laryngeal cancer	HEp-2	Mitogenesis	Apoptosis
(Rosso 2008)	Human gastrointestinal adenocarcinoma	23132/87, SW-403	Mitogenesis	Inhibited growth
(Bigioni 2005)	Human breast carcinoma	MDA-MB-231	Mitogenesis	Inhibited growth
(Akazawa 2009)	Human glioblastoma	U373	Reduce apoptosis	Apoptosis

(Huang 2010)	Human breast cancer	T47D	Mitogenesis	Apoptosis
(Lang 2004)	Human breast carcinoma	MDA-MB-468	Migration	-
(Lang 2004)	Human prostate carcinoma	PC-3	Migration	-
(Drell 2003)	Human breast carcinoma	MDA-MB-468	Migration	-
(Korcum 2009)	Murine melanoma	B16F10, B16LNAD	Inhibit growth	-
(Nagakawa 1998)	Human prostate cancer	PC-3	Inhibit migration	-
(Nagakawa 2001)	Human prostate cancer	DU-145	No effect on migration	-
(Ogasawara 1997)	Murine colon adenocarcinoma	26L.5	Inhibit migration	-
(Palma 1998)	Human astrocytoma	U373 MG, SNB-19, DBTRG-05 MG	Increased cytokine secretion	Blocked cytokine secretion
(Ruff 1985)	Human lung carcinoma	Calu-3 , SK-MES-1, NCI-H69, A549	Chemotaxis	-
(Munoz 2004a)	Human neuroblastoma	SK-N-BE(2)	-	Inhibited growth
(Munoz 2004a)	Human glioma	GAMG	-	Inhibited growth
(Munoz 2010b)	Human melanoma	COLO 858 MEL HO and COLO 679	-	Apoptosis
(Singh 2000)	Human breast cancer	ZR-75–30, BT-474, T-47D, MDA-MB-330,184B5, CP-96 345-1, DU4475, BT 483	-	Inhibited growth
(Munoz	Human acute	T-ALL BE-13,	-	Apoptosis

2012)	lymphoblastic Leukaemia	B-ALL SD-1		
(Munoz 2010a)	Human glioma	GAMG	-	Apoptosis
(Munoz 2010a)	Human neuroblastoma	SKN-BE(2), IMR-32, KELLY	-	Apoptosis
(Munoz 2010a)	Human retinoblastoma	Y-79,WERI-Rb-1	-	Apoptosis
(Munoz 2010a)	Human larynx carcinoma	HEp-2	-	Apoptosis
(Munoz 2010a)	Human colon carcinoma	SW-403	-	Apoptosis
(Munoz 2010a)	Human gastric carcinoma	23132-87	-	Apoptosis
(Munoz 2010a)	Human embryonic kidney	HEK 293	-	Apoptosis
(Munoz 2010a)	Human pancreatic carcinoma	PA-TU 8902, CAPAN-1	-	Apoptosis

Table 1.4 Substance P (SP) and NK1 antagonist effects on cancer in vivo

Reference	Cancer type	Cell line	Species	SP beneficial	NK1 antagonist beneficial
(Korcum 2009)	Melanoma	B16F10	C5BL/6 mice	No	-
(Palma 2000)	Astrocytoma grade III	U373 MG	Female nude mice	No	Yes
(Palma 2000)	Ovarian carcinoma	A2780	Female nude mice	-	No
(Bigioni 2005)	Human breast carcinoma	MDA-MB-231	Female nude mice	-	No
(Pagan 2010)	Colon carcinoma	intracolonic, then systemic, administration of trinitrobenzene sulfonic acid	Sprague-Dawley rats	-	-
(Manske 2005)	Melanoma	K1735	Mice	Yes	-
(Harris 2003)	Lung carcinoma	exposure to side stream cigarette smoke	Inbred C57BL mice	Yes	-
(Erin 2008)	Breast carcinoma	4THMpc	Balb-c mice	No	-
(Erin 2006)	Breast carcinoma	4T1 cells, 4THMpc	Female Balb-c mice	Yes	-
(Erin 2004)	Breast carcinoma	Syngeneic 4T1	Adult mice	Yes	-

1.11.6 Substance P effects on angiogenesis

The stimulatory effect of SP on cell growth and the presence of NK1 receptors on many capillary endothelial cells have made NK1 antagonism an attractive target for inhibition of cancer-associated angiogenesis. SP has been shown to cause endothelial cell migration and proliferation in cell culture studies (Ziche 1990; Wang 2009). SP injected into the synovium of rat knees stimulated endothelial cell proliferation when compared to their saline injected counterparts (Seegers 2003). Growth of new capillaries was also seen in a rabbit avascular cornea with the application of a 1-5 microgram pellet of SP (Ziche 1990). Furthermore, NK1 antagonist treatment inhibited the angiogenesis seen in this model (Ziche 1990). Similarly, NK1 antagonism at least partially ameliorated the increased blood vessel growth seen when capsaicin injection induced angiogenesis in rat knees (Mapp 2012). Therefore it is possible that SP mediates tumour-initiated angiogenesis and that NK1 antagonist treatment may be effective in reducing tumour growth by decreasing available blood supply, although more studies are required to confirm this in tumour models.

1.11.7 Substance P interactions with radiotherapy of cancer

Radiation therapy for the treatment of cancer may cause alterations in SP expression in and around tumours, although the mechanism and implications of this is yet to be determined. In vitro, irradiation caused an increase in SP secretion by a subpopulation of SP immunostained MDA-MB-231 breast carcinoma cells (Aalto 1998). In contrast to this study, B16F10 melanoma cells implanted into C5BL/6 mice showed delayed tumour growth by three weeks, when treated with ionising radiation. In conjunction, the radiation reduced SP expression in and surrounding the skin lesions, whilst improving animal survival (Korcum 2009). Thus the role of SP in relation to radiotherapy is unclear.

1.11.8 Potential effects of Substance P on tumour cell extravasation into the brain

Since SP has the ability to increase the permeability of the BBB, it is a potential candidate involved in tumour cell extravasation into the brain parenchyma to form secondary brain tumours. It is therefore proposed that tumour cells may secrete SP or cause a release of SP from cerebral capillary endothelial cells or the primary sensory neurons that surround the blood vessels in the brain. This would result in a decrease in the barrier properties of the BBB and may allow tumour cells to metastasise to the brain.

1.12 Conclusion

The mechanism of tumour cell extravasation from the cerebral circulation into the brain parenchyma through the BBB is not yet understood. However, it is known that SP acts to increase the permeability of the BBB in other pathologies and therefore it may play a role in the metastatic progression of tumour cells to the brain. If this is the case, treatment with NK1 receptor antagonists may be able to prevent the formation of metastatic tumours in the brain. Furthermore, SP had previously been shown to cause an increase in oedema and a worse clinical outcome in animal models of stroke and traumatic brain injury. Therefore it is proposed that SP may play a role in the increased permeability of the BBB that leads to oedema formation surrounding metastatic brain tumours. This will be investigated in the hope that novel treatments for peritumoral oedema in the brain may be developed in order to prevent the complications that often arise as a result of cerebral oedema.

A brief introduction will precede each experimental investigation, along with a summary of the experimental protocol; these are outlined in detail in Chapter 2. While each chapter reports results specific to that chapter, many of the results have implications not only for the immediate point being considered, but often for other aspects raised in the thesis. For this reason, there will be some overlap in interpretation and discussion. A concluding general discussion will thereafter integrate the major conclusions drawn from each chapter.

2 Materials and Methods

2.1 Cell culture

Walker 256 rat breast carcinoma cells were obtained from two cell banks, the American Type Culture Collection (ATCC), and the Cell Resource Centre for Medical Research at Tohoku University, Japan (CRCTU). Both cell populations were cultured according to the instructions from the respective cell banks. These cells were not synchronised before experiments, as this may impair cell replication. In each experiment treatment groups were performed concurrently where possible to compensate for different tumour cell passage number.

2.1.1 Walker 256 cells from American Type Culture Collection

Walker 256 cells from the ATCC were cultured in growth medium made up of Sigma 199 M4530 culture medium containing 5% sterile normal horse serum and 1% penicillin and streptomycin (Sigma 10,000 units of penicillin and 10 mg of streptomycin/mL). Culture flasks of 150cm^2 were used to grow the cells and once >90% confluence was reach the cells were detached with addition of 3.5 mL of 1% trypsin (Sigma). The cells were spun down in a centrifuge (5 minutes at 1500 RPM) and then resuspended in serum free culture medium. The number of cells was calculated using a haemocytometer and then diluted, so that the correct number of cells suspended in serum free culture medium were ready for inoculation. The cell suspension was mixed prior to inoculation to maintain tumour cell concentration throughout the entire volume. The specific concentrations used are described in the relevant results chapters.

2.1.2 Walker 256 cells from Cell Resource Centre at Tohoku University

Walker 256 cells from the CRCTU were cultured in growth medium made up of Sigma RPMI-1640 culture medium containing 10% sterile fetal bovine serum and 1 mL of penicillin and streptomycin (Sigma 10,000 units penicillin and 10 mg of streptomycin/mL) for each 100 mL volume. Culture flasks of 150 cm^2 were used to grow the cells and once >90% confluence was reached the cells were detached with

addition of 3.5 mL of 0.02% EDTA. The cells were spun down in a centrifuge (5 minutes at 1500 RPM) and then resuspended in serum free culture medium. The number of cells was calculated using a haemocytometer and then diluted, so that the correct number of cells suspended in serum free culture medium were ready for inoculation. The cell suspension was mixed prior to inoculation to maintain tumour cell concentration throughout the entire volume. The specific concentrations used are described in the relevant results chapters.

2.1.3 Cell viability assay

The cell viability assay was performed on Walker 256 cells from the CRCTU and as such 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). Fosaprepitant dimeglumine (Emend^{R}; MERCK & CO), n-acetyl $_{L}$-tryptophan (NAT) and dexamethasone sodium phosphate (DBL) were used as treatments in this assay. To assess the response of Walker 256 tumour cells to differing doses of NK1 receptor antagonists (Emend and NAT) and dexamethasone, a trypan blue cell viability assay was used. 10^5 cells were seeded into each well of a 12 well tissue culture plate with 2 mL of complete culture medium. Cells were allowed to grow for 24 hours, after which the drugs of interest or saline as a vehicle control were added for a further 24 hours. Each treatment was applied to 3 wells on 4 different occasions (n=12), with three concentrations, 10, 100 and 1000µg / mL for each of the three agents. Cells were detached using 0.02% EDTA and transferred into tubes that were subsequently centrifuged for 5 minutes at 1500 RPM. The cells were then resuspended in 1 mL of fresh medium with 1 mL of 0.4% trypan blue. Cells capable of excluding the trypan blue dye were counted as viable cells. The percentage of viable Walker 256 cells was calculated in relation to the total cell count using a haemocytometer.

2.2 Animals

The experimental procedures described throughout this project were performed within the National Health and Medical Research Council (NHMRC) guidelines and were

approved by the University of Adelaide Animal Ethics Committee (approval numbers M-018-2008a and M-2011-53) and the IMVS animal ethics committee (approval numbers 39a-09 and 104-09).

Animals were group housed in the IMVS Animal Facility in a standard rodent room with a 12 hour day-night cycle. These animals were supplied with a diet of rodent pellets and water ad libitum. All experiments were performed on adult male Wistar rats weighing 250-350g. Male animals were utilised to eliminate the possible confounding effects of oestrogen, a known neuroprotective agent (De Nicola 2012). The number of animals in each experimental group is detailed in the relevant chapters that follow.

2.3 Internal carotid artery injection

For internal carotid artery injection of either tumour cells or culture medium as controls, anaesthesia was induced in a transparent 5L container by 5% isoflurane in oxygen. Animals were then intubated and anaesthesia maintained using 2 % isoflurane in oxygen, delivered by an endotracheal tube attached to a small animal ventilator. Following this, the animal was placed in the supine position on a heat pad and the front paws taped down to expose the neck region. The skin was shaved and then swabbed with 70% alcohol, before a subcutaneous injection of local anaesthetic, bupivacaine, was administered.

A longitudinal skin incision was made in the midline of the neck from the chin to the upper end of the sternum. The right neurovascular bundle was exposed and the vagus nerve identified and separated from the carotid vessels (Fig 2.1 A & B). The ophthalmic and superior thyroid arteries were sacrificed using a cautery unit, and the pterygopalatine artery, which branches off the internal carotid artery, was occluded using 3–0 silk suture (Fig. 2.1 C & D). The external carotid artery was divided between two ligatures and the proximal stump was turned inferiorly (Fig. 2.1 E).

The common carotid artery was temporarily occluded using a silk sling (Fig. 2.1 D & E). Using micro-scissors, a small hole was cut in the proximal external carotid stump through which a cannula was threaded into the internal carotid artery and tied in place. Tumour cell suspension or culture medium (0.2 mL) was slowly injected into the internal carotid artery, after which the blood flow through the common carotid artery was established by removal of the sling. The wound was closed with sutures and animals allowed to recover.

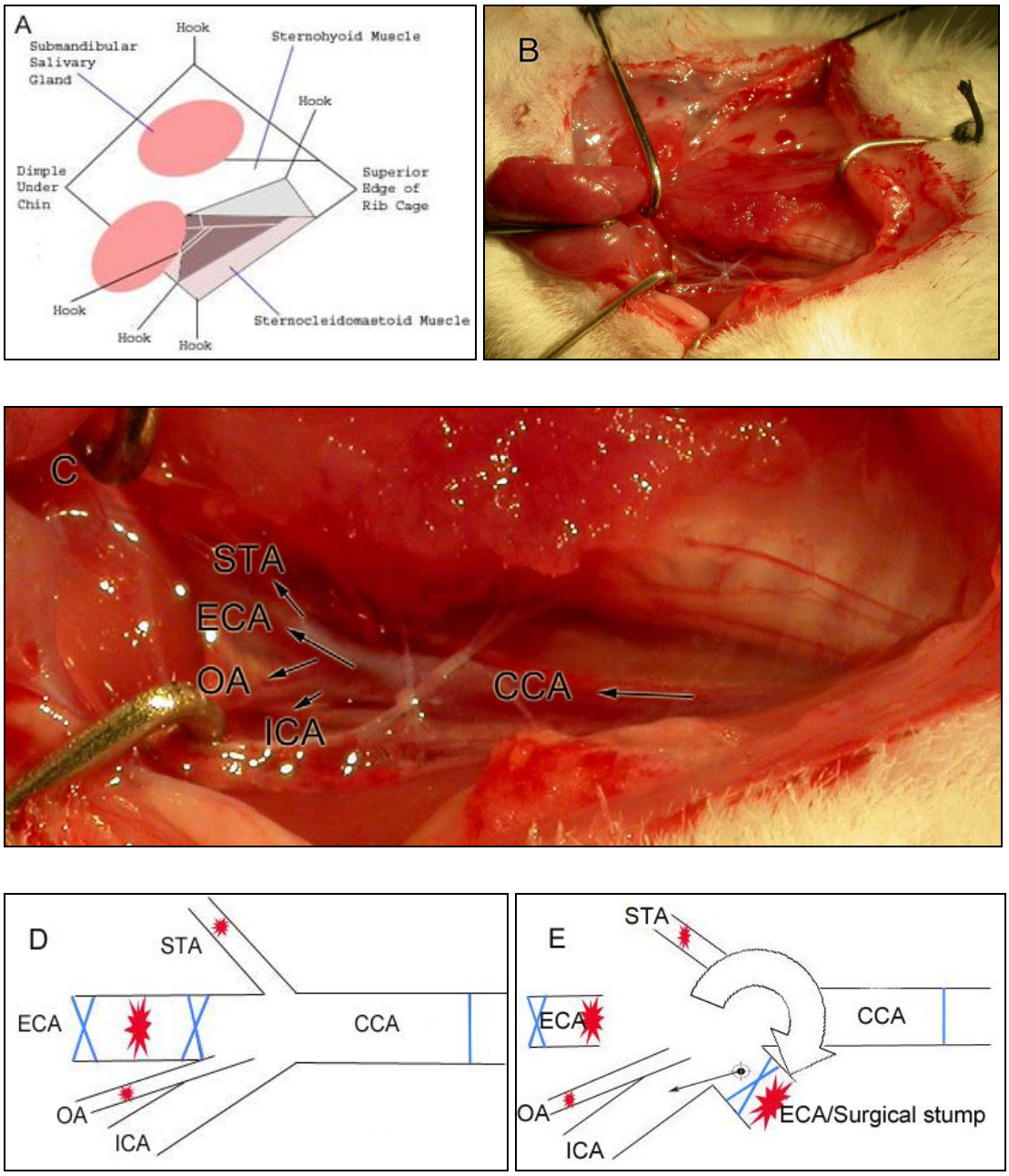

Figure 2.1 Internal Carotid Artery injection

(A) Diagram of the surgical field showing the location of the carotid bifurcation (B) Photograph of the surgical field (C) Close up of image B showing the common carotid artery (CCA), superior thyroid artery (STA), external carotid artery (ECA), ophthalmic artery (OA) and internal carotid artery (ICA) (D) Diagram of the blood vessels shown in image C demonstrating the location of sutures (blue lines) and sites of cauterisation (red starburst) (E) Diagram of carotid bifurcation during internal carotid artery cannulation, small black arrow indicates the cannulation point and direction in which the tube was threaded.

2.4 Direct intracerebral inoculation

For the direct inoculation model of tumour cells, anaesthesia was induced with 5% isoflurane in oxygen in a transparent 5L container, and maintained at 3% via a nose cone. The animal was placed in a stereotaxic frame, local anaesthetic bupivacaine 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.

2.5 Drug treatments

In treatment studies animals were randomly assigned to receive fosaprepitant dimeglumine (Emend^R; MERCK & CO), n-acetyl $_L$-tryptophan (NAT), dexamethasone sodium phosphate (DBL) or equal volumes of vehicle saline. The treatment regimen for each study is described in the relevant results chapters. All agents were dissolved in 0.9% saline solution and delivered via intraperitoneal injection with a 30gauge ½ inch needle attached to a 1mL syringe.

2.5.1 Emend

The NK1 receptor antagonist Emend was used at a dose of 3 mg/kg/day in all treatment studies described, although the duration and timing varied. This dose was determined based on the dose recommended clinically and that shown to previously elicit a central effect in rodents (Watanabe 2008). Emend was prepared by mixing 15mg of the drug with 5ml of 0.9% saline until dissolved and was stored at 4ºC for a maximum of 4 days.

2.5.2 NAT

The NK1 receptor antagonist, NAT, was used in all treatment studies at a dose of 7.5 mg/kg/day. Dosage was determined based on previous studies from our laboratory describing the dose response required to reverse the increased BBB permeability evident following traumatic brain injury (Donkin 2009). NAT was prepared by mixing 150mg of the drug with 20 mL of 0.9% saline, with the addition of 3 drops of 5N NaOH to aid in dissolving the NAT powder. Once the solution was clear, the pH was adjusted to between 7.3 and 7.6. This solution was refrigerated at 4ºC and used within 4 days of preparation.

2.5.3 Dexamethasone

Dexamethasone, a synthetic glucocorticoid, was obtained as a 4mg/mL solution in 2mL ampoules from the Royal Adelaide Hospital pharmacy and stored at 4ºC. A high dose of 8 mg/kg/day dexamethasone was chosen as determined by a previous study that had successfully used dexamethasone to treat tumour-associated oedema (Gu 2007a).

2.6 Assessment of brain histology

For histological analysis, animals were transcardially perfused with 10% formalin under terminal anaesthesia induced by intraperitoneal administration of pentobarbitone sodium (60 mg/kg). Animals were decapitated 1 hour following perfusion and the brains submerged in 10% normal buffered formalin for 24 hours.

Once the cerebellum and olfactory bulb have been removed, the remaining tissue was placed into a cassette and exposed to increasing concentrations of ethanol for 20 minutes each (50%, 70%, 80%, 90%, 95%, 100%, 100%). Following this, the tissue was placed in two baths of xyline each of 90 minutes duration and then bathed in paraffin for increasing periods of time (30, 60, 60 and 90 minutes).

Brains were subsequently embedded in paraffin wax and 5 µm coronal sections were cut. This was done every 2mm in preliminary studies and then every 400µm in subsequent experiments. The brain sections were then mounted on glass slides (Menzel-Glaser, Superfrost Plus) and stored in slide boxes in a 37ºC humidified oven until required for staining.

2.7 Tumour volume

Haematoxylin and eosin stained slides were scanned using a Nanozoomer (Hammamatsu, Hammamatsu City, Japan) and images used to calculate tumour volume. This was achieved by determining the area of tumour in each section using the NDP viewer programme and multiplying the area by the distance between sections.

2.8 Immunohistochemistry

Immunostaining for albumin (ICN Pharmaceuticals, 1:20,000 anti goat), SP (Santa Cruz Biotechnology, 1:2000 anti goat), NK1 receptors (Biocore, 1:8000 anti rabbit), endothelial barrier antigen (EBA Chemicon, 1:5000 anti mouse), glial fibrillary acidic protein (GFAP Dako 1:40,000 anti rabbit) and ionized calcium binding adaptor molecule 1 (IBA1 Dako 1:50,000 anti rabbit) was performed. The specific stains used in each study are described in the relevant results chapters. Tumour cells were also grown onto cover slips in vitro to be immunostained for cytokeratin18 (Gene Tex 1:3000 anti mouse).

Immunohistochemistry was performed using the standard streptavidin procedure used regularly in our laboratory with 3,3`-diaminobenzidine (DAB) for visualization and haematoxylin counterstaining. All immunostained slides were scanned using the Nanozoomer. Analysis of immunostaining for each antibody is described in the relevant results chapters.

2.9 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:

$$\% \text{ of brain water} = ((\text{wet weight} - \text{dry weight}) / \text{wet weight}) \times 100$$

2.10 Evans blue extravasation

Evans blue was used as an indicator of BBB permeability, since it binds to serum albumin and is only able to leak into the brain tissue from the vasculature under pathological conditions that increase the permeability of the BBB. 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). 10 minutes before perfusion, animals were administered an intraperitoneal injection of 1000 IU of heparin in 0.5mL using a 30 gauge ½ inch needle.

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.

2.11 Statistical analysis

Data are expressed as mean±SEM throughout. To determine statistical significance, an unpaired two tailed 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 Tumorigenicity of Walker 256 breast carcinoma cells from two different tumour cell banks as assessed using two models of brain metastases

3.1 Abstract

Metastatic brain tumours are a common end stage of breast cancer progression, with significant associated morbidity and high mortality. Walker 256 is a rat breast carcinoma cell line syngeneic to Wistar rats and commonly used to induce metastatic brain tumours. In this study internal carotid artery injection and direct cerebral inoculation models of metastatic brain tumour were used to determine the tumorigenicity of Walker 256 cells obtained from the American Type Culture Collection (ATCC), and the Cell Resource Centre for Medical Research at Tohoku University (CRCTU). Tumour incidence and volume, plus immunoreactivity to albumin, IBA1 and GFAP, were used as indicators of tumorigenicity and tumour interaction with the host brain microenvironment. CRCTU Walker 256 cells showed greater incidence, larger tumour volume, pronounced blood-brain barrier (BBB) disruption and prominent glial response when compared to ATCC cell line. These findings indicate that immortalised cancer cell lines obtained from different cell banks may have diverse characteristics and behaviour in vivo.

3.2 Introduction

Cancer research has received much attention and funding over the past decades, reflecting its increased incidence and significance as a public health problem. Carcinogenesis is a multifaceted and complex disease process, making malignancies inherently difficult to treat, while at the same time presenting multiple pathways for investigation as management options. Novel treatments targeting these different pathways can then be assessed, although tumours in the brain have been excluded from many clinical trials due to the restrictive nature of the BBB, often making brain metastases not accessible to novel treatments (Puduvalli 2001; Harford-Wright 2011). Metastatic brain tumours are present in 22-30% of patients diagnosed with breast cancer (Schuette 2004; Heyn 2006; Hines 2008), therefore making animal models of brain metastases important tools to explore adequate treatment options for this aspect of the disease.

The process of brain metastases involves cells from a primary tumour entering blood vessels, avoiding death signals in the circulation, then undergoing extravasation through the BBB (Marchetti 2003). The BBB is a dynamic interface between the cerebral circulation and brain tissue, and acts to protect the brain microenvironment (Hawkins 2005). While investigating metastases, many scientists using cell culture presume that tumour cell lines will behave indefinitely in a uniform manner, although several studies have demonstrated that this is not the case. Changes exhibited with extended in vitro growth time, high passage number and cross contamination with other cell lines have been frequently described in the literature (Sacchi 1984; Chang-Liu 1997; Buehring 2004; Liscovitch 2007), particularly when cancer cell lines are obtained from sources other than reputable major cell libraries (Reid 2011). There is the assumption that well characterised cell lines available from cancer cell repositories are verified and maintained at a high standard, meaning that researchers do not need to authenticate these cell lines before commencing their experiments (Cree 2011). However, the current publication reports differential characteristics of the same cancer cell line obtained from two different reputable cell banks, suggesting that researchers cannot assume that cells obtained from reputable cancer cell repositories will all behave identically.

3.3 Method

3.3.1 Cell Culture

Walker 256 breast tumour cells (rat) were obtained from two cell banks, the American Type Culture Collection (ATCC), and the Cell Resource Centre for Medical Research at Tohoku University (CRCTU). Both cell populations were cultured according to the instructions from the respective cell bank. This has been described previously in sections 2.1.1 and 2.1.2 on page 42.

Culture flasks of 150cm^2 were used to grow the cells and once >90% confluence was reached, the cells were detached by the addition of 3.5 mL of 1% trypsin (Sigma) or 3.5 mL of 0.02% EDTA for ATCC and CRCTU Walker 256 cells, respectively. The cells were spun down in a centrifuge (5 minutes at 1500 RPM) and then resuspended in serum free culture medium. The number of cells was calculated using a haemocytometer and then diluted, so that there was between 10^5 and10^6 cells in every 0.2 mL of cell suspension for internal carotid artery injection, or the same number of cells in 8 μL for direct inoculation into the brain.

3.3.2 Animals

The experimental procedures were performed as described in section 2.2 on page 43. Animals were randomly selected for either the internal carotid injection procedure or the direct inoculation procedure and then were further divided into culture medium only control group, Walker 256 tumour CRCTU group and Walker 256 tumour ATCC group.

3.3.3 Internal Carotid Artery Injection

Animals allocated to the internal carotid injection procedure were sacrificed at 24 h (early, n=5), 6 days (intermediate, n=5) and 9 days (late, n=9) for the CRCTU Walker 256 cells, and at 24 h (early, n=5), 4 weeks (intermediate, n=5) and 10 weeks (late, n=9) for the ATCC Walker 256 cells. The selected late time points were determined

after a pilot study of tumour burden and animal weight loss for both cell lines. The method for internal carotid artery injection of tumour cells to induce metastatic brain tumour growth has been previously described in detail in section 2.3 on page 44.

3.3.4 Direct Inoculation

Animals that received direct intraparenchymal inoculation were sacrificed at 7 days and 4 weeks for the CRCTU and ATCC Walker 256 cells, respectively (n=6/group). Direct stereotaxic inoculation of tumour cells into the right striatum for induction of metastatic brain tumour has been previously described in detail in section 2.4 on page 47.

3.3.5 Tumour Volume

For histological analysis, animals were transcardially perfused with 10% formalin under terminal anaesthesia induced by intraperitoneal administration of pentobarbitone sodium (60 mg/kg) as described in section 2.6 page 48. Brains were embedded in paraffin wax and sequential 5 µm coronal sections were cut from blocks 2mm thick in a rostro-caudal direction, to be used for haematoxylin and eosin staining and immunohistochemistry. The haematoxylin and eosin stained slides were scanned using a Nanozoomer (Hammamatsu, Hammamatsu City, Japan) and images used to calculate tumour volume. This was performed by determining the area of tumour in each section using the NDP viewer programme and multiplying the area by the distance between sections as previously described in section 2.7 page 49 (Corrigan 2012).

3.3.6 Immunohistochemistry

Slides from each model were stained for albumin (ICN Pharmaceuticals, 1:20,000), GFAP (Dako 1:40,000) and IBA1 (Dako 1:50,000). Tumour cells were also grown on cover-slips in vitro to be immunostained for cytokeratin18 (Gene Tex 1:3,000). Immunohistochemistry was performed as described in section 2.8 page 49. Slides

were scanned using the Nanozoomer. Albumin immunostaining, expressed as the weighted %DAB in each coronal section, was estimated using colour deconvolution techniques, as described previously (Harford-Wright 2010; Helps 2012). For GFAP and IBA1 immunoreactivtiy, 4 fields of view were taken from the cortex and striatum for the internal carotid artery injection model and the direct inoculation model. The immunolabelled cells in these images were counted and the mean number calculated for all images from each brain.

3.3.7 Statistical Analysis

Results were expressed as mean±SEM and an unpaired two tailed t test (for two groups) or a one-way analysis of variance followed by a Bonferroni post test (for more than two groups) performed. Values of $p<0.05$ were designated as significant.

3.4 Results

3.4.1 Cell Morphology

In cell culture, both the Walker 256 cell populations received from the CRCTU and the ATCC grew very effectively, although with very different cell morphology. The cells from the CRCTU were small and spicular in appearance with deeply stained nuclei (Fig. 3.1A), whereas the cytoplasm of the ATCC cells was abundant and the cells had a larger, flatter appearance with open-face lighter stained nuclei (Fig. 3.1B). Nuclei of the two cell populations were comparable in size (Fig. 1a and b). Both the CRCTU and ATCC Walker 256 cell populations stained positively for cytokeratin 18, a marker of breast cancer cells (Fig. 3.1A and B).

When the CRCTU and ATCC Walker 256 breast carcinoma cells were delivered to the brain through internal carotid artery injection, the resultant tumours also showed differential cell morphology. The tumours from CRCTU Walker 256 cells that grew 9 days following internal carotid artery injection showed cells with large nuclei and scanty cytoplasm (Fig. 3.1C). In contrast, the single tumour that grew 10 weeks following internal carotid artery injection of ATCC Walker 256 cells showed spindle-shaped cells with smaller nuclei and a larger cytoplasmic component (Fig. 3.1D). Furthermore, there was no evidence of mitotic figures in the ATCC tumour, whereas CRCTU tumours exhibited several cells undergoing replication (Fig 3.1C).

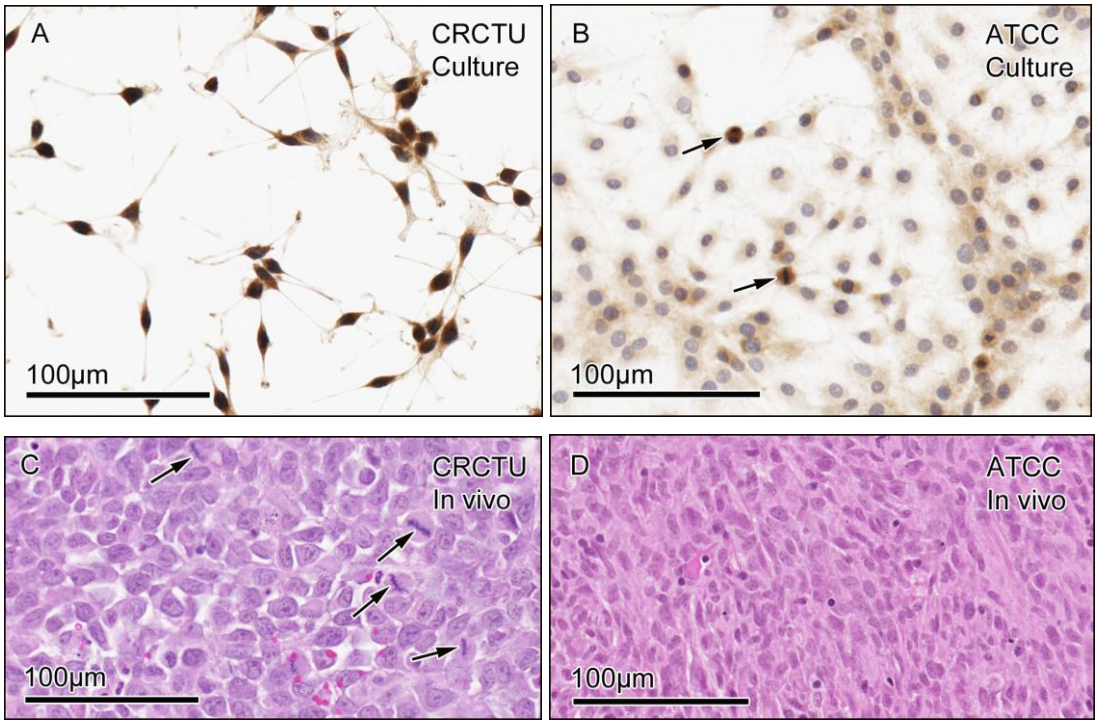

Figure 3.1 Tumour cell morphology in vitro and in vivo

(A) CRCTU Walker 256 cells in culture stained for cytokeratin 18, showing spicular appearance with deeply-stained nuclei. (B) ATCC Walker 256 cell in culture stained for cytokeratin 18 showing flattened cells with a large cytoplasmic component and lightly stained nuclei and mitotic figures (arrows) (C) CRCTU Walker 256 tumour cells in vivo, 9 days following internal carotid artery injection, stained with haematoxylin and eosin showing large nuclei and scanty cytoplasm with many mitotic figures (arrows) (D) ATCC Walker 256 tumour cell in vivo, 10 weeks following internal carotid artery injection, showing smaller nuclei and abundant elongated cytoplasmic component, giving the section an eosinic appearance

3.4.2 Tumorigenicity

The CRCTU Walker 256 cells grew much more aggressively in vivo than the ATCC population as indicated by the earlier sacrifice time required for the CRCTU injected animals in both models. Following internal carotid artery injection, only one animal of 9 injected with ATCC cells developed a metastatic brain tumour at the 10 week time point, whereas 8 out of the 9 animals injected with the CRCTU cells showed tumours at the late time point of 9 days (Table 3.1). Furthermore, the CRCTU internal carotid artery injected animals also showed metastatic brain tumours in one out of the 5 animals killed at the intermediate time point of 6 days following surgery (Table 3.1). Neither the CRCTU nor the ATCC Walker 256 injected animals showed any evidence

of tumour growth at the early time point of 24 hours post internal carotid artery injection (Table 3.1). The single tumour that resulted from inoculation with ATCC Walker 256 cells was located in the striatum. In contrast, the masses in the CRCTU Walker 256 inoculated animals were predominantly found in the lateral ventricles.

Similar to the internal carotid artery injection model, the CRCTU cells were effective in producing metastatic brain tumours when inoculated directly into the brain, whilst the ATCC cells were not (Table 3.1). Direct inoculation of CRCTU tumour cells into the striatum resulted in development of large neoplastic masses in the brain tissue of 100% of the animals, whereas none of the ATCC Walker 256 inoculated animals showed any evidence of tumour growth (Table 3.1). Comparison of the two models used in this study revealed that direct injection of CRCTU Walker 256 cells into the brain resulted in larger and more consistent location of tumour growth in the striatum with a mean volume of 55.28 mm^3, compared with an average tumour volume of 36.61mm^3 following internal carotid artery injection of the same CRCTU Walker 256 cells (Fig. 3.2A and B).

All the animals that developed metastatic brain tumours in the 9 day CRCTU group showed a concurrent growth of a tumour in the right eye (Fig. 3.3A). Also 44.4% of these animals had small tumour nodules in the right temporalis muscle, and 33.3% developed lung tumours (Fig. 3.3B and C). None of these features were seen in the animals injected with ATCC Walker 256 cells, or with animals inoculated directly into the striatum with the CRCTU Walker 256 cells.

Table 3.1 Tumour incidence in animals injected via the internal carotid artery or directly inoculated into the brain with Walker 256 breast tumour cells obtained from the Cell Resource Centre for Medical Research at Tohoku University (CRCTU) or the American Type Culture Collection (ATCC)

Internal Carotid Artery injection	**CRCTU**		**ATCC**	
Early time point (n=5)	24 hours	0% (0/5)	24 hours	0% (0/5)
Intermediate time point (n=5)	6 days	20% (1/5)	4 weeks	0% (0/5)
Late time point (n=9)	9 days	89% (8/9)	10 weeks	11% (1/9)
Direct inoculation	**CRCTU**		**ATCC**	
(n=6)	7 days	100% (6/6)	4 weeks	0% (0/6)

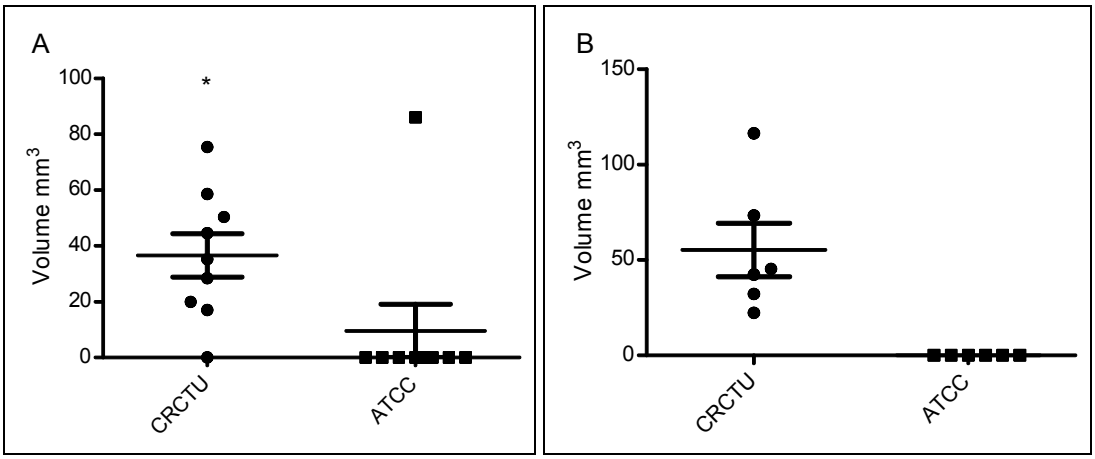

Figure 3.2 Tumour volume in models of brain metastases

(A) Tumour volume following internal carotid artery injection with CRCTU and ATCC Walker 256 rat carcinoma cells at 9 days and 10 weeks, respectively, following surgery showing. Only a single ATCC Walker 256 inoculated animal exhibited tumour growth (*$p<0.05$) (B) Tumour volume following direct inoculation of CRCTU and ATCC cells into the right striatum 7 days and 4 weeks respectively following surgery. Only CRCTU Walker 256 inoculated animals grew metastatic brain tumours of substantial volume

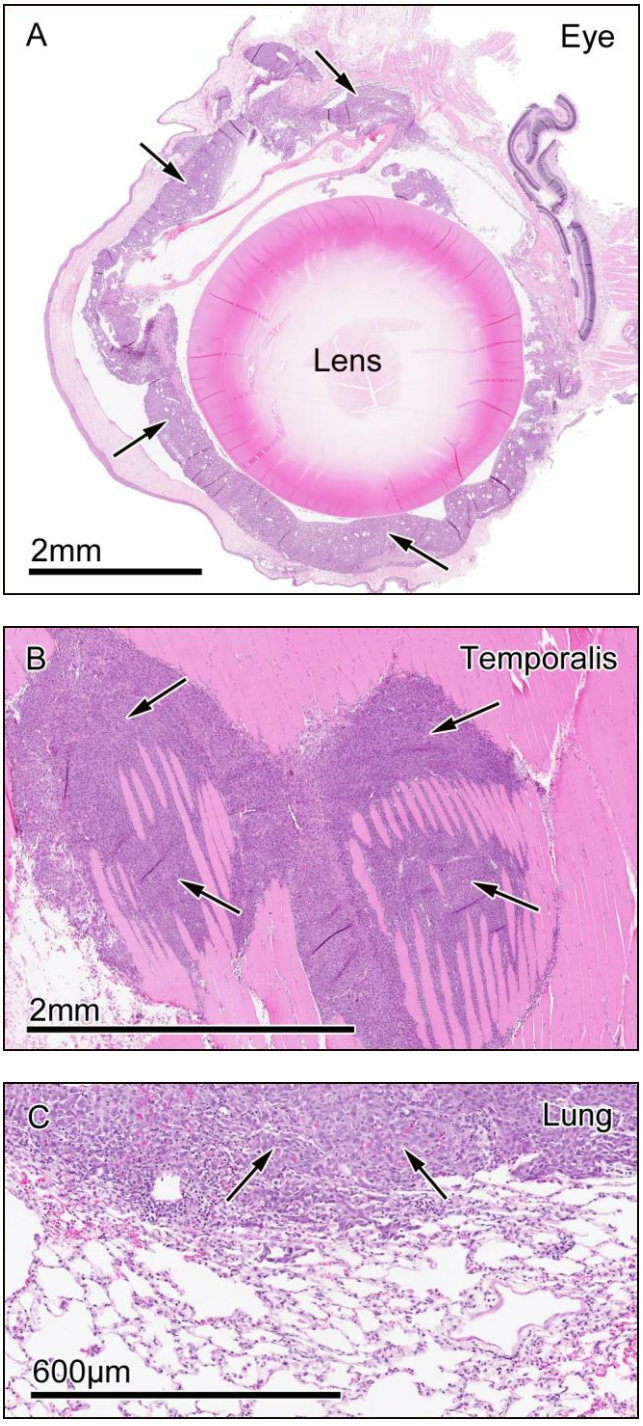

Figure 3.3 Extracranial tumour growth following internal carotid artery injection of CRCTU Walker 256 cells

(A) CRCTU Walker 256 tumour growth (arrows) in the eye 9 days following internal carotid artery injection stained with haematoxylin and eosin (B) CRCTU Walker 256 tumour growth (arrows) invading the temporalis muscle 9 days post internal carotid artery injection, stained with haematoxylin and eosin (C) Lung section stained with haematoxylin and eosin, showing a large tumour mass (arrows) 9 days following CRCTU Walker 256 cells carotid injection

3.4.3 Tumour Interactions with the BBB

In the present study, the large plasma protein albumin was used as an endogenous marker of BBB permeability, given that serum albumin is confined to blood vessels under normal conditions. However, when the BBB is substantially compromised, albumin leaks out of the blood vessels into the surrounding neuropil. Separate control groups for different time points were required for the direct inoculation model, due to the invasive nature of the surgery. In contrast, injection of culture medium into the internal carotid artery did not cause variation of BBB disruption over time, and only one control group was used for all time points.

Neither CRCTU nor ATCC Walker 256 tumour injection into the internal carotid artery caused a significant increase in albumin immunoreactivity 24 h following surgery, when compared to the culture medium control group (Fig 3.4A). A similar pattern of immunoreactivity was evident at the intermediate time point following internal carotid artery injection of Walker 256 cells from both cell banks (Fig 3.4A). In contrast, by 9 days following CRCTU Walker 256 internal carotid artery injection there was a significant increase in albumin immunoreactivity in the brain coronal sections when compared to the culture medium control group ($p<0.001$; Fig. 3.4A). Similarly, only CRCTU Walker 256 inoculated and not ATCC Walker 256 inoculated brains showed a significant increase in albumin immunoreactivity following direct injection of tumour cells into the striatum when compared to the respective culture medium control group ($p<0.001$; Fig. 3.4B).

Widespread albumin immunoreactivity was evident throughout the brains in animals that grew tumours after receiving CRCTU Walker 256 cells by the internal carotid artery injection or via direct inoculation into the brain (Fig. 3.4C). This indicates that the tumours that result from CRCTU tumour cell had widespread effects on BBB permeability. In contrast, the increase in BBB permeability was more concentrated in the immediate vicinity of the single tumour that formed after ATCC Walker 256 tumour injection into the internal carotid artery (Fig. 3.4D).

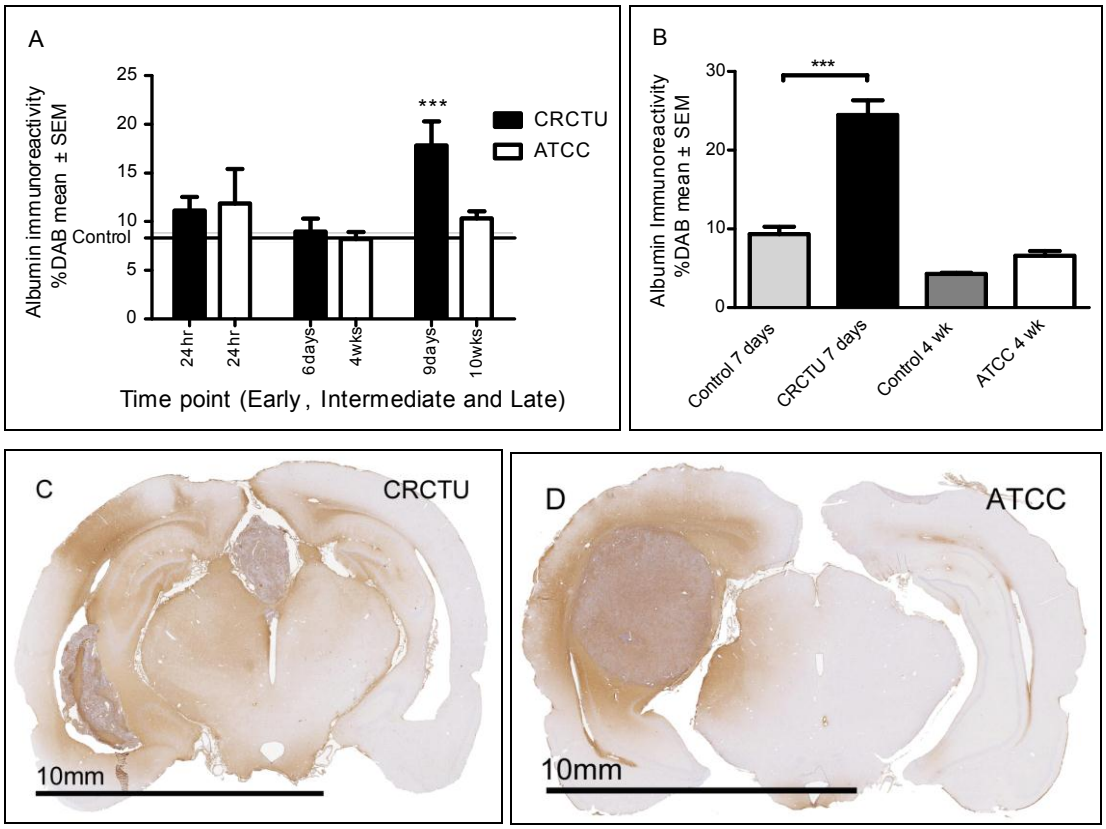

Figure 3.4 Albumin immunoreactivity in metastatic brain tumour models

(A) Graph showing %DAB in albumin immunostained coronal sections of the brain at early, intermediate and late time points following internal carotid artery injection of CRCTU and ATCC Walker 256 tumour cells when compared to culture medium control brains; ***p<0.001. (B) Graph showing %DAB in albumin immunostained brain coronal sections 7 days and 4 weeks following CRCTU and ATCC Walker 256 tumour inoculation respectively, compared to culture medium control brains; ***p<0.001. (C) Coronal brain section stained for albumin 9 days following internal carotid artery injection of CRCTU Walker 256 breast carcinoma cells showing widespread immunoreactivity mainly in the right hemisphere. (D) Albumin immunostained brain coronal section 10 weeks post internal carotid artery injection with ATCC Walker 256 breast carcinoma cells showing peritumoral immunoreactivity.

3.4.4 Brain Microenvironment

Both models of metastatic tumour induction caused changes in the brain microenvironment when CRCTU Walker 256 breast carcinoma cells were utilised (Fig. 3.5 and 3.6). There was a significant increase in the number of GFAP positive cells in the cortex of animals 9 days following internal carotid artery injection of CRCTU Walker 256 cells when compared to the culture medium control group ($p<0.01$; Fig. 3.5A). Correspondingly, there was a significant increase in the number of astrocytes immunostained for GFAP in the striatum surrounding the tumour mass 7 days following direct injection of CRCTU Walker 256 cells ($p<0.01$; Fig. 3.5B). In contrast, ATCC Walker 256 cells administered via either the internal carotid artery injection or direct inoculation into the striatum did not significantly alter the number of GFAP labelled cells when compared to the same location in culture medium inoculated brains (Fig. 3.5A, B).

GFAP immunoreactivity was absent within the tumour masses for both the CRCTU and ATCC Walker 256 internal carotid artery models indicating the absence of astrocytes within the tumours (Fig. 3.5C and D). However, the single tumour that grew 10 weeks following internal carotid artery injection of ATCC Walker 256 tumour cells showed an increase in GFAP labelled cells in the peritumoral area (Fig. 3.5D) and some infiltrating labelled cells within the periphery of the tumour. The astrocytes surrounding the tumour mass exhibited short, blunt, thickened processes, with the flattened cells creating a limiting rim (Fig. 3.5D). The tumours that grew 9 days following internal carotid artery injection of CRCTU Walker 256 cells within the lateral ventricles, had limited contact with the neuropil and lacked the GFAP positive astrocytic border that was evident around the ATCC Walker 256 tumour (Fig. 3.5C).

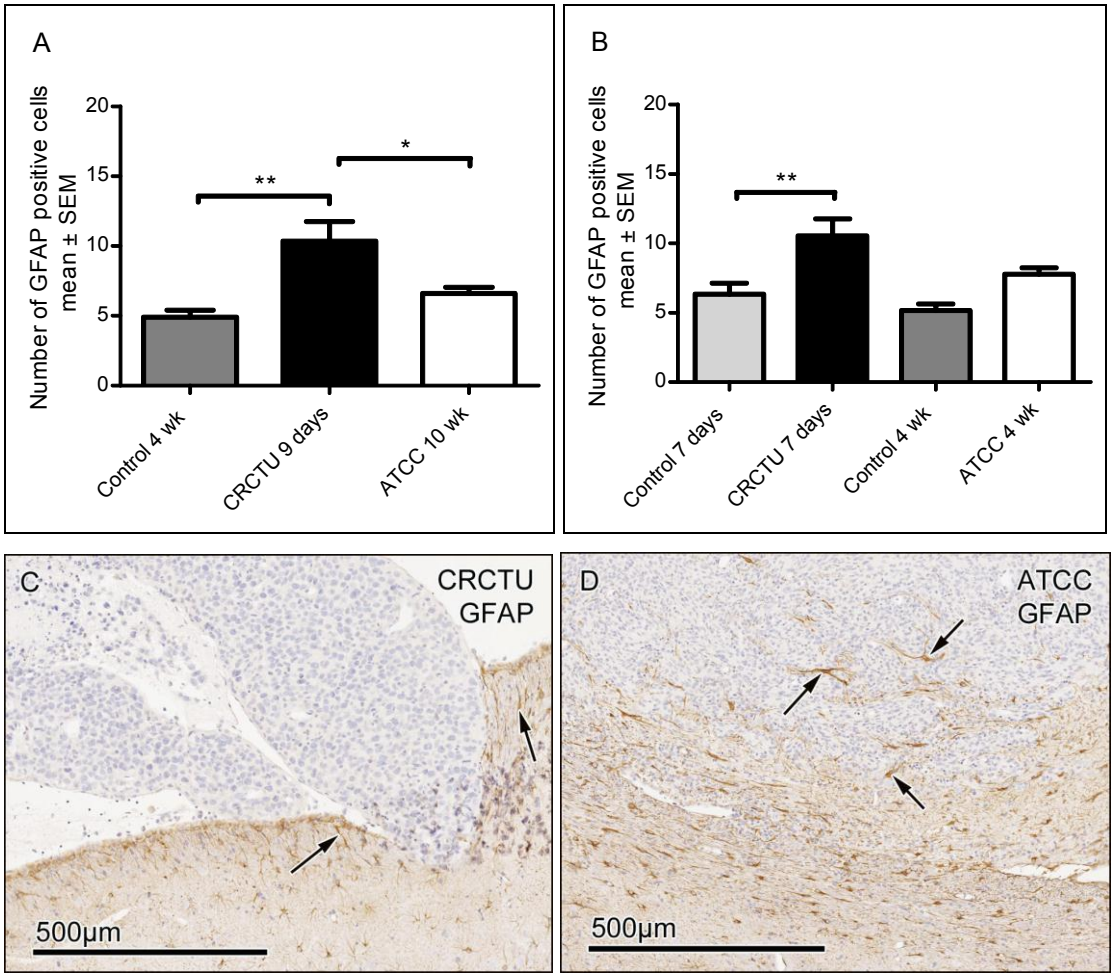

Figure 3.5 GFAP immunoreactivity in metastatic brain tumour models

(A) Graph showing the average number of GFAP positive cells in 4 areas of the cortex (0.0678 mm^2) in animals injected with culture medium, or injected with CRCTU or ATCC Walker 256 cells into the internal carotid artery; **p<0.01; *p<0.05 (B) Graph showing the average number of GFAP positive cells in 4 areas of the striatum (0.0678 mm^2) following direct inoculation of culture medium, CRCTU or ATCC Walker 256 cells into the brain; **p<0.01 (C) GFAP immunostained section 9 days following CRCTU Walker 256 internal carotid artery injection, showing absence of staining within the tumour, but showing GFAP labelled cell in peritumoral area (arrows) (D) Brain section stained for GFAP 10 weeks following carotid ATCC Walker 256 tumour cell injection. Labelled astrocytes (arrows) are seen in the peritumoral area and in the periphery of the tumour mass between the tumour cells

Tumour cell inoculation caused an increase in the number of microglia, as indicated by IBA1 labelling, in the cortex of brains 9 days following internal carotid artery injection of CRCTU Walker 256 cells in comparison to control brains (Fig. 3.6A). Similarly, 7 days after direct injection of CRCTU Walker 256 cells into the brain, there was a significant increase in IBA1 positive cells in the striatum surrounding the

tumour mass, when compared to the same location in the culture medium control group ($p<0.01$; Fig. 3.6B). However, the increase in microglia seen with CRCTU Walker 256 cell inoculation, was not replicated by ATCC Walker 256 cells when injected into the internal carotid artery or inoculated directly into the striatum (Fig. 3.6A and B).

Examination of brain sections immunolabelled for IBA1 showed a distinct pattern of staining for each Walker 256 cell type. The CRCTU tumours showed sparse but specific discrete labelling of infiltration by microglia (Fig. 3.6C). In contrast, the ATCC tumour showed more widespread ill-defined labelling throughout the tumour mass (Fig. 3.6D). Furthermore, there was a halo of IBA1 labelled cells surrounding the tumour mass following internal carotid artery inoculation of ATCC Walker 256 cells, a feature that was not present surrounding the CRCTU Walker 256 induced tumours (Fig. 3.6C and D).

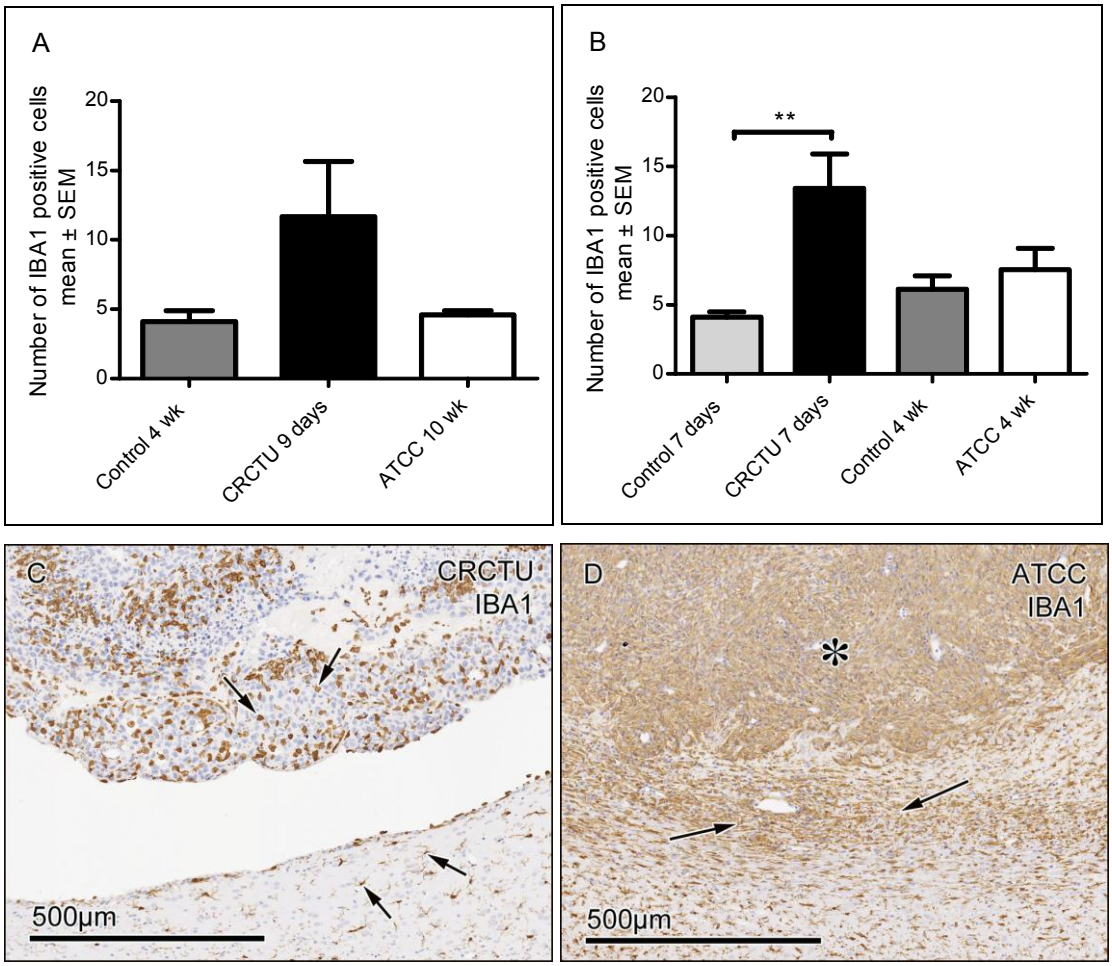

Figure 3.6 IBA1 immunoreactivity in metastatic brain tumour models

(A) The average number of IBA1 positive cells /0.0678 mm^2 of the cortex in animals injected with culture medium, CRCTU or ATCC Walker 256 breast carcinoma cells into the internal carotid artery (B) The average number of IBA1 positive cells /0.0678 mm^2 of the striatum following direct inoculation of culture medium, CRCTU or ATCC Walker 256 cells into the brain; **p<0.01 (C) IBA1 immunostained brain 9 days following internal carotid artery inoculation with CRCTU Walker 256 cells, arrows showing labelled cells dispersed between cancer cells and in the peritumoral area (D) IBA1 immunostained brain section showing extensive labelling within the tumour mass (asterisk) and in the peritumoral area (arrows) 10 weeks following ATCC Walker 256 breast carcinoma cell injection into the internal carotid artery

3.5 Discussion

In the current study, Walker 256 cells obtained from the CRCTU had potent tumorigenic properties when compared to the ATCC Walker 256 breast carcinoma cells. Evidence of this includes the substantially increased incidence of tumour growth and tumour volume after CRCTU Walker 256 inoculation in the two tumour models used in this study, as well as the fact that only CRCTU Walker 256 internal carotid artery injected animals developed tumours in the eye, temporalis muscle and lung. It has been shown in previous studies that different tumour cell lines cloned from the same neoplasm may have different tumerogenic properties when implanted in vivo (Kripke 1978; van Lamsweerde 1983). However, cell lines developed from a single mouse mammary tumour that showed differing culture morphology and growth characteristics in vitro, resulted in tumours that displayed similar histology to each other and comparable tumerigenicity when injected into syngeneic hosts (Dexter 1978).

Despite the fact that both populations of Walker 256 breast carcinoma cells were obtained from reputable tumour cell banks that described the Walker 256 cell line as tumorigenic in Wistar rats, there was considerable variability in both their growth behaviour in vivo and morphology in vitro. ATCC has been instrumental in the push to develop a standard method of cell line verification involving short tandem repeat profiling along with the development of a database of short tandem repeat profiles for commonly used cell lines (Barallon 2010; A.T.C.C.S.D. Organisation 2010).

Control of cancer cell tumorigenicity has been extensively studied, predominantly in relation to genetic control of cancer growth in vivo. For example, p75 has been linked to reduced neuroblastoma tumorigenicity (Schulte 2009). However, characteristics of tumour cells in culture have also been investigated, with shorter doubling time, reduced monolayer density, poor motility and lower incidence of focus formation in vitro linked to decreased tumorigenicity of cell lines when used in vivo (Reynolds 1987; Gildea 2000), although these experiments were generally comparing different

cell lines. In contrast, the current study aimed to determine the differences between the same cell line obtained from two different sources.

The CRCTU Walker 256 breast carcinoma cells, found to be more tumorigenic than their ATCC counterparts, showed darker nuclear staining and increased nucleus to cytoplasm ratio when compared to the flatter more eosinophilic ATCC Walker 256 cells. There have been few previous studies to determine the relationship between cell morphology and cancer cell tumorigenicity. Further investigation is required to determine if the characteristics observed in this experiment are related to the tumorigenicity of the cells described. Furthermore, previous studies have suggested that behaviour of cancer cell lines in vitro is poorly correlated with tumorigenicity in vivo (Reynolds 1987). Despite this, in the current study morphological features seen in vitro for Walker 256 cells from both the CRCTU and ATCC were closely associated with the morphology evident in vivo.

There are many plausible explanations for the differential characteristics evident for CRCTU and ATCC Walker 256 breast carcinoma cells in this study. It is possible that variations in storage methods, extended culture times and high passage number may have contributed to the differences seen in the same cell line obtained from the CRCTU and the ATCC. Immortalised tumour cell lines evolve over time in animal models where malignancies are induced by inoculation with a homogenous population of tumour cells (Poste 1982b). Conversely, human neoplastic tissue is not a uniform entity. Within a tumour mass, there exists various heterogeneous subpopulations of tumour cells with different metastatic potential and diverse propensity to metastasise to various organs (Fidler 1978; Poste 1982a).

Tumour cells harvested from a neoplasm in vivo have been known to develop characteristics over time in vitro that are distinct from those evident in the original cancerous tissue (van Lamsweerde 1983). The proposed reason for this phenotypic change is that more aggressive or mitotic properties are favoured by clonal selection

in vitro, with highly metastatic varieties more phenotypically stable (Chambers 1981; Hiraiwa 1997). Long term passage of Walker 256 cells has previously been shown to alter chemotactic behaviour in vitro (Oda 1984).

Walker 256 carcinoma is rat mammary tumour cell line that originally occurred spontaneously in a pregnant albino Wistar rat (Buffon 2007). The Walker 256 cell line has been used previously to establish experimental brain metastases through an internal carotid artery injection and direct implantation into the cerebral cortex (Blasberg 1984a; b; Blasberg 1986; Hiesiger 1986; Jamshidi 1992).

Tumour growth evident following both inoculation methods of CRCTU Walker 256 cells showed larger tumour volume in a shorter period of time, when compared to previous experiments described in the literature using the Walker 256 cell line, although the incidence was comparable (Hasegawa 1979; Hasegawa 1983; Morreale 1993; Felix 2012). In contrast, the ATCC Walker 256 cells showed a much lower incidence and longer incubation period required to form only a single tumour when compared to these previous studies. Therefore, neither the CRCTU, nor the ATCC Walker 256 breast carcinoma cells behaved exactly as previously studies have described, although the CRCTU population were more analogous to the literature.

Despite the consistency of the direct injection model of tumour induction, the ATCC Walker 256 cells did not grow any tumours through the use of this method. Thus the extravasation process through the BBB is not the limiting factor for ATCC Walker 256 tumour growth in the brain. Furthermore, 11% of animals grew metastatic brain tumours 10 weeks following ATCC Walker 256 inoculation into the internal carotid artery, meaning that at least some of the tumour cells were able to complete the extravasation process.

The CRCTU Walker 256 inoculated animals for both the internal carotid artery and the direct inoculation model showed a significant increase in albumin immunoreactivity when compared to the culture medium group. It is likely that albumin immunoreactivity was increased in response to the substantial tumour growth evident in the CRCTU Walker 256 tumour inoculated groups and subsequent angiogenesis. Angiogenic blood vessels that grow within brain metastases of breast cancer are more permeable than BBB microvessels, as they are characteristic of the breast tissue origin of the tumour cells (Cornford 1992; Shuto 2008). The process of angiogenesis has been shown to take place once tumours have exceeded 2mm in diameter (Blasberg 1984a; Fidler 1990; Zhang 1992).

The ATCC tumour cell inoculated animals only grew one tumour in either model of metastatic brain tumour induction, which was not sufficient to cause a significant difference in albumin immunoreactivity from vehicle level and thus did not increase the permeability of the BBB. This shows that the presence of tumour cells with low tumorigenicity in the brain microcirculation do not cause an inflammatory reaction disrupts the normal function of the BBB. Furthermore, ATCC Walker 256 localisation in the neuropil of the striatum did not cause long term damage to the brain sufficient to increase the permeability of the BBB 4 weeks following direct injection.

A rim of reactive glial cells is often evident surrounding metastatic brain tumours in human surgical tissue (Zhang 1995), as was also apparent surrounding tumours grown in this study. The pattern of glial cell reaction was different surrounding CRCTU and ATCC Walker 256 tumours that grew following internal carotid artery inoculation. The location of CRCTU tumours within the lateral ventricles may be the cause of these differences, as the mass is in less direct contact with the neuropil. In contrast, the single tumour that grew 10 weeks following internal carotid artery inoculation of ATCC Walker 256 cells, showed much more extensive microglial infiltration along with increased microglia and astrocytes surrounding the tumour. Astrocytes and microglia may proliferate and become activated in response to contact with serum

proteins, such as albumin which are present in oedematous fluid that accumulates around the tumour (Jamshidi 1992; Hooper 2009).

The low tumorigenicity of ATCC Walker 256 cells may be the reason that these cells did not show the same influence on the brain microenvironment as CRCTU Walker 256 growth. This is demonstrated by the significant increase in IBA1 and GFAP labelled cells following both internal carotid artery and direct injection of CRCTU Walker 256 tumour cells when compared to the culture medium injected groups. However this phenomenon was not evident following ATCC Walker 256 tumour inoculation for either model used in this study. Thus, the presence of low tumorigenicity cancer cells in the brain microcirculation or the neuropil, did not show significant interaction with the host microenvironment.

In conclusion, this study has demonstrated that the Walker 256 tumour cells obtained from two reputable sources have different tumorigenicity, growth characteristics and interactions with the host brain. Such variability should be considered when comparing studies using the same cell line obtained from different sources.

4 Walker 256 tumour cells increase Substance P immunoreactivity locally and modify the properties of the blood-brain barrier during extravasation and brain invasion

4.1 Abstract

It is not yet known how tumour cells traverse the blood-brain barrier (BBB) to form brain metastases. Substance P (SP) release is a key component of neurogenic inflammation which has been recently shown to increase the permeability of the BBB following CNS insults, making it a possible candidate as a mediator of tumour cell extravasation into the brain. This study investigated the properties of the BBB in the early stages of tumour cell invasion into the brain, and the possible involvement of SP. Male Wistar rats were injected with Walker 256 breast carcinoma cells via the internal carotid artery and euthanised at 1, 3, 6 and 9 days post tumour inoculation. Culture medium-injected animals served as controls at 1 and 9 days. Evidence of tumour cell extravasation across the BBB was first observed at three days post inoculation, which corresponded with significantly increased albumin ($p<0.05$) and SP immunoreactivity ($p<0.01$) and significantly reduced endothelial barrier antigen labelling of microvessels when compared to culture medium control animals ($p<0.001$). By day 9 after tumour cell inoculation, 100% of animals developed large intracranial neoplasms that had significantly increased albumin in the peri-tumoral area ($p<0.001$). The increased SP immunoreactivity and altered BBB properties at three days post inoculation that coincided with early tumour invasion may be indicative of a mechanism for tumour cell extravasation into the brain. Thus, extravasation of tumour cells into the brain to form cerebral metastases may be a SP-mediated process.

4.2 Introduction

It is estimated that cancer is responsible for more than 6 million deaths each year (WHO 2002). Approximately 20-40% of patients suffering from systemic cancer will also have a metastatic brain tumour at autopsy, whereas clinical studies generally report an incidence of about 10-20% (Posner 1978; Cifuentes 1979; Soffietti 2002; Gavrilovic 2005; Lin 2008a). Breast cancer is one of the most common malignancies to metastasise to the brain, occurring in late stages of the disease (Lorger 2010). Nearly 50% of patients with metastatic triple-negative breast cancer, which do not express estrogen receptors, progesterone receptors or human epidermal growth factor receptor 2, will develop a metastatic brain tumour, with a median survival time of 4.9 months from diagnosis (Lin 2008b). There is a predilection for metastatic brain tumours and central nervous system (CNS) recurrence amongst node positive, estrogen receptor negative, young patients with high-grade breast cancer, with no evidence that there is any benefit from an early diagnosis and treatment for their intracranial malignancy (Pestalozzi 2006). Therefore there is a pressing need for improved treatment and prevention strategies for brain metastatic breast cancer.

Metastatic brain tumours are caused by cancerous cells that detach from their primary site, migrate through the blood stream, attach to cerebral endothelium and cross the BBB to colonise the brain (Marchetti 2003). Despite the high incidence and clinical impact of metastatic brain tumours, the exact mechanism of tumour cell extravasation across the BBB has not been elucidated. This information is vital for the development of treatments that aim to prevent invasion of the CNS by cancer cells.

The BBB is a dynamic structure composed of endothelial cells joined by tight junctions, encompassed by pericytes and supported by astrocytic end feet (Hawkins 2005). This arrangement is able to be modified and respond to external stimuli or disease states, allowing for differential barrier properties. For example, macrophages are able to infiltrate the brain neuropil through an intact BBB, which remains impermeable to serum proteins (Schackert 1988b).

Extravasation of tumour cells through the BBB is most likely a multifactorial process, and few studies have investigated substances proposed to be involved in metastatic spread to the brain. Vascular endothelial growth factor (VEGF) has been considered as a driving factor for tumour cell extravasation, using the VEGF receptor antagonist cediranib AZD2171 (Juanyin 2009). Other substances that are under investigation are serine proteases released by melanoma cells, which disrupt tight junctions between cerebral capillary endothelial cells (Fazakas 2011) and matrix metalloproteinase 2 (MMP2), as breast cancer cells transfected with tissue inhibitor of MMP2 have been shown to have reduced propensity for metastases to the brain (Mendes 2007).

Substance P (SP) is an excitatory tachykinin that is predominantly released from primary sensory nerve endings in the CNS, but is also localized in brain endothelial cells (Cioni 1998; Ribeiro-da-Silva 2000). It is a potent mediator of neurogenic inflammation and preferentially binds to NK1 receptors to induce microvascular leakage in the brain and throughout the body (Harrison 2001; Nimmo 2004). Neurogenic inflammation is characterized by the release of vasoactive peptides from neurons, driving vasogenic oedema in the CNS through increased BBB permeability. In vitro, SP treatment of rat cortical capillary endothelial cells causes an increase in intracellular free calcium ions and is postulated to cause modulation of the BBB by endothelial cell contraction (Paemeleire 1999). Furthermore, cytokine stimulated endothelial cells caused release of SP, stimulating a NK1 receptor dependent increase in BBB permeability (Cioni 1998; Annunziata 2002). Moreover, SP alters expression of tight junction proteins by a NK1 receptor mediated mechanism (Annunziata 1998; Lu 2008).

SP has also been implicated in cancer cell migration (Drell 2003; Lang 2004), chemo-attraction (Ruff 1985), DNA synthesis, replication and cytokine secretion (Palma 1999). NK1 receptors are expressed by many tumour cell lines (Prasad 2007; Munoz 2008; Huang 2010). Antagonism of the actions of SP on tumour cells in vitro has been an active area of research in recent times. NK1 receptor antagonists have been found

to inhibit tumour cell mitogenesis and induce apoptosis of human melanoma, breast cancer, glioma, neuroblastoma, colon cancer and prostate cancer cell lines in cell culture studies (Munoz 2004b; Munoz 2005a; Huang 2010; Munoz 2010b; Mayordomo 2011). Many tumour cells release SP in culture, acting in an autocrine fashion to promote survival (Mayordomo 2011). However, because these studies are performed in vitro, the effect of SP secretion on the peri-tumoral tissue in vivo is yet to be investigated. Furthermore, studies comparing in vitro brain metastatic invasion models to their in vivo counterparts demonstrated that there was a poor correlation between the two (Lorger 2011).

Endothelial barrier antigen (EBA) is a protein triplet used as an immunological marker of the BBB in rats. Although its role in the structure and function of the BBB is not entirely understood, it has been shown that its immunological deactivation by intravenous injection of a monoclonal antibody to EBA, results in increased permeability of the BBB to horseradish peroxidase through the widening of junctional complexes and an increase in cytoplasmic vesicles (Ghabriel 2000; Ghabriel 2002). Therefore EBA is integral to the normal functioning of the BBB in rats. Furthermore, EBA expression in rat cerebral capillary endothelial cells is altered in models of several pathological conditions that are known to disrupt the BBB, such as traumatic brain injury, stroke and glioma (Lin 2001; Chekhonin 2007; Park 2010).

The actions of SP on both cancer cells and the BBB make it a possible candidate for involvement in the mechanism of tumour cell extravasation into the brain. The aim of this study was to investigate the early interactions of Walker 256 carcinoma cells with the brain vasculature and to elucidate the role of SP in the modification of the BBB in response to tumour invasion.

4.3 Methods

4.3.1 Animals

This project was performed as described in section 2.2 on page 43. Animals were randomly selected for carotid inoculation with Walker 256 cells or injection with culture medium only as controls. Tumour-inoculated animals were sacrificed at 1, 3, 6 and 9 days (n=5 per group). Culture medium-injected animals were sacrificed at 1 and 9 days (n=5 per group).

4.3.2 Cell Culture

Walker 256 rat breast tumour cells were obtained from the Cell Resource Centre for Medical Research at Tohoku University and cultured as described previously 2.1.2 on page 42. The number of cells was calculated using a haemocytometer and then diluted, so that in every 0.2 mL of cell suspension, there was between 10^5 and10^6 cells ready for inoculation.

4.3.3 Internal Carotid Artery Inoculation

The internal carotid artery inoculation procedure has been detailed in section 2.3 page 44.

4.3.4 Tumour Volume

For histological study of the brain, animals were perfused via the heart at 1, 3, 6 and 9 days with 10% formalin under general anaesthesia, then brains were removed and embedded in paraffin wax. The brain was divided into 2 mm coronal blocks in a cranio-caudal sequence. 5 μm sequential sections were cut from each block and used for haematoxylin and eosin (H&E) staining and immunolabelling. The H&E stained sections were scanned (Nanozoomer, Hamamatsu, Hamamatsu City, Japan) and tumour volume calculated as described in section 2.7 page 49.

4.3.5 Immunolabelling

Brain sections were stained for albumin (ICN Pharmaceuticals, polyclonal, 1:20,000), SP (Santa Cruz Biotechnology N-18:9758, polyclonal, 1:2000), NK1-receptor (Biocore Pty Limited AB-N-33AP, polyclonal, 1:8000), and EBA (Chemicon SMI-71R-100, monoclonal, 1:5000). Immunolabelling was done as described in section 2.8 page 49. Objective assessment of the immunolabelling was achieved through colour deconvolution techniques, to reveal the % of DAB in the scanned slides. Immunolabelling was performed on slides from the brain corresponding to a position at 0.8 mm posterior to bregma (Paxinos 1998), the location showing maximal blood vessel invasion by tumour cells at 3 days after tumour cell inoculation.

4.3.6 Immunolabelling analysis

Virtual dissection was completed for all scanned immunolabelled slides. Albumin immunoreactivity was analysed by using stained whole coronal sections of the brain, whereas analysis of substance P and NK1 receptor immunolabelling required images to be taken from the cortex, striatum, the tumours and in the peri-tumoral areas due to the specificity and localised nature of these stains. These exported files were run through colour deconvolution software and expressed as DAB wt % total, using a technique previously described in detail (Harford-Wright 2010; Helps 2012). This process involves the removal of background stining intensity variations (Helps 2012). EBA immunoreactivity was evaluated by counting the blood vessels that were negative for EBA immunoreactivity as a percentage of the total number of blood vessels in the virtually dissected areas. Data were expressed as mean ± SEM. Statistical differences were determined using an unpaired t-test (for 2 groups) or one way analysis of variance (ANOVA) followed by a Bonferroni post test (for more than 2 groups), as applicable.

4.4 Results

Invasion of microvessels by tumour cells was first evident in 60% of animals at 3 days after tumour inoculation (Table 4.1). Thus, tumour cells begin passing from the circulation, through the BBB and into the brain tissue between 1 and 3 days after inoculation in this model of secondary brain tumours. Tumour cell invasion across the BBB occurred in the right cerebral hemisphere, ipsilateral to the injected carotid artery, mainly in the brain segment located at a coordinate 0.8mm posterior to bregma.

In this study, a tumour mass is defined as the formation of more than 3 layers of tumour cells around the circumference of microvessels. This was initially seen by 6 days post tumour inoculation in 20% of animals, increasing to 100% by day 9 (Table 4.1). Tumour growth was not allowed to progress past this time point because extensive tumour burden was evident with a mean tumour volume of 36.61 mm^3 (Fig. 4.1A). Although, the most common location of tumour cell invasion of brain microvessels was within the cortex (Fig. 4.1B), large tumour masses were seen within the lateral ventricles (Fig. 4.1C) and in the striatum.

Table 4.1 Tumour Incidence over Time

Time post tumour inoculation	**Percentage of animals showing invasion of microvessels**	**Percentage of animals showing mass lesion**
1 day	0%	0%
3 days	60%	0%
6 days	80%	20%
9 days	100%	100%

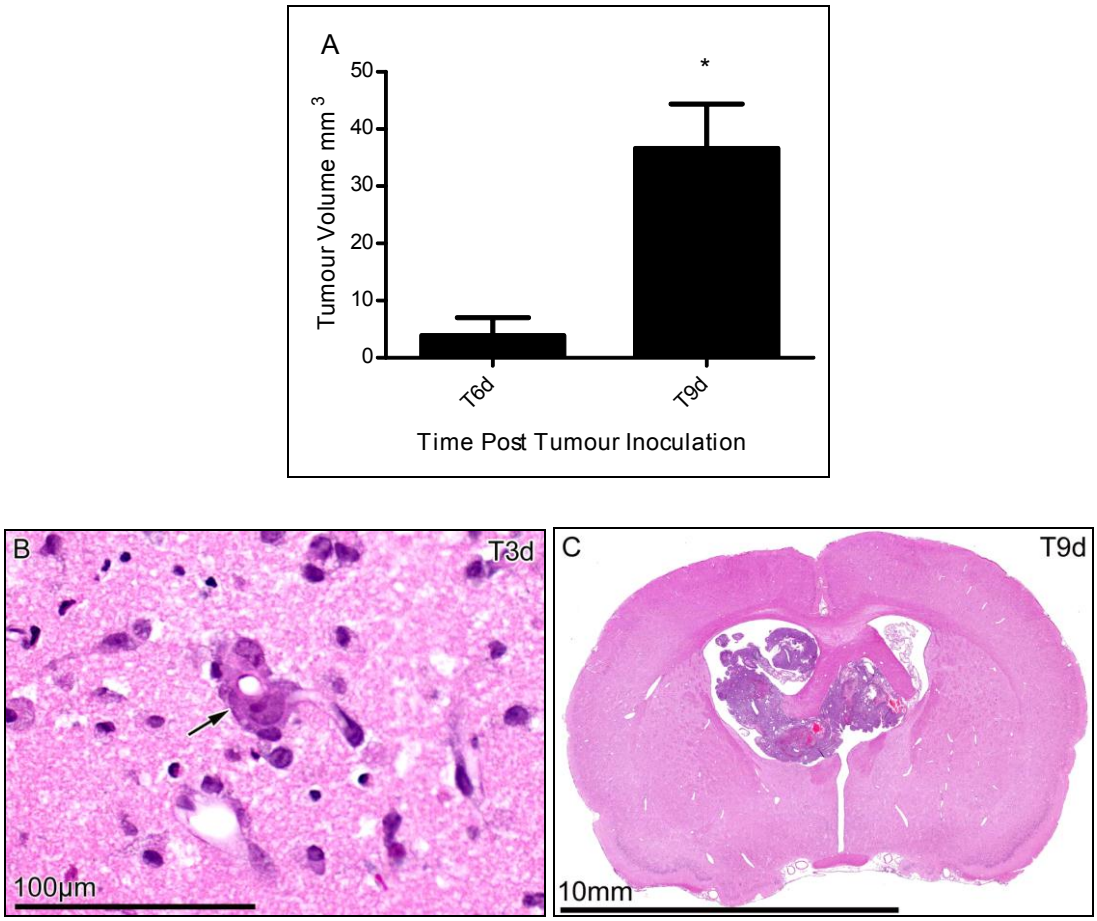

Figure 4.1 Tumour growth over time

(A) Tumour volume in mm^3 over time following tumour (T) inoculation at 6 and 9 days, * $p<0.05$. (B) H&E stained section of cortex 3 days post tumour inoculation (T3d) showing tumour cells around a microvessel (arrow). (C) H&E stained coronal section of rat brain 9 days after tumour cell inoculation (T9d) showing a large tumour mass within the lateral ventricles.

Albumin immunoreactivity was not observed in any of the brains of culture medium-injected control animals at 1 and 9 days post-inoculation (Fig. 4.2A and B), indicating that the injection procedure did not cause any long-term disruption of the BBB. Sections from 9 day control animals were subsequently used in the quantitative analysis. In control animals, albumin immunoreactivity was only seen in the choroid plexus and the meninges (Fig. 4.2B). In tumour-inoculated animals, albumin immunoreactivity in the brain was significantly increased compared to vehicle control levels with widespread staining at 3 and 9 days post tumour inoculation ($P<0.05$ and

$p<0.001$, respectively; Figs. 4.2A, C and E). Six days following tumour inoculation, weaker immunolabelling for albumin was seen where tumour cells had invaded across microvessels (Fig. 4.2D).

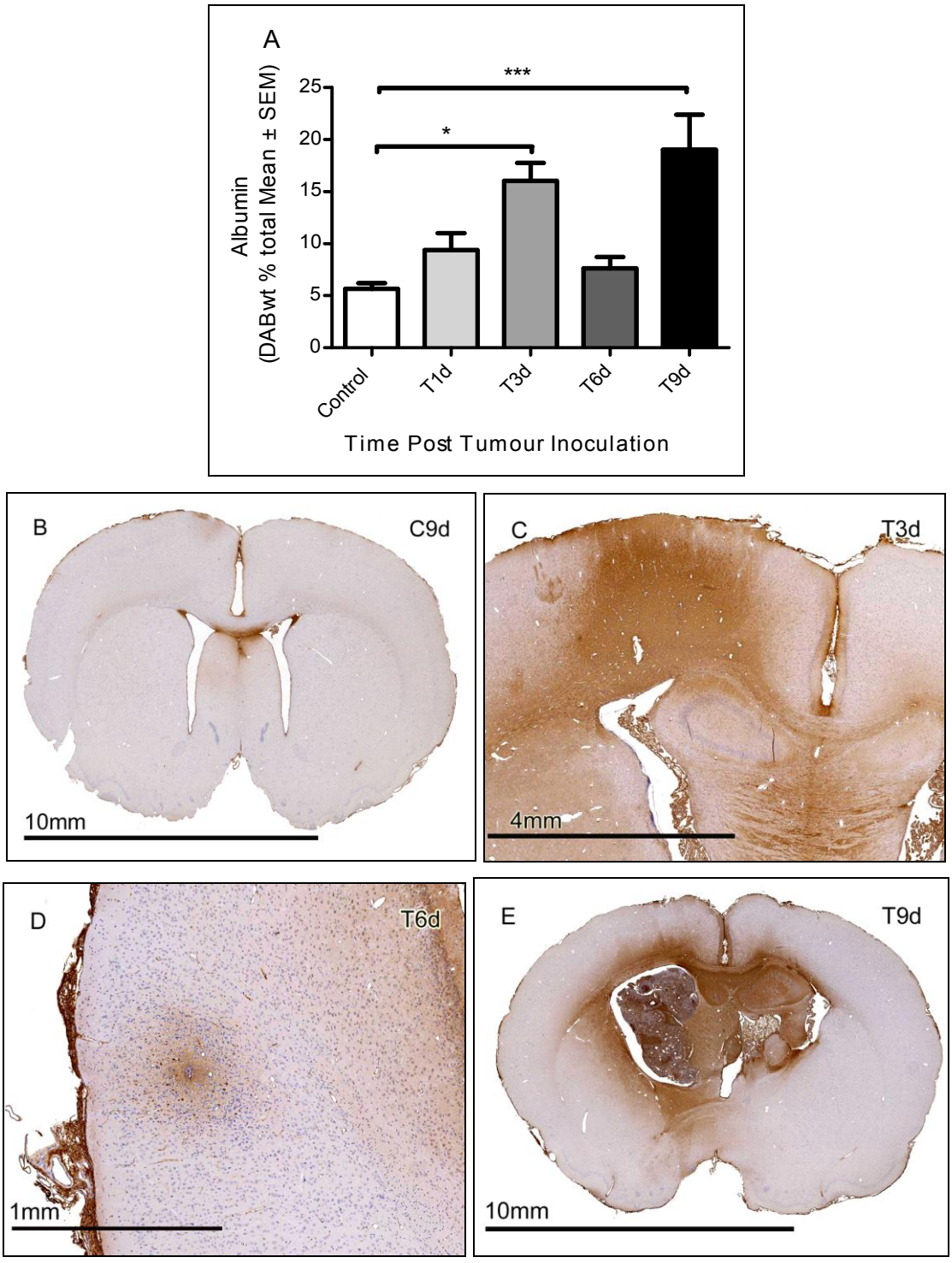

Figure 4.2 Albumin immunoreactivity over time

(A) Albumin immunoreactivity over time post tumour (T) inoculation at 1, 3, 6 and 9 days post-inoculation, compared to culture medium-injected animals, *p<0.05, **p<0.01 (B) Brain coronal section from a culture medium- injected control animal 9 days following surgery (C9d), showing minimal albumin immunoreactivity in the meninges and ependymal lining of the ventricles (C) Brain coronal section 3 days post tumour inoculation (T3d) showing widespread albumin immunoreactivity, appearing as dark brown reaction product, indicating breakdown of the BBB (D) Albumin

immunolabelled section of cortex 6 days post tumour inoculation (T6d) showing focal albumin immunoreactivity (E) Coronal section of rat brain, 9 days post-inoculation (T9d) stained for albumin showing extensive labelling surrounding an intra-ventricular tumour

EBA staining at 3 days was absent in tumour-invaded blood vessels when compared with non-invaded blood vessels within the same brain $p<0.001$ (Fig.4.3). Absence of EBA staining is indicative of compromised BBB function. Almost all microvessels in culture medium-injected animals were labeled for EBA. In well-developed tumours, EBA immunolabelling was absent in 100% of blood vessels within the tumour, indicating that these blood vessels no longer retain the characteristics of the BBB. EBA labelled blood vessels were present in the peri-tumoral area, with similar incidence of labeled vessels in the same location in culture medium control brains but significantly greater unlabelled vessels in the tumours (Fig. 4.3C and D).

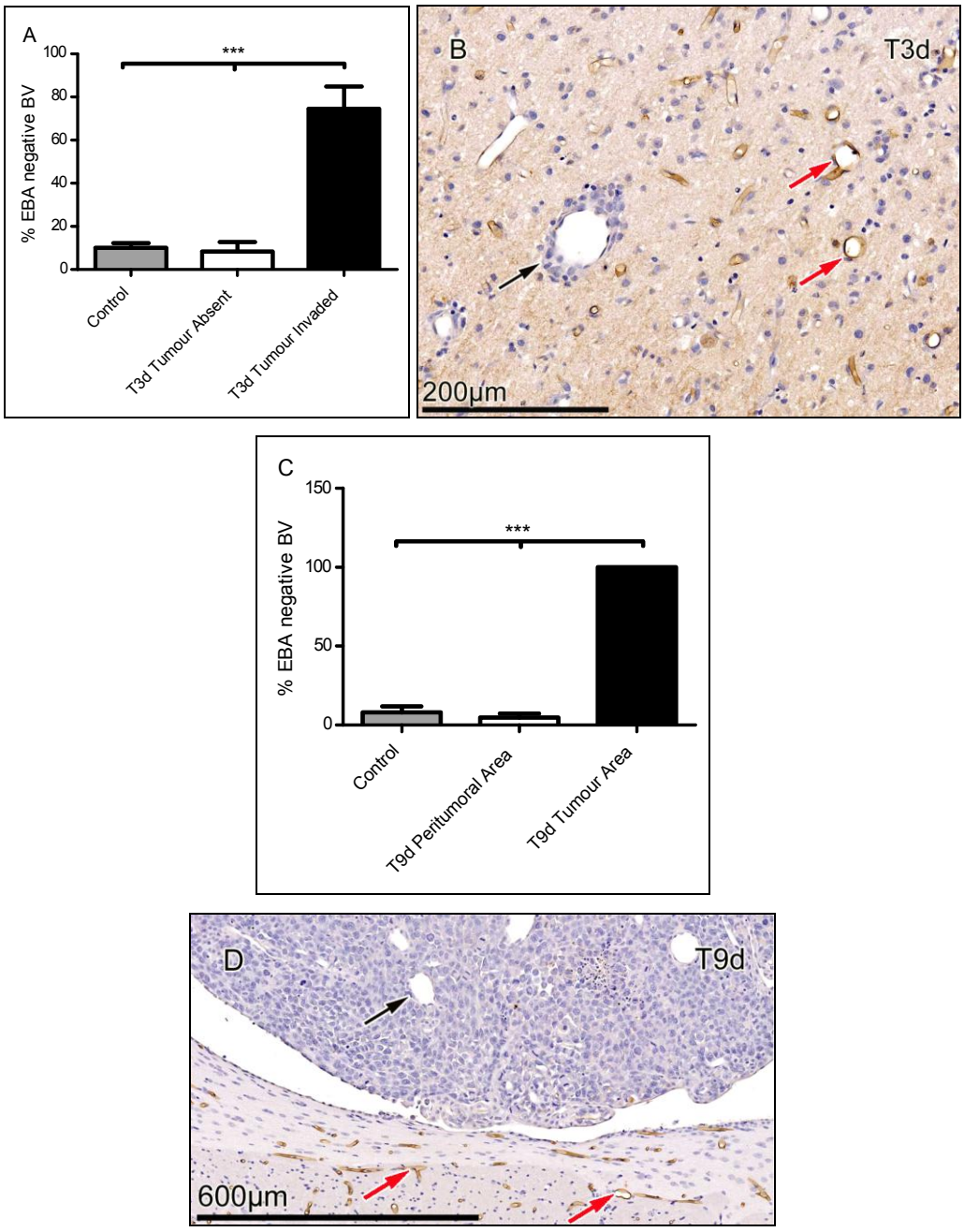

Figure 4.3 Endothelial barrier antigen (EBA) immunoreactivity

(A) Graph showing % of EBA unlabelled blood vessels (BV), in control and tumour-inoculated animals at 3 days (T3d), in brains were tumour masses were absent or present, *** $p<0.001$. (B) EBA immunolabelled section of cerebral cortex 3 days post tumour inoculation (T3d) showing tumour invaded blood vessel with absent EBA labelling (black arrow), whilst surrounding non-invaded blood vessels show clear EBA immunoreactivity (red arrows). (C) Graph showing the percentage of unlabelled vessels for EBA at 9 days in culture medium-injected control and tumour inoculated animals in the peri-tumoral and tumour areas, ***$p<0.001$ (D) EBA immunolabelled section showing prominent labelled vessels in the peri-tumoral area (red arrows) but unlabelled vessels within the tumour (black arrow) in a tumour inoculated animal 9 days following surgery (T9d)

Similar to the increase in albumin immunoreactivity associated with tumour cell invasion, an increase in SP immunoreactivity was evident in the right cortex at 3 days following tumour inoculation (Fig. 4.4A and B). Due to the variability of SP staining among brains, the left hemisphere cortex was used as an internal control for each brain. Furthermore, a significant increase in SP immunoreactivity was apparent surrounding tumour invaded blood vessels when compared to blood vessels from culture medium control animals ($p<0.01$; Figs. 4.4C, D and E). There was no alteration in NK1 receptor immunoreactivity within or surrounding the tumours (data not shown), suggesting that receptor down-regulation was not occurring. By 9 days post tumour inoculation, with large tumour mass development, there was a significant increase in SP immunoreactivity in the peri-tumoral area when compared to the same location in culture medium control animals (Fig. 4.5).

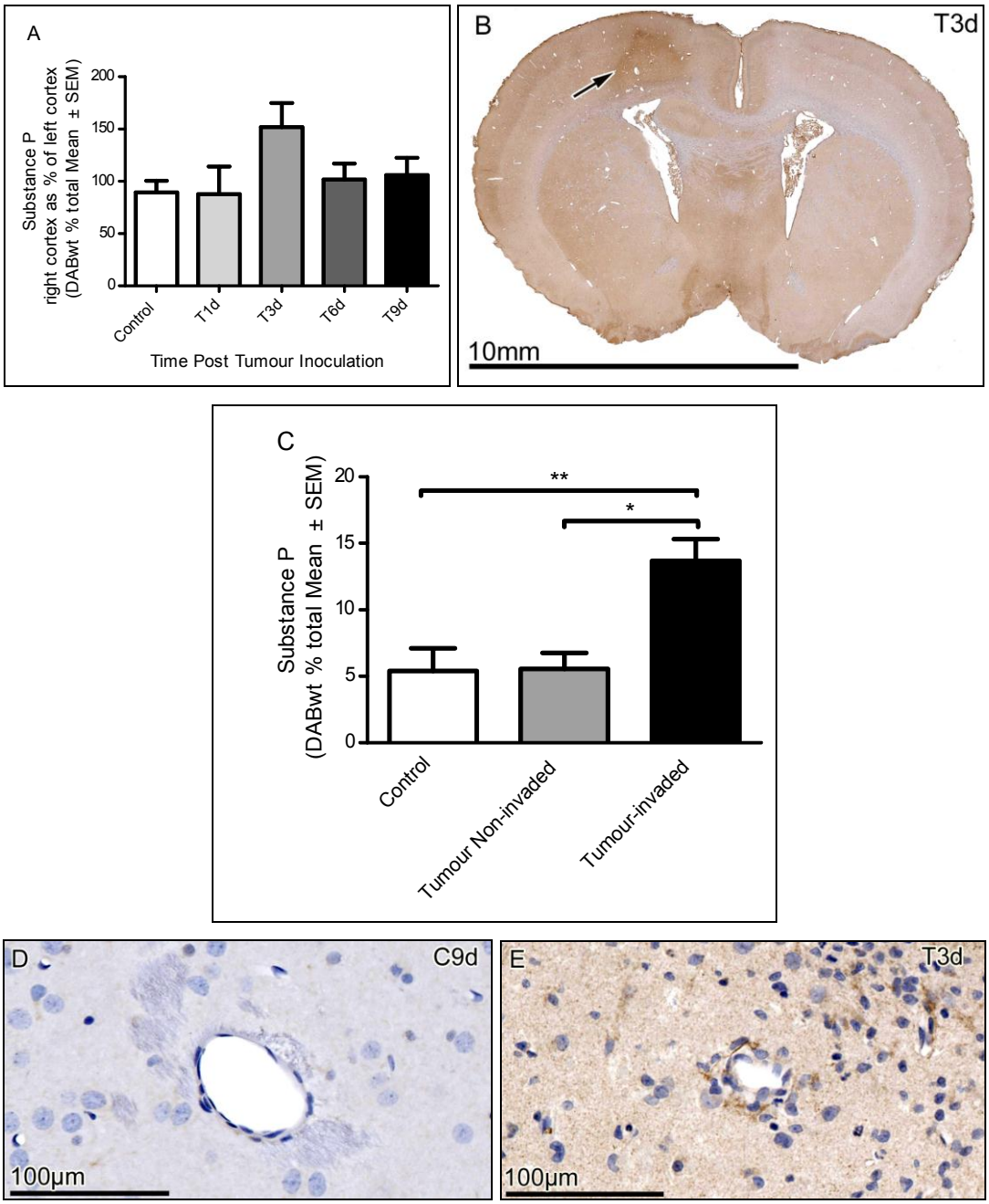

Figure 4.4 Substance P (SP) immunoreactivity with tumour invasion

(A) SP Immunoreactivity over time post tumour inoculation, right cortex as a percentage of the left cortex (B) SP immunolabelled coronal section 3 days post tumour inoculation showing increased immunoreactivity in the right cortex (arrow) (C) Graph showing SP immunoreactivity 3 and 6 days post tumour inoculation in areas of tumour-invaded compared with non-invaded blood vessels from the same animal and culture medium-injected control animals, *$p<0.05$, ** $p<0.01$ (D) Brain section from culture medium control animal 9 days following surgery (C9d), showing faint SP immunoreactivity around cortical blood vessels (E) Brain section from a tumour inoculated animal 3 days after inoculation (T3d) showing increased SP immunoreactivity in the neuropil around cortical microvessels.

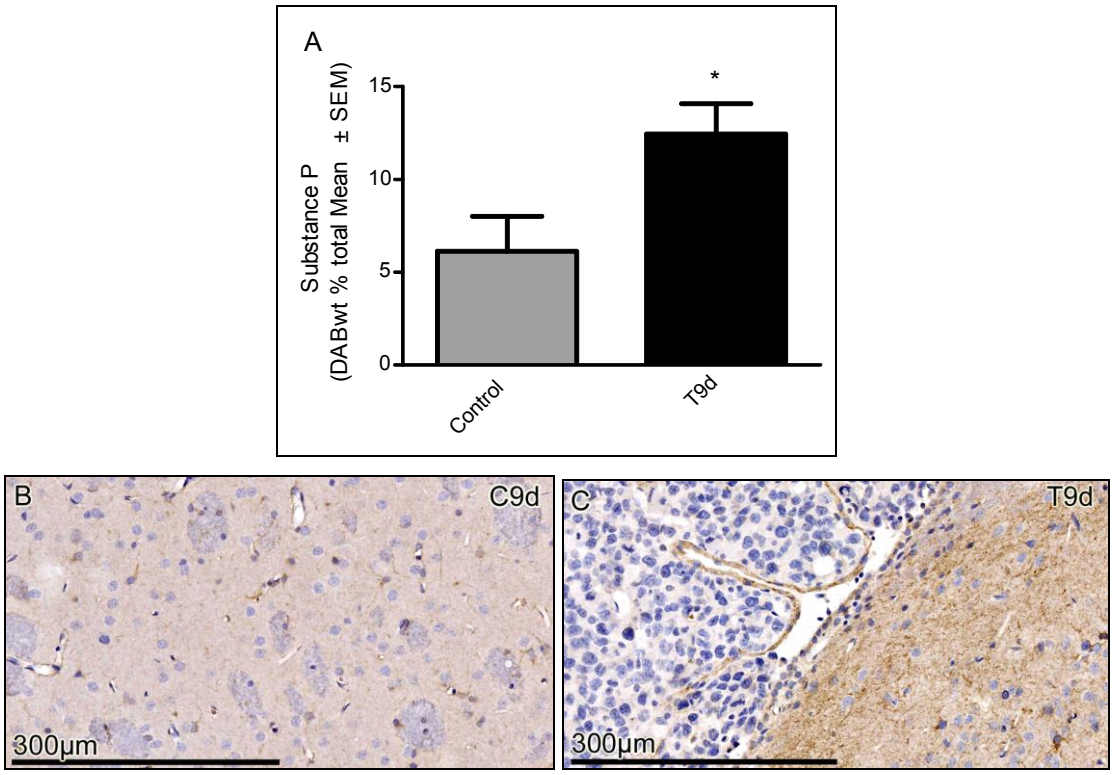

Figure 4.5 Substance P (SP) immunoreactivity surrounding established brain metastases

(A) Graph showing SP Immunoreactivity in the peri-tumoral area 9 days following tumour inoculation compared to control animals, *$p<0.05$ (B) SP immunolabelled section showing the striatum 9 days post culture medium injection (C9d), showing no increase in SP immunoreactivity (C) SP immunostained section showing a tumour and the increased SP staining in the peri-tumoral area 9 days post tumour inoculation (T9d)

4.5 Discussion

The model of brain metastases used in this study produced tumour invasion across the BBB by day 3 following internal carotid artery inoculation. This time frame is consistent with other studies using either internal or common carotid artery injection methods of tumour induction (Ballinger 1979; Kienast 2010; Lorger 2011). Despite the fact that previous studies have shown that tumour cells reach the brain microvasulature within 15 minutes of internal carotid artery inoculation, tumour cells still take at least 3 days to invade the BBB (Lorger 2010). Thus, tumour cells appear to remain arrested in the vasculature when metastasising to the brain compared to other organs like the liver or adrenals, which have been shown to have a tumour extravasation time of 6 hours following intravenous inoculation of lung cancer cells in nude mice (Paku 2000). In that study, initial tumour cell replication was located along the inside of blood vessel walls which has been well established in the literature as the method of tumour growth, regardless of tumour cell type or method of inoculation (Carbonell 2009).

The fate and behavior of tumour cells within the circulation prior to forming brain metastases has been the subject of several investigations. Some studies showed that tumour cells replicate within the brain microvasculature before invading the brain across the BBB, with one study reporting arrested non-proliferating breast cancer cells by 33 days post intra-cardiac inoculation that had not been removed from the circulation (Heyn 2006; Lorger 2011). On the other hand, it has been shown that human lung and melanoma tumour cells that do not cross the BBB before day 3 following tumour inoculation do not go on to form brain metastases in an experimental setting (Kienast 2010). The presence of intravascular tumour cells was not investigated in the present study as all animals were fixed by intracardiac perfusion before tissue processing.

The intra-ventricular location of the tumours growth in the current study is not uncommon, with several other studies reporting tumour growth within the lateral

ventricles along with the more common malignant growth within the cortex (Schackert 1988b; Schackert 1990). The preference for growth in the choroid plexus may be a specific feature of the Walker 256 cell line, although the extravasation of tumour cells also occurred across the cortical microvessels. Other investigators reported that 58.3% of animals inoculated with Walker 256 cells into the internal carotid artery demonstrated choroid plexus and meningeal tumours (Hasegawa 1983).

Coinciding with the first evidence of Walker 256 tumour cell invasion across the BBB at 3 days post tumour inoculation, there was widespread albumin immunoreactivity and an increase in SP immunoreactivity. The invaded microvessels showed significantly increased perivascular SP immunoreactivity along with a significantly reduced number of EBA labelled blood vessels. This indicates that tumour cell extravasation across the BBB causes a modification to the properties of the BBB. It is possible that the altered BBB permeability seen in the current study is induced by the increased SP levels and the process of neurogenic inflammation. Alternatively, tumour cells may preferentially invade brain microvessels that lack EBA immunoreactivity and thus are more susceptible to barrier disruption.

The role of neurogenic inflammation and SP in tumour cell extravasation across the BBB has not been investigated previously. However, the effects of neurogenic inflammation in other BBB altering pathologies has been well characterised. SP is known to be associated with BBB dysfunction leading to oedema in acute brain injury (Donkin 2011; Turner 2011). The effects of SP on BBB permeability may be a mechanism by which tumour cells modify the BBB to allow for extravasation. The increase in SP seen in this study may have originated from cerebral endothelial cells, being triggered to secrete SP upon interaction with tumour cells, or it may have been secreted by the tumour cells themselves (Cioni 1998; Mayordomo 2011).

The increased SP immunoreactivity evident with tumour cell extravasation supports further investigation into the possible use of NK1 antagonists as a preventative

treatment for brain metastatic breast cancer. The effects of SP on increased tumour cell mitogenesis (Luo 1996; Friess 2003; Munoz 2004b; Munoz 2005b; Munoz 2007; Munoz 2008; Rosso 2008; Huang 2010), migration (Ruff 1985; Drell 2003) and angiogenic initiation (Ziche 1990; Wang 2009) have been well characterized, all effects that lead to increased propensity for metastases (Munoz 2011a). Therefore, it is not only possible that NK1 receptor antagonist treatment is able to reduce BBB permeability and prevent tumour cell extravasation into the brain, but may also provide additional protection from SP mediated initiation of a tumour cell phenotype with increased metastatic potential.

This is the first study to report alterations in EBA labelling of brain microvessels in response to tumour cell extravasation across the BBB. At early stages of tumour invasion, angiogenesis is yet to occur, thus the lack of EBA staining reflects a modification of the existing BBB rather than the growth of new blood vessels with different properties to the BBB.

Albumin immunoreactivity decreased at 6 days following tumour cell inoculation compared to 3 days post inoculation, but then increased again by day 9. This partial resolution of increased BBB permeability at day 6 implies that the mechanisms of BBB disruption are different at days 3 and 9 post tumour inoculation. It is likely that the increase in BBB permeability, observed at 9 days, was caused by angiogenic blood vessels within the tumour mass. Therefore, the absence of EBA labelling on the angiogenic vessels that develop within the Walker 256 brain metastases at 9 days was to be expected, as secondary brain tumours grow blood vessels that are characteristic of their organ of origin rather than having the BBB characteristics of the host vessels. Previous studies have gathered substantial evidence supporting the theory that blood vessels within metastatic brain tumours are more permeable than the normal cerebral vasculature (Greig 1983; Zhang 1992; Andersen 1998; Lockman 2010).

In contrast, the increase in albumin immunoreactivity at day 3 following tumour inoculation, indicates increased permeability of the host BBB, and is likely to be due to the interaction of tumour cells with endothelial cells of the BBB. This may be consistent with the presumption that tumour cell extravasation into the brain occurs through the paracellular pathway (Kienast 2010; Lorger 2010), and the increased permeability of the BBB may aid tumour cell passage between cerebral capillary endothelial cells. Pranlukast, a leukotriene receptor antagonist, has been successful in reducing brain metastatic colon cancer development in other studies, but only when the BBB was pre-treated with arachidonic acid causing increased BBB permeability (Nozaki 2010). Therefore the modification of the BBB leading to increased permeability may play a role in metastatic brain tumour extravasation in humans. However, no studies have yet investigated preventative treatments for metastatic brain tumours that impede tumour cell extravasation across the BBB under physiological conditions in vivo.

With maximal tumour growth seen at 9 days post tumour inoculation, SP immunoreactivity was increased in the peri-tumoral area. The compressive nature of metastatic brain tumours means that damage to the host microenvironment commonly occurs (Zhang 1997). The increased SP expression surrounding brain metastases may be implicated in peri-tumoral oedema. SP has been linked to vasogenic oedema in the brain and throughout the body following acute injury (Alves 1999; Donkin 2009), and the use of NK1 receptor antagonists ameliorate this effect. A similar mechanism may drive vasogenic oedema formation surrounding metastatic brain tumours. There has been limited experimentation on the in vivo effect of NK1 receptor antagonists on cancer, aiming to inhibit malignant growth and progression. Human glioma cells injected subcutaneously into the flank of nude mice showed a decrease in tumour volume when treated with a NK1 receptor antagonist (Palma 2000). However, this study does not accurately replicate human gliomas as the tumour was grown in a non-neural environment, thus preventing the study of the interactions of the NK1 receptor antagonists with the BBB or the brain microenvironment.

In conclusion, the present study has demonstrated that the properties of the BBB are altered during early stages of tumour cell extravasation, which presents a potential window for therapeutic intervention to prevent the formation of metastatic brain tumours. The increase in SP expression surrounding brain vessels associated with tumour cells, combined with its known effects of increasing BBB permeability, warrants further investigation into the role of SP in the formation of secondary brain tumours.

5 NK1 antagonist treatment is not sufficient to prevent Walker 256 breast carcinoma extravasation and metastatic brain tumour development

5.1 Abstract

Metastatic brain tumours are increasing in incidence, however the exact mechanism by which tumour cells traverse the blood-brain barrier (BBB) remains to be elucidated. It is currently unclear why the BBB is unable to prevent the entry of tumour cells into the brain. Substance P (SP) is an excitatory tachykinin that acts preferentially on NK1 receptors and has been shown to increase the permeability of the BBB. Previous studies have shown that SP is increased in the perivascular area of Walker 256 breast carcinoma invaded microvessels, along with an increase in albumin immunoreactivity. This suggests that SP may be involved in the tumour extravasation process and thus NK1 antagonists may be a promising treatment for the prevention of metastatic brain tumour formation. As such, the NK1 antagonists Emend and NAT were tested in a haematogenic model of breast cancer-induced brain metastasis. Walker 256 breast carcinoma cells were injected into the internal carotid artery and Emend, NAT or saline vehicle treatment were administered on days 0-3 following surgery. Tumour incidence and volume were used to determine the efficacy of the NK1 antagonist treatment. There was no significant difference in tumour incidence or volume with either NK1 antagonist treatment when compared to the vehicle treated group. Therefore, the increase in SP with tumour invasion of brain microvessels reported in previous studies is not the predominate factor driving brain colonisation by cancer cells. It is likely that the extravasation process is multifactorial and that blocking only a single factor is not sufficient to prevent tumour cells from crossing the BBB.

5.2 Introduction

Secondary brain tumours occur in up to 30% of breast cancer cases (Schuette 2004; Hines 2008). The development of increasingly effective targeted therapies for primary breast cancer has resulted in increased survival time following initial diagnosis and consequently an increase in the incidence of metastatic brain tumour. However, even with the best available multimodal treatments, the prognosis remains poor. This is because the location of metastases in the brain and the restricting function of the BBB, making these neoplasms inherently difficult to treat.

Many factors contribute of metastatic progression of breast cancer to the brain, including migration, angiogenesis and extravasation potential of cancer cells. Several studies have shown that specific cancer types have a predilection for metastatic brain tumour formation, for example melanoma, lung cancer and breast cancer (Barnholtz-Sloan 2004; Villa 2011). Furthermore, cancer cells may acquire characteristics, which increase propensity for brain invasion as they develop (Schackert 1988a; Fidler 1990; Schackert 1990; Kusters 2001).

The development of metastasis in the brain is inherently dissimilar to metastases to other organs because of the presence of the BBB. Extravasation into the brain requires cancer cells to pass through the tight junctions between cerebral capillary endothelial cells, and then through the basement membrane surrounding endothelial cells (Petty 2002). The exact mechanism of tumour cell extravasation into the brain is yet to be elucidated and may lead to the development of treatment strategies to prevent brain metastasis.

SP is a potent neurogenic inflammatory mediator, a process which is characterised by passage of plasma proteins accompanied by fluid from the vasculature into surrounding tissue (Harford-Wright 2011). Acting preferentially on NK1 receptors, SP is able to increase the permeability of the BBB via endothelial cell contraction

along with decreased expression of tight junction proteins (Annunziata 1998; Paemeleire 1999; Lu 2008).

Studies of a haematogenous model of brain metastasis have demonstrated increased SP immunoreactivity surrounding tumour invaded microvessels in correlation with albumin immunoreactivity in the neuropil, indicative of increased BBB permeability (Lewis 2012b). These results, along with the known actions of SP on the BBB, make SP a likely mediator of tumour cell extravasation into the brain. Therefore the current study aims to test the efficacy of NK1 antagonists to prevent breast cancer extravasation through the BBB and thus, metastatic brain tumour development.

5.3 Method

5.3.1 Animals

The current study was performed as detailed in section 2.2 page 43. They were randomly selected for culture medium injection, tumour inoculation and treatment groups, with all animal sacrificed 9 days following surgery (n=6 per group).

5.3.2 Cell culture

The Walker 256 breast carcinoma cell line was obtained from the Cell Resource Centre for Medical Research at Tohoku University and cultured as detailed in section 2.1.2 page 42. Using a haemocytometer, the cell suspension was diluted so that 10^5-10^6 walker 256 cells/0.2 mL of serum free media was ready for tumour inoculation

5.3.3 Internal carotid artery inoculation

The internal carotid inoculation procedure was previously described in section 2.3 page 44.

5.3.4 Treatment

The NK1 antagonists fosaprepitant dimeglumine (Emend, MERCK & CO) 3 mg/kg/day and n-acetyl L-tryptophan (NAT) 7.5 mg/kg/day were dissolved in 0.9% saline solution and administered IP on days 0-3 following tumour inoculation. Equal volumes of saline were used as a vehicle control. The treatment concentration of NAT was determined based on a previous dose response, in which the dose required to elicit maximal decrease in BBB permeability following diffuse traumatic brain injury was evaluated (Donkin 2009). The dose for Emend was determined based on that used clinically and previously shown to have a central effect in rodents (Watanabe 2008).

5.3.5 Immunostaining

Animals were euthanized and brains processed as detailed in 2.6 page 48. Slides from each group were stained for SP (Santa Cruz Biotechnology, 1:2000) and albumin immunoreactivity (ICN Pharmaceuticals, 1:20,000) using the method described in section 2.8 page 49. These slides were scanned using a Hammamatsu nanozoomer (Hammamatsu City, Japan) and then run through the colour deconvolution software. This was done to estimate the %DAB in coronal sections of the brain for albumin immunostaining and in four 0.0678 mm^2 areas in the right cortex for SP immunostaining. The use of colour deconvolution software has been described previously (Helps 2012).

5.3.6 Tumour volume

Slides from each group were stained with haematoxylin and eosin and scanned using a Hammamatsu nanozoomer so that the tumour area could be determined in each slide using NDP viewer software as detailed in section 2.7 page 49.

5.3.7 Statistical analysis

A one-way analysis of variance followed by a Bonferroni post test performed to determine statistical significance, with p values of <0.05 designated significant. All data are expressed as mean ± SEM.

5.4 Results

Inoculation of walker 256 breast carcinoma caused microvascular invasion in 100% of vehicle treated animals and evidence of a tumour mass in 83.33% of animals 9 days following surgery (Table 5.1). There was no significant difference in either microvascular invasion or tumour mass development with either Emend or NAT treatment. Similarly, neither Emend nor NAT treatment significantly altered the metastatic brain tumour volume (Fig. 5.1).

Table 5.1 Effect of treatment on incidence of metastatic brain tumours

Treatment	Microvascular Invasion	Tumour Mass
Vehicle	100%	83.33%
Emend	100%	100%
NAT	100%	83.33%

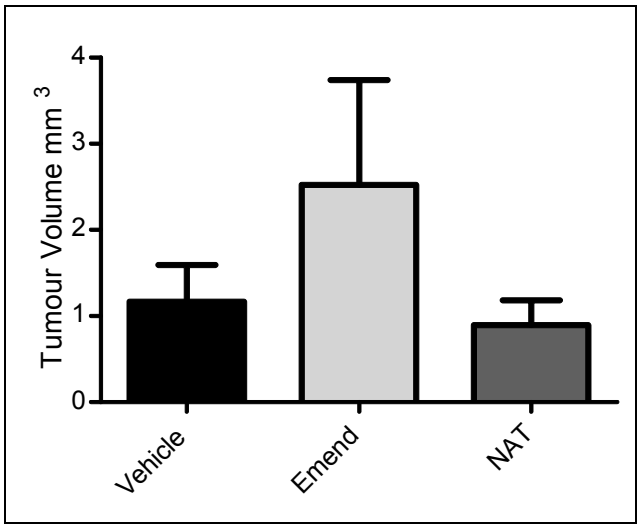

Figure 5.1 Effect of treatment on tumour volume in mm^3 showing no significant difference in tumour volume among the groups

In this study albumin immunoreactivity was used as an indicator of increased BBB permeability. Albumin is located within the vasculature, but under pathological conditions that disrupt the function of the BBB, it is able to leak into the perivascular neuropil. Tumour inoculation and subsequent growth of metastatic brain tumours resulted in substantially increased albumin immunoreactivity in brain coronal sections ($p<0.01$, Fig. 5.2A). This phenomenon remained unaffected when animals were treated with either Emend or NAT (Fig. 5.2A).

SP immunoreactivity was substantially elevated in the cortex following tumour inoculation when compared with the control group, which was not exposed to tumour inoculation (Fig. 5.2B). Similar to albumin immunoreactivity, there was no detectable effect of NK1 antagonist treatment on SP expression using either Emend or NAT, 9 days following Walker 256 inoculation (Fig. 5.2B).

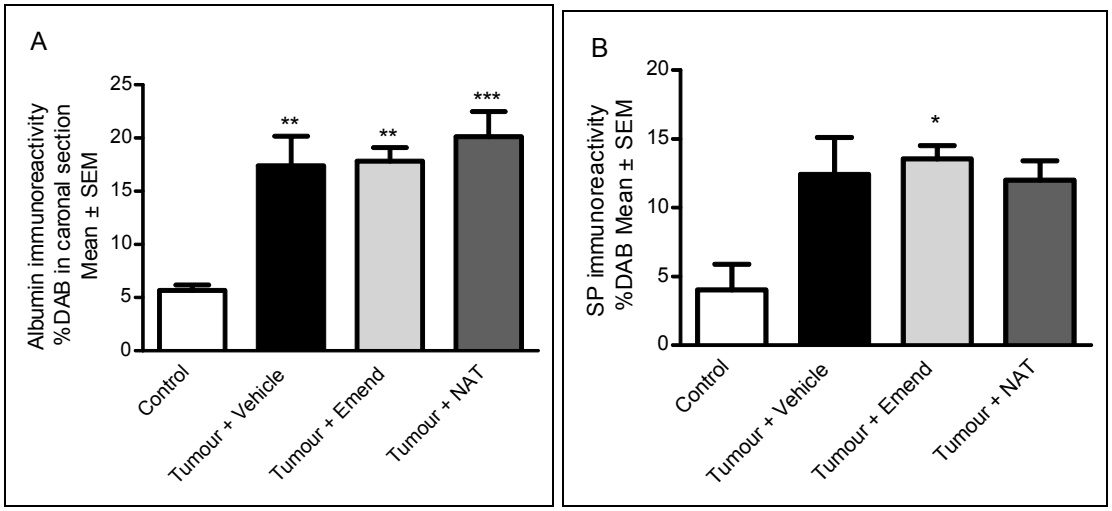

Figure 5.2 Albumin and substance P (SP) immunoreactivity

(a) Effect of tumour inoculation and treatment on %DAB representing albumin immunoreactivity in brain coronal sections when compared to the control group, **p<0.01, ***p<0.001 (b) Average Substance P (SP) immunoreactivity in four areas of 0.0678 mm^2 in the right cortex, expressed as %DAB following tumour inoculation and NK1 antagonist treatment *p<0.05

On average, animals injected with culture medium alone as controls showed 19.9 g of weight gain 9 days following surgery (Fig. 5.3). In contrast, tumour inoculation caused a mean weight loss of 0.92 g during the same time period (Fig. 5.3). Treatment with both NK1 antagonists reversed this, such that the animals gained approximately 8 g 9 days following surgery (Fig. 5.3).

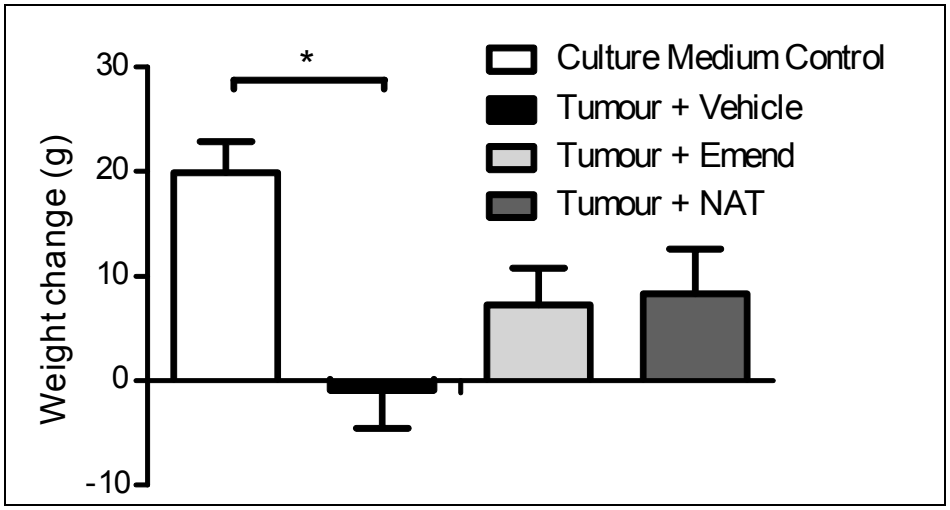

Figure 5.3 Animal weight change following tumour cell inoculation and treatment *p<0.05

5.5 Discussion

In this study using an internal carotid artery model of Walker 256 breast carcinoma inoculation, treatment with NK1 antagonists did not prevent metastatic brain tumour growth. This was despite the previous findings that SP immunoreactivity was increased locally surrounding tumour invaded cerebral microvessels, which was associated with increased BBB permeability as indicated by albumin immunoreactivity in the neuropil (Lewis 2012b). Therefore the SP immunoreactivity in conjunction with tumour cell extravasation is not the primary mediator of BBB compromise that permits cancer cells to invade the brain microenvironment.

SP has previously been implicated in many processes involved in cancer growth and metastatic progression. For example, the well-characterised effects of SP on tumour cells in vitro, such as induction of tumour cell mitogenesis, migration, chemotactic behaviour and cytokine secretion (Ruff 1985; Palma 1998; Lang 2004; Bigioni 2005; Munoz 2005a). Furthermore, NK1 antagonist treatment has resulted in decreased oedema in rodent models of traumatic brain injury and stroke through resolution of BBB disruption (Donkin 2009; Turner 2011).

In contrast, the results of the current study suggest that extravasation is a multifactorial process, in which the role of SP is not of sufficient magnitude for its blockade by NK1 antagonist treatment to result in prevention of metastatic brain tumour formation. Tumour progression involves the expression of multiple adhesion molecules such as integrins and selectins, and matrix metalloproteases (Li 1993; Lu 2001; Mendes 2005; Mendes 2007). Furthermore, vasoactive inflammatory substances may be required for extravasation into the brain to take place. It is plausible that blocking any one of these activities would be ineffective for metastatic brain tumour prevention, rather multiple pathways may need to be targeted.

Although this study deals solely with the neurogenic inflammatory peptide SP, there are many other vasoactive substances that may contribute to increased BBB permeability along with tumour cell extravasation into the brain microenvironment (Lewis 2012b). Classical inflammation, characterised by signals for accumulation and proliferation of microglia and perivascular macrophages, has been implicated in BBB disruption that results in oedema development associated with metastatic brain tumours (Lewis 2012a), making bradykinin a possible candidate for involvement in the passage of tumour cells between cerebral capillary endothelial cells.

Similarly, vascular endothelial growth factor has also been proposed to play a role in the progression of cancer to the brain, due to the evidence of its involvement in angiogenesis and oedema development in animal models of brain metastases (Wang 1996; Batchelor 2007). However, blockade of vascular endothelial growth factor also failed to reduce the incidence of secondary brain tumours (Juanyin 2009). It is possible that multiple vasoactive inflammatory mediators are involved in brain metastases development and as such, agents that act through multiple pathways may be more effective as prophylactic agents for secondary brain tumour progression.

In the current study, albumin immunoreactivity, which was elevated with tumour inoculation, was not altered with either Emend or NAT treatment. This is likely a reflection of the similar tumour volume evident in the treatment groups, as the link between tumour volumes and vascular permeability is well documented (Zhang 1992). This result also reflects that SP alone is not responsible for the increased BBB permeability evident with metastatic brain tumour growth in this model.

By 9 days following walker 256 breast carcinoma inoculation into the internal carotid artery, animals lost weight, contrasting sharply with the culture medium inoculated animals and also both NK1 antagonist treatment groups which gained weight following surgery. The reversal of tumour associated weight loss by NK1 antagonist treatment has not been reported previously. However, both weight loss and weight

gain have been reported as a rare side effect of Emend treatment when co-administered with other agents for the treatment of chemotherapy induced emesis (Kramer 2004).

In conclusion, NK1 antagonist treatment was not sufficient to prevent metastatic brain tumour formation in this model. Therefore, this study demonstrates that SP does not mediate Walker 256 breast cancer extravasation into the brain following internal carotid artery inoculation. This is despite the increased SP expression evident in association with tumour invasion of brain microvessels and the associated increase in BBB permeability to albumin. Thus, it is plausible that extravasation of Walker 256 tumour cells across the BBB is a multifactorial process, the inhibition of which would require multiple pathways to be targeted.

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

6.1 Abstract

Cerebral metastases are associated with the development of substantial vasogenic peritumoral oedema formation, which is the source of many neurological complications associated with secondary brain malignancies. The current standard treatment for peritumoral brain oedema is dexamethasone, which inhibits classical inflammation, however it is associated with numerous adverse side effects. Neurogenic inflammation has been implicated in vasogenic oedema formation following animal models of reperfusion ischemic stroke and traumatic brain injury through the actions of substance P (SP). SP acts preferentially on NK1 receptors, the blockade of which may provide an alternative treatment to dexamethasone for peritumoral oedema. Thus, this study investigates the efficacy of NK1 receptor antagonist treatment, in comparison with dexamethasone treatment, for the management of peritumoral brain oedema in a rat model of intrastriatal inoculation of Walker 256 rat breast carcinoma cells. Brain water content, albumin immunoreactivity and Evan's blue extravasation were evaluated at 7 days following tumour inoculation with culture medium injected animals used as controls. Dexamethasone was effective in reducing brain water content and caused a reduction in Evan's blue extravasation, whilst NK1 receptor antagonist treatment groups had no effect on oedema formation. Thus, we conclude that classical inflammation rather than neurogenic inflammation drives peritumoral oedema in this model.

6.2 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, whilst clinical studies commonly describe an incidence of approximately 10% (Posner 1978; Cifuentes 1979; Gavrilovic 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 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 localized ischemia and brain herniation through compression adjacent structures. The mass effect of tumour and the addition of oedematous fluid often cause many neurological symptoms. These may include blindness, cognitive deficit, weakness, headaches and aphasia (Mukand 2001; Ayata 2002; Shinoura 2010).

Angiogenic blood vessels that grow within metastatic brain tumours have the same characteristics of their tissue of origin (Cornford 1992; Shuto 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 1984; Zhang 1997). As such, peritumoral oedema is related to the size of the neoplasm and the extent of angiogenesis (Zhang 1992). Both the BBB and BTB are dynamic structures with barrier properties that may vary in response to external stimuli, including vasoactive substances (Zhang 1997; Turner 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 1967). Approximately 70% of metastatic brain tumour patients receive dexamethasone treatment whilst they undergo radiotherapy (Hempen 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 1994a; Heiss 1996; Andersen 1998; Sinha 2004). Unfortunately, dexamethasone treatment is associated with many harmful side-effects including insomnia, immune suppression (Lesniak 2004), hyperglycemia (McGirt 2008)and occasional psychosis (Alpert 1986)..

Neurogenic inflammation is characterised by vasoactive neuropeptide release causing vasodilatation, plasma extravasation and subsequent oedema formation (Woie 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 1998; Paemeleire 1999; Annunziata 2002; Lu 2008). The modulatory effect of SP on the BBB is involved in the pathogenesis of peri-lesional cerebral oedema in ischemic stroke and traumatic brain injury. Treatment with NK1 receptor antagonists in these conditions results in reduction of water content and improved functional outcome (Nimmo 2004; Vink 2004; Turner 2006; Turner 2007; Donkin 2009; Harford-Wright 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 2000) . The aim of the current study was to use an intracerebral injection 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.

6.3 Methods

6.3.1 Animals

Animal procedures were performed as described in section 2.2 on page 43. Six animals for each treatment group were randomly selected for the immunostaining study, brain water content study and Evans blue extravasation study.

6.3.2 Cell culture

Walker 256 rat breast carcinoma cells were obtained from the Cell Resource Centre for Medical Research at Tohoku University, Japan and cultured according to the description in section 2.1.2 page 42. 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 media were used for direct injection into the brain.

6.3.3 Tumour inoculation

The direct inoculation of tumour cells has been described in section 2.4 page 47.

6.3.4 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) 3mg/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 2007a). 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 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 2008).

6.3.5 Immunostaining

Brains were analysed for histology as described in section 2.6 page 48. Slides from each treatment group were stained for substance P (Santa Cruz Biotechnology, 1:2,000) and albumin (ICN Pharmaceuticals, 1:20,000) as detailed in section 2.8 page 49. 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 %DAB in the scanned slides. The colour deconvolution technique has been described previously (Harford-Wright 2010; Helps 2012). Whole coronal sections for albumin-stained brain sections, and peritumoral areas ($0.0678mm^2$) from SP immunostained sections were taken and run through the colour deconvolution software to automatically estimate the % of brown stain in the selected area and through this process removes background staining variations.

6.3.6 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 as described in section 2.9 on page 50.

6.3.7 Evans blue extravasation

Animals were injected intravenously with 0.8 ml of 4% Evans blue (MW 69,000; Sigma, E-2129) 30 minutes before they were perfused transcardially with saline under general anaesthesia induced by pentobarbitone sodium (60mg/kg). This has been described in detail in section 2.10 page 50.

6.3.8 Statistical Analysis

Data were expressed as mean ± SEM. To determine statistical significance, an unpaired two tailed 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.

6.4 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 1983; Jamshidi 1992; Morreale 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 2007a). Days 4, 5 and 6 following tumour inoculation were chosen for treatment so that tumours were well established prior to its commencement.

6.4.1 SP immunoreactivity

Tumour inoculation caused a significant increase in SP immunoreactivity 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 peri-tumoral area was not altered by any of the treatment regimes used in this study (data not shown).

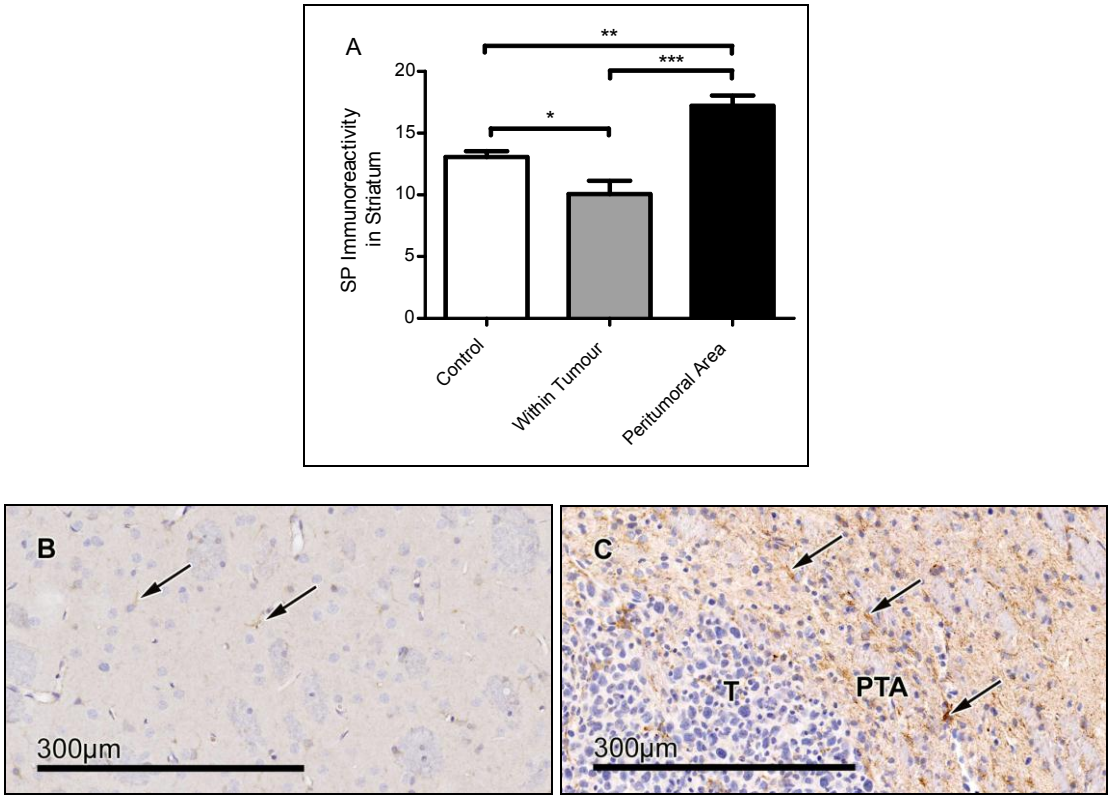

Figure 6.1 Substance P (SP) immunoreactivity with metastatic brain tumour growth

(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 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)

6.4.2 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).

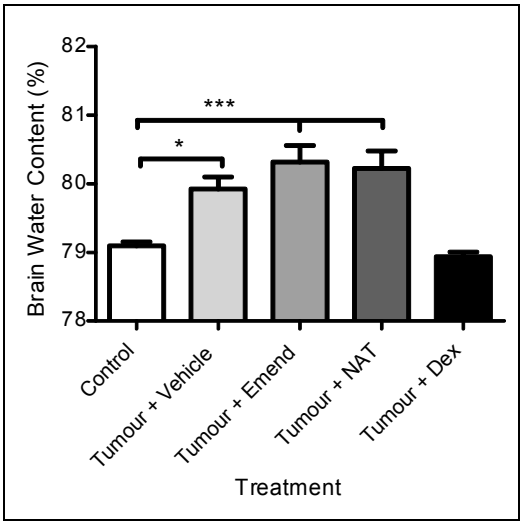

Figure 6.2 Brain water content

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; *$p<0.05$; ***$p<0.001$

6.4.3 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). Figure 4E and 4D show 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. However, there was no significant difference seen between these groups using colour deconvolution of albumin immunoreactivity.

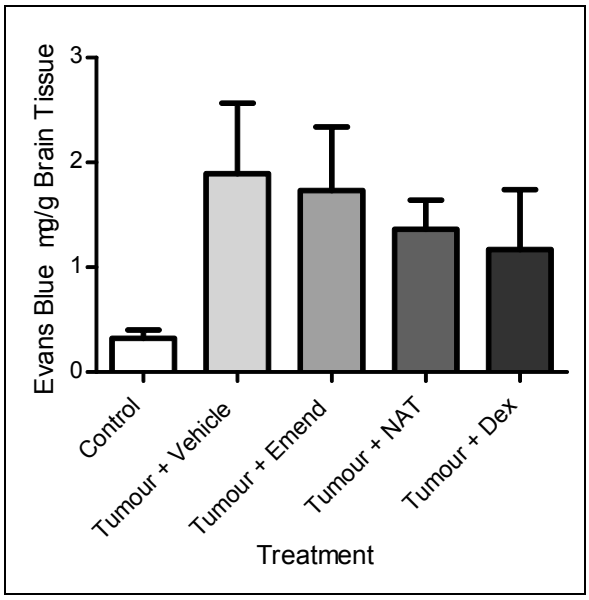

Figure 6.3 Evans blue extravasation

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

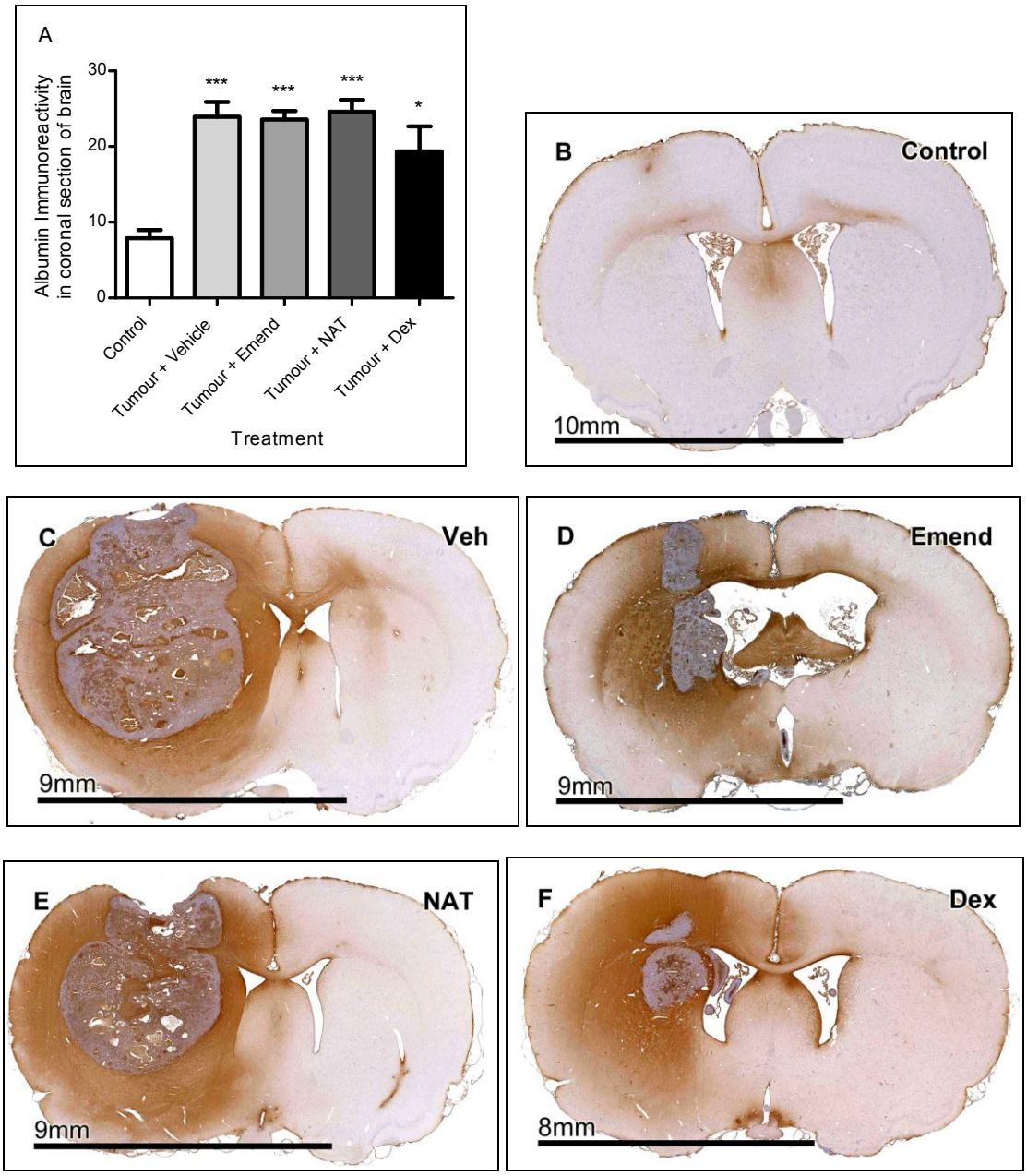

Figure 6.4 Albumin immunoreactivity with tumour inoculation and treatment

(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 peritumoral 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

6.5 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 antagonism 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 1983; Jamshidi 1992; Engelhorn 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 often produce multiple secondary brain tumours without predictable location (Ushio 1977; Hasegawa 1983; Song 2011; Budde 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 2004; Turner 2006; Li 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 2011). The substantially larger percentage change in brain water content for models of trauma and reperfusion ischemic 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 ischemic reperfusion stroke (Donkin 2009; Donkin 2011; Turner 2011). In contrast, NK1 antagonists have not been shown to be effective in reducing vasogenic oedema associated with subarachnoid haemorrhage (Barry 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 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 2010). In the CNS, classical inflammation is characterised by accumulation and proliferation of microglia along with perivascular macrophages (Graeber 2011). This leads to blood vessel alterations driven by classical inflammatory mediators like bradykinin (Donkin 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 previously been used effectively in models of vasogenic oedema to reduce brain water content, BBB permeability and other measures of cerebral oedema (Betz 1990; Guerin 1992). The outcomes associated with dexamethasone treatment are thought to be through its actions on glucocorticoid receptors, with modulation of VEGF (Heiss 1996; Kim 2008) and occludin (Forster 2006; Gu 2009a) proposed to play a role in its activity. Despite the undefined mechanism of action, dexamethasone has been shown to decrease transendothelial fluid movement and extravascular fluid volume (Nakagawa 1987; Andersen 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 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 proteins 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. This suggests that the pathogenesis of peritumoral oedema may be more related to classical inflammation, rather than neurogenic inflammation driven by substance P.

7 NK1 receptor antagonists and dexamethasone as anticancer agents in vitro and in a model of brain tumours secondary to breat cancer

7.1 Abstract

Emend, a NK1 antagonist, and dexamethasone are used to treat complications associated with metastatic brain tumours and their treatment. It has been suggested that these agents have anti-cancer effects apart from their current use. Effects of the NK1 antagonists, Emend and n-acetyl $_{L}$-tryptophan (NAT), and dexamethasone on tumour growth were investigated in vitro and in vivo at clinically relevant doses. For animal experiments, a stereotaxic injection model of Walker 256 rat breast carcinoma cells into the striatum of Wistar rats was used. Emend treatment caused a decrease in tumour cell viability in vitro, although this effect was not replicated by NAT. Dexamethasone did not decrease tumour cell viability in vitro but decreased tumour volume in vivo, likely to be through a reduction in tumour oedema, as indicated by the increase in tumour cell density. None of the agents investigated altered tumour cell replication or apoptosis in vivo. Inoculated animals showed increased GFAP and IBA1 immunoreactivity indicative of astrocytes and microglia in the peritumoral area, while treatment with Emend and dexamethasone reduced the labelling for both glial cells. These results do not support the hypothesis that NK1 antagonists or dexamethasone have cytotoxic action on tumour cells, although these conclusions may be specific to this model and cell line.

7.2 Introduction

Malignancies of the CNS have shown an increased incidence in recent years, although mortality rates have plateaued (Bray 2010). Breast cancer is the most prevalent cancer type in women and the second most common cancer type to cause metastatic brain tumours after lung cancer, accounting for approximately 20% of all secondary brain tumours (Schouten 2002; AIHW 2010; Villa 2011) and causing a significant patient morbidity and mortality, with survival time commonly in the order of months (Sperduto 2012).

While curative treatments for metastatic brain tumours remain elusive, anti-inflammatory agents are commonly prescribed to patients with secondary brain tumours, to control symptoms associated with tumour complications and treatment side effects. Dexamethasone is a synthetic glucocorticoid administered to patients with brain metastases to reduce neurological symptoms related to the mass effect of peri-tumoral oedema. Since it was first used in 1962 it contributed to a significant reduction in mortality in brain tumour patients (Jelsma 1967). However, the beneficial effects of dexamethasone treatment are limited by its associated side effects, including suppression of the immune system (Lesniak 2004), hyperglycaemia (McGirt 2008) andoccasionallypsychosis (Alpert 1986).

Aprepitant, also known as L-754,030 and its intravenous prodrug fosaprepitant diglutemide also termed L-758,298 (Emend), is an NK1 receptor antagonist used as an antiemetic, to control chemotherapy induced nausea in many cancer patients; it is the only NK1 antagonist that has been approved for use in humans (Hesketh 2003; Herrstedt 2005; Warr 2005; Ruhlmann 2012). The mechanism of chemotherapy-induced nausea is thought to be through neurotransmitter release in the gastrointestinal tract and in the central nervous system, with the vomiting centre and chemoreceptor trigger zone in the medulla oblongata being particularly affected (Navari 2004a). It is thought that NK1 antagonism has inhibitory activity on this process both centrally and peripherally (Navari 2004b). Emend is commonly co-

administered with dexamethasone, and maximum benefit is seen when combined with 5-HT3 receptor-antagonist (Hesketh 2006). Despite the common use of dexamethasone and Emend in cancer patients, their effect on tumour growth remains controversial.

It has been suggested that dexamethasone may also act to control cancer growth (Villeneuve 2008; Moroz 2011). Dexamethasone has been shown to reduce brain tumour volume in vivo in murine models of brain tumours (Guerin 1992; Wolff 1993; Badruddoja 2003; Villeneuve 2008; Moroz 2011), although it remains unclear if this results from decreased oedematous fluid or decreased tumour cell viability and proliferation (Guerin 1992; Wolff 1993; Badruddoja 2003; Villeneuve 2008). Indeed, the effect of dexamethasone on tumour cell proliferation and apoptosis in vivo has not been investigated. Several in vitro studies have found that dexamethasone has an anti-proliferative, pro-apoptotic effect on cancer cells (Bavaresco 2007; Piette 2009; Tazik 2009), while in other apparently conflicting in vitro studies dexamethasone was not always able to inhibit tumour cell growth (Wolff 1993; Villeneuve 2008). Despite the consistent use of dexamethasone as the standard treatment for peritumoral brain oedema for many years, its exact mechanism of action remains unclear. Furthermore, dexamethasone treatment is often investigated in conjunction with other chemotherapy treatments, meaning that its mechanism of action alone remains unclear (Kim 2004; Sur 2005; Zhang 2006; Moroz 2011).

Several studies have demonstrated an increase in NK1 receptor expression in human surgical specimens of astrocytoma and brain metastases from breast carcinomas and melanoma (Allen 1985; Hennig 1995; Khare 1998; Singh 2000; Schulz 2006; Huang 2010). Substance P (SP) is a pro-inflammatory tachykinin that acts preferentially on NK1 receptors. Recent studies have implicated SP in the proliferation and progression of many cancer types (Munoz 2011b). In cell culture studies, SP has consistently been shown to induce tumour cell mitogenesis, with NK1 antagonists causing apoptosis (Friess 2003; Munoz 2004a; Bigioni 2005; Munoz 2005a; Munoz 2005b; Munoz 2007; Rosso 2008; Akazawa 2009; Huang 2010) and decreased mitogenesis,

particularly on NK1 receptor-expressing human cancer cell lines, while non-neoplastic cell lines did not exhibit these effects (Singh 2000). Thus, it has been suggested that the NK1 receptor antagonist Emend may not only be useful in the treatment of chemotherapy-induced emesis, but may also inhibit SP-induced tumour cell proliferation. Therefore, Emend may aid in the treatment of cancer itself, as well as the side effects of chemotherapeutic agents (Kast 2009; Munoz 2010b; Harford-Wright 2011). However, the literature shows conflicting results. While some studies showed that NK1 antagonists or inhibition of SP decrease tumour growth (Palma 2000; Erin 2008; Korcum 2009), nearly an equal number of studies demonstrated that SP inhibits tumour growth through its stimulatory effects on the immune system (Harris 2003; Erin 2004; Manske 2005; Erin 2006). The current study was undertaken to determine the effects of NK1 antagonism and dexamethasone on breast carcinoma cell growth in vitro and in vivo.

7.3 Materials and Methods

7.3.1 Cell Viability Assay

Walker 256 rat breast carcinoma cells were obtained from the Cell Resource Centre for Medical Research at Tohoku University were cultured as described in section 2.1.2 page 42. A cell viability assay was then performed as detailed in section 2.1.3 on page 43.

7.3.2 Cell Culture for Inoculation

Cells growing in 150 cm^2 culture flasks were passaged once >90% confluence was reached using 3.5 mL of 0.02% EDTA. The cells were spun down in a centrifuge (5 minutes at 1500 RPM) and resuspended in serum free media. The cells were counted using a haemocytometer and then diluted, so that between 10^5 and 10^6 cells / 8 µL used for direct injection into the brain.

7.3.3 Animals

Animal procedures were performed as detailed in section 2.2 page 43. Six male albino Wistar rats between 250-350 g were randomly selected for each treatment group.

7.3.4 Tumour Inoculation

The direct inoculation of tumour cells into the striatum has been described previously in section 2.4 page 47.

7.3.5 Treatment

All drugs used were dissolved in 0.9% sodium chloride solution, and this was also used as a vehicle control. In each study, each animal group was treated with an IP injection of either the NK1 antagonist Emend^R (3 mg/kg/day), the NK1 antagonist NAT (7.5 mg/kg/day), DBL dexamethasone sodium phosphate (8 mg/kg/day) or equal

volume saline on days 4-6 following tumour inoculation. All animals were sacrificed on day 7 following tumour inoculations. The dose used for dexamethasone treatment was determined by that previously reported in the literature for use on murine models of brain tumours (Gu 2007a; Ewing 2008). The Emend dose was based on that recommended for intravenous injection clinically and previously shown to have a central effect in animal models (Watanabe 2008), while the NAT dose was determined based on that previously used to alter the permeability of the blood-brain barrier (BBB) (Donkin 2009).

7.3.6 Tumour Volume

To determine tumour volume, animals were transcardially perfused with 10% formalin as described in 2.6 and 2.7 on pages 48 and 49. The same technique was performed to determine the volume of haemorrhage and necrosis within the tumour mass.

7.3.7 Immunostaining

Slides from each treatment group were stained for NK1 receptors (Biocore 1:8000), Ki67 (Abcam 1:2000), caspase 3 (Bio Vision 1:400), GFAP (Dako 1:40,000) and IBA1 (Dako 1:50,000) as described in 2.8 page 49.

7.3.8 Analysis of NK1 receptor, GFAP and IBA1 immunostained sections

Images were exported from the Nanozoomer files for all immunostained sections. From each slide, four images were taken from each of the following areas: the tumour, the peritumoral area and the striatum. Non-subjective estimation of the immunocytochemical staining was achieved through colour deconvolution techniques to reveal the %DAB in the scanned slides as described previously (Harford-Wright 2010; Helps 2012). The %DAB from the four fields of view were averaged to determine the mean immunoreactivity in each area for each stain used. For GFAP and IBA1 immunostained slides, in addition to colour deconvolution, labelled cells in the

images were counted to determine the effect of treatment on the number of GFAP and IBA1 labelled cells.

7.3.9 Tumour cell replication, density and apoptosis

Ki67 labelled cells were counted in four fields of view, each equalling 0.0678 mm^2 as representative for the tumour and the percentage of labelled cells in relation to the total number of tumour cells in the tumour mass was calculated. The same method was used to determine the percentage of caspase 3 labelled cells indicative of apoptotic tumour cells. Similarly, the density of tumour cells was determined by counting tumour cells within six fields of view 0.0678 mm^2 each within the tumour, from haematoxylin and eosin stained slides for each brain.

7.3.10 Statistical Analysis

Data were expressed as mean ± SEM. To determine statistical significance either an unpaired two tailed t-test (2 groups) or a one-way analysis of variance (more than two groups) followed by Bonferroni post tests was performed as appropriate. A value of $p<0.05$ was considered significant.

7.4 Results

7.4.1 Cell Viability Assay

Treatment with NK1 antagonists in vitro showed inconsistent results on Walker 256 cell viability. While Emend treatment at both 100 and 1000 μg/mL caused a significant reduction in viable cells that excluded trypan blue dye ($p<0.001$; Fig. 1), NAT treatment had no effect on the percentage of viable tumour cells at any concentration (Fig. 1). Similarly, dexamethasone did not alter the percentage of viable tumour cells after 24 hours of treatment at any of the concentrations used (Fig. 1).

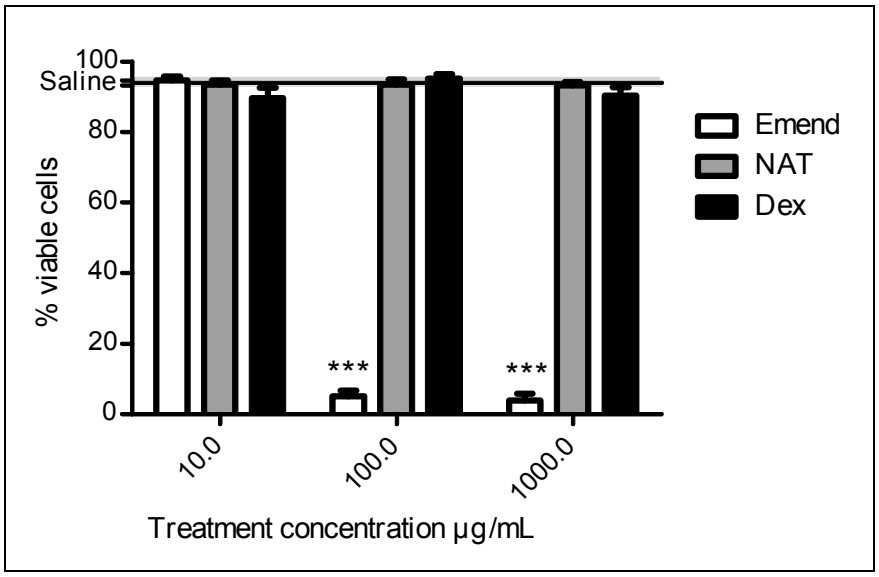

Figure 7.1 Cell viability assay

Walker 256 cell viability as indicated by trypan blue exclusion assay. NAT, n-acetyl L-tryptophan; Dex, Dexamethasone; ***p<0.001

7.4.2 NK1 receptor expression

The induced Walker 256 tumours expressed NK1 receptors in vivo, as evidenced by the significant increase in NK1 receptor immunoreactivity within the tumour mass when compared to the peritumoral area of tumour inoculated brains 7 days following surgery ($p<0.05$; Fig 2A & B). The immunostaining was localised within the tumour cell cytoplasm (Fig 2B).

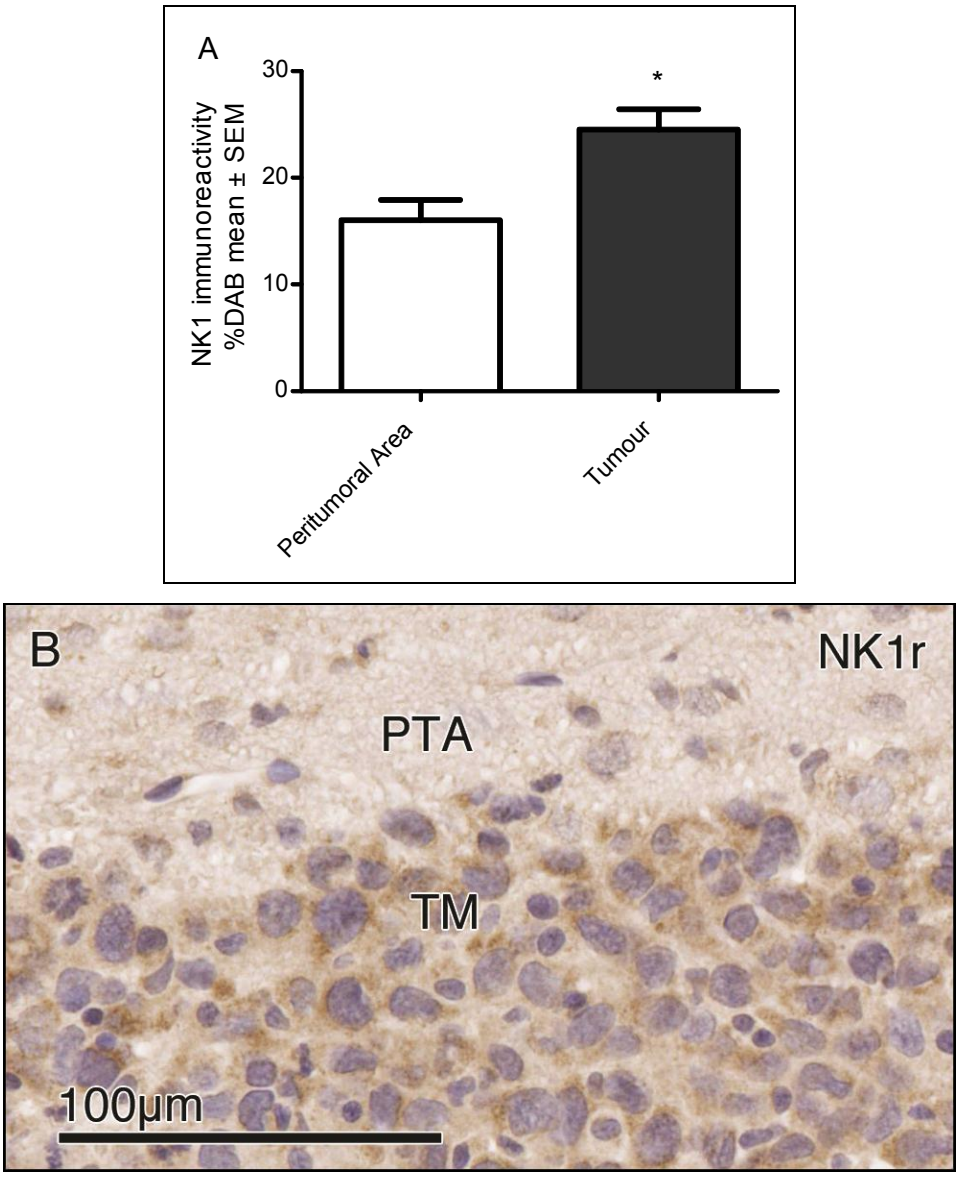

Figure 7.2 NK1 receptor immunoreactivity

(A) Graph showing NK1 receptor immunoreactivity in the peritumoral area and within the tumour mass in tumour inoculated animals 7 days following surgery, *p<0.05 (B) NK1 receptor immunostained brain section showing tumour cells labelled brown, 7 days following tumour inoculation; TM, tumour mass; PTA, peritumoral area

7.4.3 Tumour Growth

Following Walker 256 tumour cell inoculation, treatment with the NK1 receptor antagonists Emend or NAT, did not cause a significant difference in tumour volume when compared to vehicle treated animals (Fig. 3A, C, D & E). Conversely,

dexamethasone treatment resulted in a significant decrease in tumour volume when compared to the NAT treated group ($p<0.05$; Fig. 3A, E & F). Furthermore, dexamethasone treatment also caused a significant reduction in necrosis within the tumour mass when compared to the NAT treated group ($p<0.05$; Fig. 3B). NK1 antagonists did not have any effect on the percentage of necrosis or haemorrhage within the tumours compared to vehicle control (Fig.3B).

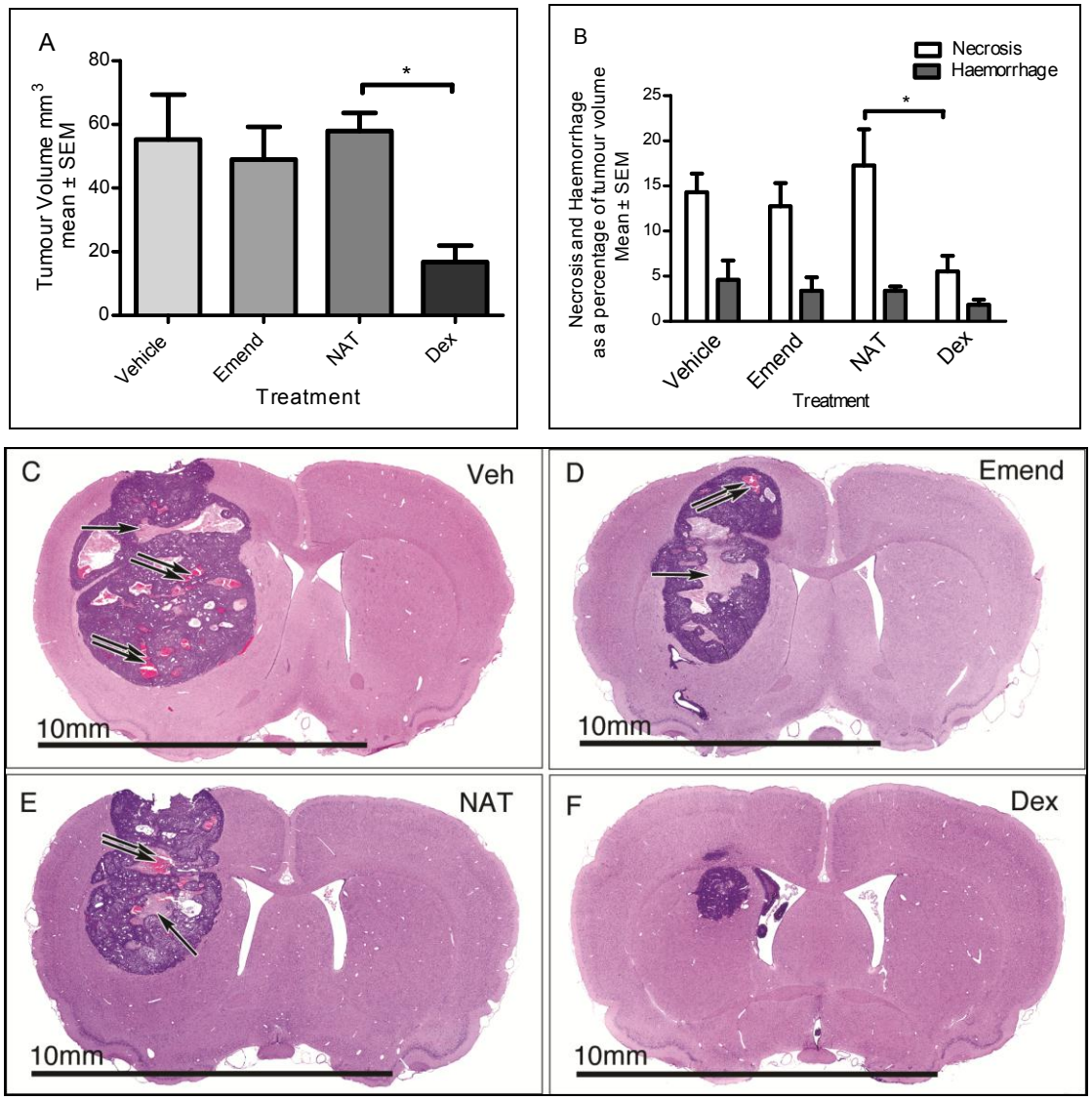

Figure 7.3 Tumour growth

(A) Tumour volume (mm^3) in Emend, n-acetyl $_L$-tryptophan (NAT), and dexamethasone (Dex) treated animals compared to vehicle control treatment; *$p<0.05$ (B) The volume of haemorrhage and necrosis within the tumour mass as a percentage of the tumour volume in Emend, n-acetyl $_L$-tryptophan (NAT) and dexamethasone (Dex) compared to vehicle treated animals; *$p<0.05$ (C-F) Haematoxylin and eosin stained coronal section from animals treated with Vehicle (Veh, C), Emend (D), n-acetyl $_L$-tryptophan (NAT, E), and dexamethasone (Dex, F). The sections are showing large tumour masses in the right hemisphere, with apparent reduction in size in the dexamethasone-treated animals (F). Necrosis, single arrow; haemorrhage, double-arrow

Neither of the NK1 receptor antagonists, Emend or NAT, had any effect on tumour density or Ki67 immunoreactivity, which indicates replicating cells (Fig. 4A & B).

Similarly, neither Emend, nor NAT altered the percentage of caspase 3 positive cells, which indicates apoptotic tumour cells (Fig. 4C). Dexamethasone treatment caused a significant increase in the density of tumour cells within the tumour mass when compared to the vehicle treated group ($p<0.01$; Fig. 4A). However, dexamethasone treatment did not alter the percentage of Ki67 positive tumour cells or caspase 3 positive cells (Fig. 4B & C). Therefore, the dexamethasone treated group exhibited tumour masses with cancer cells more tightly packed together, but with no change in replication or apoptosis.

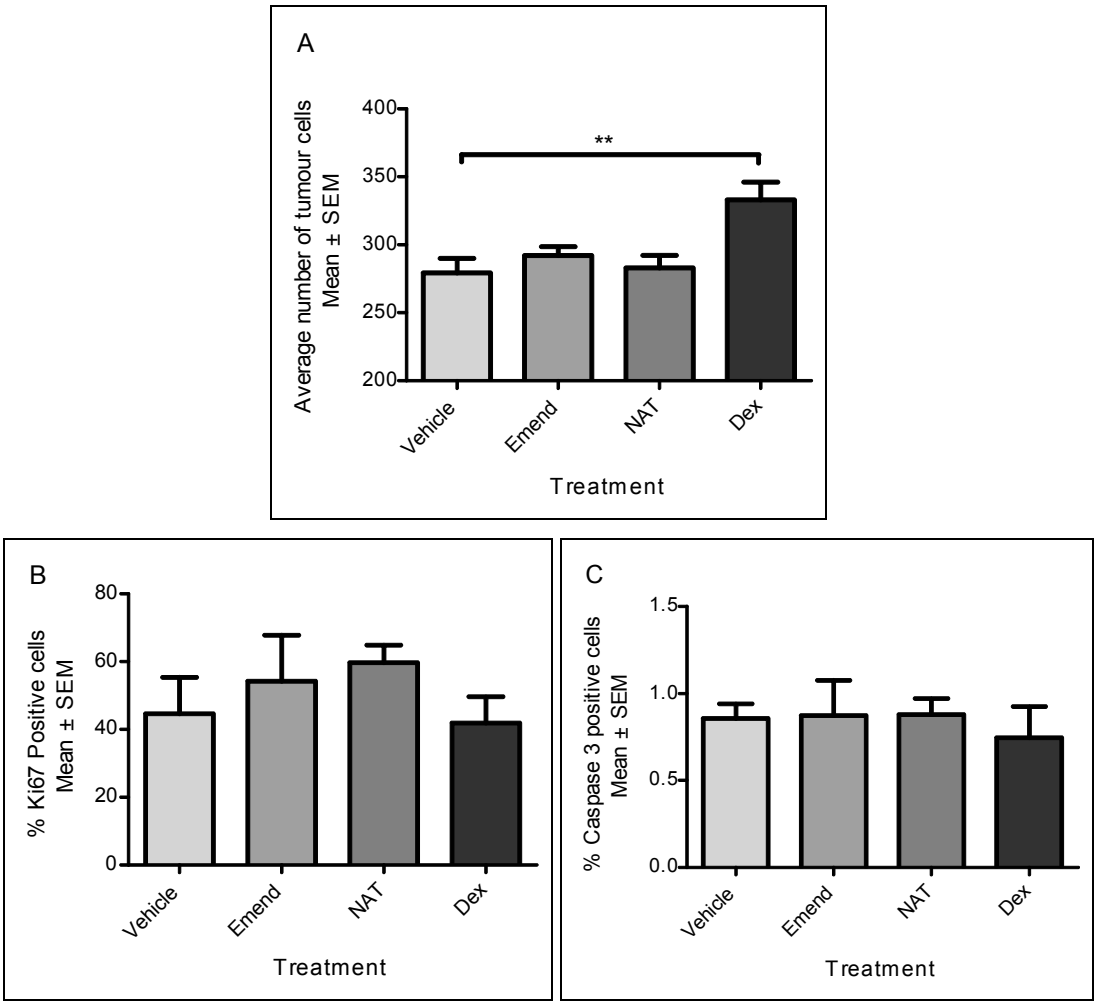

Figure 7.4 Tumour growth characteristics

(A) The effect of treatment on tumour cell density. The average number of cells was obtained from four areas within the tumours in animals treated with Vehicle, Emend, n-acetyl $_{L}$-tryptophan (NAT), and dexamethasone (Dex); **p<0.01 (B) The percentage of replicating tumour cells as indicated by Ki67 immunoreactivity in animals treated with vehicle, Emend, n-acetyl $_{L}$-tryptophan (NAT) and dexamethasone (Dex) (C) The percentage of caspase 3 immunopositive cells, indicative of apoptosis, in tumour masses from animals treated with vehicle, Emend, n-acetyl $_{L}$-tryptophan (NAT) and dexamethasone (Dex)

7.4.4 Brain microenvironment

GFAP and IBA1 immunoreactivity were used as indicators of astrocytic and microglial response, respectively, and thus represented the interaction of the tumour cells and treatment agents with these components of the brain microenvironment. Inoculation with Walker 256 breast carcinoma cells and subsequent tumour growth, caused a significant increase in GFAP and IBA1 immunoreactivity in the peritumoral

area when compared to the same location in culture medium control animals ($p<0.001$ and $*p<0.05$, respectively; Fig. 5A-E). GFAP immunoreactivity was not present within the tumour, and thus showed significantly reduced %DAB when compared to the peritumoral area ($p<0.001$; Fig. 5A & B). Furthermore, the tumour mass also showed less GFAP immunoreactivity than the same location in brains of control animals, injected with culture medium without Walker 256 tumour cells (Fig. 5A). However, IBA1 immunoreactivity was significantly elevated within the tumour mass, when compared with that evident in the striatum of brains from the culture medium control group ($p<0.01$; Fig. 5C, D & E).

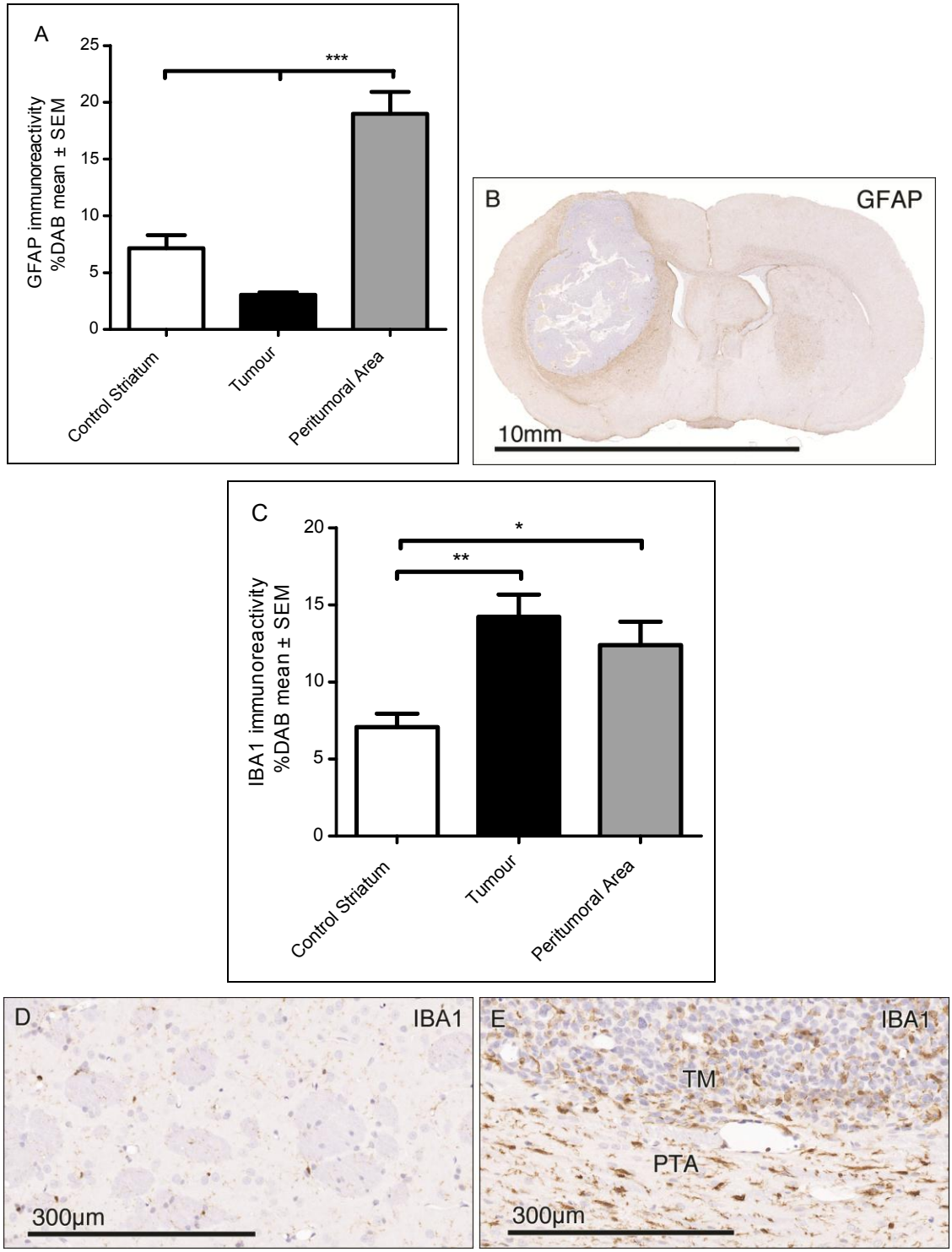

Figure 7.5 Tumour and peritumoral glial reaction

(A) GFAP immunoreactivity in the striatum of culture medium control animals compared with the tumour mass and the peritumoral area of the right hemisphere of tumour inoculated brains 7 days following surgery, ***$p<0.001$ (B) Coronal section of GFAP immunostained brain 7 days following tumour inoculation showing increased immunoreactivity in the peritumoral area when compared to similar location in the contralateral hemisphere (C) IBA1 immunoreactivity in the striatum of the culture medium control group compared with the tumour mass and in the peritumoral

area of the right hemisphere of tumour inoculated brains 7 days following surgery, **$p<0.01$, *$p<0.05$ (D) Section from the right striatum of a culture medium control brain stained for IBA1 antibody, showing minimal microglial immunoreactivity (E) IBA1 immunostained brain 7 days following tumour inoculation showing increased immunoreactivity in the peritumoral area (PTA) and tumour mass (TM)

In conjunction with the color deconvolution results, GFAP and IBA1 immunolabelled cells were also counted to determine the number of astrocytes and microglia respectively. The growth of Walker 256 breast carcinoma tumours caused an increase in the number of astrocytes and microglia in the striatum surrounding tumour masses, when compared to similar brain locations in the culture medium control group, although this difference was only significant for IBA1 ($p<0.05$; Fig. 6A & B). Treatment with Emend caused a reduction in the number of GFAP and IBA1 positive cells to levels comparable to the culture medium injected control group (Fig. 6A & B). Dexamethasone caused a similar phenomenon to Emend, particularly in regards to the number of IBA1 positive cells (Fig. 6A & B). Furthermore, there was a small reduction in the number of astrocytes and microglia present in the peritumoral area of NAT treated animals (Fig. 6A & B).

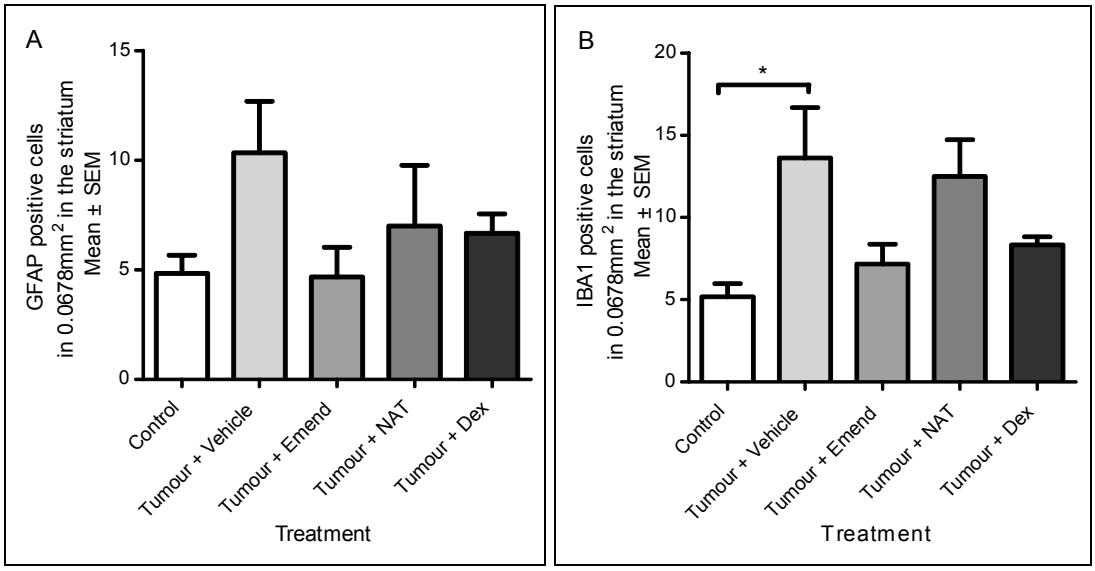

Figure 7.6 Brain microenvironment reaction to tumour growth

(A) The effect of treatment on the number of GFAP immunolabelled cells in the striatum of culture medium control animals and the striatum surrounding the tumours in inoculated animals 7 days following surgery; NAT, n-acetyl $_{L}$-tryptophan; Dex, Dexamethasone (B) The effect of treatment on number of IBA1 immunolabelled cells within the striatum of culture medium control animals and the striatum surrounding the tumours in inoculated animals, 7 days following surgery; NAT, n-acetyl $_{L}$-tryptophan, Dex, Dexamethasone; *$p<0.05$.

7.5 Discussion

This model of direct stereotaxic inoculation of Walker 256 tumour into the striatum of male albino Wistar rats caused consistent growth of large, spherical-shaped secondary brain tumours. These tumours showed a prominent cystic component and extensive central necrosis and haemorrhage, as described previously in the literature (Lewis 2012a). The model used in the current study is advantageous in that it is used in immune-competent animals, thus allowing the study of the interaction of tumour cells with the host microenvironment and immune system.

The Walker 256 rat breast carcinoma cells expressed NK1 receptors in vivo when injected into the striatum of male Wistar rats. It has previously been reported that many human tumour specimens and their derived tumour cell lines express NK1 receptors (Eistetter 1992; Johnson 1992; Friess 2003; Rosso 2008; Akazawa 2009; Munoz 2010b; Munoz 2012). Some cell lines have been reported to express up to 40, 000 NK1 receptors per cell (Fowler 1994), with the level of NK1 receptor expression being linked to invasiveness of the cancer cell lines (Castro 2005). Moreover, non-cancerous epithelial cell lines did not show evidence of NK1 receptor expression (Singh 2000; Ramkissoon 2007). However, NK1 receptors are by no means present on all cancer cell types, with one study demonstrating NK1 expression on SNB-19, DBTRG-05 MG and U373 MG human glioma cell lines, but not on the U138 MG or MOG-G-GCM human glioma cells (Palma 1999). Furthermore, the murine neuroblastoma cell line C1300 did not show evidence of NK1 receptor expression (Fukuhara 1996).

Despite the fact that the Walker 256 cells express NK1 receptors, only Emend was able to cause a reduction of cell viability and not NAT. A possible explanation for the differential response of Walker 256 cells to Emend and NAT is that Emend has an increased affinity for the NK1 receptor and a greater ability to penetrate the tumour cell than NAT, as; NAT is not membrane permeable unless modified to N-acetyl-$_{L}$-

tryptophan 3, 5-bis (trifluoromethyl) benzyl ester, also known as L-732-138 (Donkin 2011).

Previous studies to determine the effects of NK1 antagonist treatment on tumour cells in vitro have reported an inhibition of tumour growth, initiation of apoptosis, decreased migration and reduced cytokine secretion by tumour cells (Palma 1998; Palma 1999; Bigioni 2005; Munoz 2007; Rosso 2008; Huang 2010; Munoz 2010a; Munoz 2010b; Munoz 2012).The predominant effects of exogenous SP on tumour cells in vitro is mitogenesis, migration, cytokine secretion and chemotaxis (Ruff 1985; Luo 1996; Drell 2003; Friess 2003; Lang 2004; Munoz 2004b; Munoz 2008; Akazawa 2009). These effects are thought to be NK1 receptor dependent, as tumour cell lines with differential expression of NK1 receptors, show that the cell lines lacking NK1 receptors do not respond to NK1 antagonist treatment (Palma 1999).

Alternatively, several studies have shown evidence of inhibitory effects for SP on growth and migration of cancer cells (Ogasawara 1997; Nagakawa 1998; Korcum 2009). However, the NK1 receptor expression status of these cell lines has not been determined. In contrast, the Walker 256 cells used in the current study are positive for NK1 receptor expression, but only responded to Emend treatment and not NAT treatment in vitro. Aside from Emend having a much greater binding capacity for the NK1 receptor because of its lipophilicity, a plausible explanation for this may be that this cell line does not secrete SP into the culture medium in vitro and thus is unable to have a stimulatory autocrine effect on the NK1 expressing cells. It has previously been reported that Walker 256 breast carcinoma cells do not express SP in this model when directly injected into the brain (Lewis 2012a). However, tumours induced from this cell line do show increased SP immunoreactivity in the peritumoral neuropil in two different models of metastatic brain tumours (Lewis 2012a; Lewis 2012b). Thus it is plausible that exogenous SP from the brain microenvironment may have a stimulatory effect on these tumour cells in vivo.

Unlike previous experiments showing cytotoxic effects of dexamethasone on tumour cells (Bavaresco 2007; Piette 2009; Tazik 2009), the current study did not show any evidence of dexamethasone toxicity on Walker 256 breast carcinoma cells as measured by the trypan blue exclusion cell viability assay. Dexamethasone effects on Walker 256 cell viability have not previously been investigated in culture, although it has been shown to decrease parathyroid hormone secretion in a dose dependent manner (Schilling 1996). Previous in vitro studies have shown dexamethasone to inhibit mitogenesis and cell viability of C6 glioma cells along with decreasing migration and invasive properties of U373 MG human glioblastoma cells (Bavaresco 2007; Piette 2009). However, if dexamethasone is toxic to tumour cells in vivo, then it is also likely that non-neoplastic brain cells would also become apoptotic also (Tazik 2009).

NK1 antagonist treatment of metastatic brain tumours grown in this study did not alter tumour cell volume or the volume of necrotic haemorrhagic tissue within the tumour mass. Furthermore, levels of tumour cell replication and apoptosis remained unaffected by both NK1 antagonist treatments used. There have been few previous studies investigating the effects of NK1 antagonism on cancer growth in animal models, despite the fact that Emend is already approved for use in cancer patients for nausea associated with chemotherapy. The limited number of experiments that have been performed in vivo have been inconsistent in their findings. One published study demonstrated a beneficial effect of the NK1 antagonist MEN 11467 on subcutaneously inoculated human U373 MG astrocytoma grade III cells by arresting tumour growth, but not when used on human A2780 ovarian carcinoma lacking NK1 receptors, also inoculated into the right flank of female nude mice (Palma 2000). Another study also employed MEN 11467 on the MDA-MB-231 human breast cancer cell line, which express NK1 receptors, injected subcutaneously into the right flank of female nude mice (Bigioni 2005). This experiment showed no effect of MEN 11467 on final tumour volume when administered alone, although caused a reduction when co-administered with the NK2 antagonist nepadutant (Bigioni 2005).

In contrast, studies that have been performed in immune competent animals have suggested that SP may be beneficial in the treatment of cancer patients through its immune stimulating properties. For example, aerosolised SP caused a reduction in the incidence of lung carcinoma in C57BL mice exposed to side stream cigarette smoke (Harris 2003). Similarly, capsaicin-induced depletion of SP secreting sensory neurones, resulted in the induction of a more aggressive phenotype, including increased cardiac and lung metastases from orthotropically implanted 4T1 breast carcinoma cells, although primary tumour growth remained unaffected (Erin 2004; Erin 2006). Furthermore, K1735 melanoma cells injected subcutaneously into nude mice responded to exogenous SP with a delay in tumour growth, but only with the addition of natural killer and T cells, normally absent in immune compromised animals (Manske 2005). Therefore, the role of SP in cancer growth in vivo remains controversial, with more research required to elucidate the factors that determine if NK1 antagonist treatment is effective in the inhibition of tumour growth in animal models.

Dexamethasone treatment resulted in decreased brain tumour volume when compared to the vehicle treated group. This is likely to be due to impaired accumulation of fluid, as seen in previous studies (Lewis 2012a), rather than a decrease in the growth of the tumour cells. Evidence of this is the absence of change in tumour cell replication and apoptosis with dexamethasone treatment. Moreover, this is supported by the significantly increased tumour cell density, evident in the dexamethasone treated group when compared to vehicle-treated animals. Thus, the dexamethasone treated group may have the same number of tumour cells, packed more closely together, reducing the tumour volume. Also the decreased necrotic tissue within the dexamethasone-treated tumours indicates that although the tumour volume is reduced in this group, it is possible that the tumours had viable tumour cells comparable to the number of cells in the vehicle treated tumours.

Reduction in tumour volume has been repeatedly shown with dexamethasone treatment in the literature (Guerin 1992; Wolff 1993; Badruddoja 2003; Villeneuve

2008). These data have been used with dexamethasone's toxicity in vitro to suggest an anticancer effect for dexamethasone. However, dexamethasone has also consistently been shown to reduce BBB permeability and oedema (Nakagawa 1987; Guerin 1992; Wolff 1993; Heiss 1996; Badruddoja 2003; Ewing 2008; Gu 2009a; Gu 2009b), which may account for the volume decreases seen. Another proposed mechanism of dexamethasone inhibition of tumour growth is through manipulation of the host microenvironment. Dexamethasone causes a blockade of classical inflammation, normally favourable for tumour invasion and growth (Van Den Brenk 1974a; Van Den Brenk 1974b; Zhang 1997; Lewis 2012a).

In the current study dexamethasone treatment was accompanied by a reduction in the volume of necrotic tissue and haemorrhage in the tumours. A possible reason for the decreased necrotic tissue is the decreased distance between tumour cells and blood vessels, as a result of reduced oedema. Accumulation of tumour interstitial fluid in the vehicle treated group may cause tumour cells to become increasingly remote from blood vessels, with reduced availability of nutrients. This model of secondary brain tumour has previously been shown to cause an increase in brain water content, which is significantly reduced with dexamethasone treatment (Lewis 2012a). Furthermore, it has previously been reported that dexamethasone treatment decreased blood vessel density and volume within experimental gliomas (Wolff 1993; Badruddoja 2003).

In the current study, tumour growth caused an increase in both astrocytes and microglia, particularly in the peritumoral environment. This phenomenon has been well documented in many metastatic brain tumour models (Zhang 1995; Lorger 2010). Emend treatment decreased this glial response to tumour growth, showing that the dose used in this study was sufficient to elicit a central effect. NAT was less effective than Emend in this capacity, possibly due to its inability to cross the BBB (Donkin 2011). Emend is a BBB penetrating drug able to occupy cerebral NK1 receptors (Bergstrom 2004), therefore it is logical that it would initiate a greater effect on the brain microenvironment. Dexamethasone also reduced glial numbers to a level similar to Emend treatment, although it is unclear if this effect was due to inhibition

of tumour initiated glial recruitment, activation and proliferation, or through toxic effects on astrocytes and microglia. It has been postulated that microglia secrete growth stimulatory substances that aid metastatic brain tumour growth, as evidenced by the increase in MDA-MB-231 human breast cancer cells when co-cultured with microglia in vitro (Fitzgerald 2008). Other tumour cells have been shown to differentially activate microglia so that subpopulations express inducible nitric oxide synthase and tumour necrosis factor-alpha (He 2006). Thus with more investigation, glial regulation may provide a possible target for manipulation of metastatic brain tumour growth.

In conclusion, neither NK1 antagonist treatment acted as an anticancer agent in this animal model of metastatic brain tumours, despite the expression of NK1 receptors on Walker 256 tumour cells and the fact that Emend reduced cell viability in vitro. In conjunction, dexamethasone treatment also did not affect tumour growth, notwithstanding evident reduction of tumour volume. Therefore neither Emend nor dexamethasone is effective as anticancer agents in this model of brain metastatic breast cancer. However, further research is required using different models and cancer cells to definitively determine the exact effect of NK1 antagonists and dexamethasone treatments on tumour growth.

8 General Discussion

8.1 Purpose

The aim of this thesis was to determine the role of SP in the development, growth and complications of metastatic brain tumours caused by breast cancer, particularly in relation to the interactions between SP and the BBB. The BBB is integral to many aspects of metastatic brain tumour development and makes these types of neoplasms distinct from cancer in other organs. The progression of cancer to the brain relies on the ability of tumour cells to undergo extravasation through the BBB. Similarly, vasogenic cerebral oedema is a common complication of metastatic brain tumour growth and involves a compromised BBB function so that plasma proteins and fluid are able to pass from the vasculature into the brain microenvironment.

Aspects of metastatic brain tumour growth are also affected by the presence of the BBB as angiogenesis and often vessel co-option allow the nutritional needs of the growing neoplasm to be met (Ito 1993; Maniotis 1999; Bugyik 2011), although these new blood vessels have different properties from those of the normal BBB (Front 1984). Furthermore, the treatment of metastatic brain tumours is inherently difficult because the BBB excludes many substances from the brain microenvironment (Lien 1991), such that agents that disrupt the BBB have been investigated to improve delivery of chemotherapeutic agents to CNS tumours (Cote 2010). In conjunction, whole brain radiotherapy has been shown to cause the BBB to become more permeable (Adair 1999), which may exacerbate cerebral oedema and may lead to increased development of complications (Shamji 2009).

The effects of SP on the BBB in other neurological diseases have been well established and the blockade of its actions led to novel treatment strategies (Donkin 2009; Turner 2011). Therefore the role of SP on the BBB in association with metastatic brain tumour progression, growth and complications is of great interest. Therefore, the effects of NK1 antagonists on these parameters were investigated here

with regard to their possible benefits for patients suffering from brain metastatic breast cancer.

8.2 Models used

The Walker 256 cells used in the experiments detailed in this thesis were obtained from two different reputable cell banks. These two populations of Walker 256 cells were evaluated for their tumorigenicity and the more tumorigenic CRCTU Walker 256 cells were chosen for the remainder of the studies. CRCTU Walker 256 cells produced more reliable metastatic brain tumours in both the internal carotid artery and direct intracerebral inoculation models. Therefore using these cells required less animals in order to determine the effects of NK1 antagonist treatment. Furthermore, the consistency in growth characteristics using the CRCTU Walker 256 cells made reproducible results more likely.

Differential incidence of tumour growth was apparent, not only between the tumour cells from different cell banks, but also between the two models of inoculation. The direct injection model consistently produced a single large tumour mass in the striatum of the brain when CRCTU Walker 256 cells were utilised. In contrast, the internal carotid artery inoculation model of CRCTU Walker 256 breast carcinoma frequently resulted in multiple brain metastases, predominantly located within the lateral ventricles. These patterns of growth have been observed previously in studies comparing modes of metastatic brain tumour induction using the Lewis lung carcinoma cell line (Saito 2008).

The direct injection had the advantage of more consistent tumour size and predictable location of tumour burden, although the internal carotid artery inoculation model allows for the BBB to be investigated during the extravasation process. The internal carotid artery CRCTU Walker 256 inoculation model also elicited moderate extracranial tumour growth, although this did not lead to any increase in mortality before the euthanasia time point designated by metastatic brain tumour growth. This

is typical of haematogenously spread cancer metastases, which commonly affect multiple organs (Harrell 2012), and once again demonstrates the suitability of the internal carotid artery model of tumour induction as a model for the human metastatic spread of cancer cells to the brain.

8.3 Principal findings

In vivo, the Walker 256 breast carcinoma cells from CRCTU were positive for NK1 receptor immunoreactivity, but did not exhibit SP expression. It has previously been shown that NK1 receptor expression is necessary for SP to induce replication in culture (Palma 1999). In vitro, this cell line did not respond to NAT, but treatment with Emend reduced cell viability. Both Emend and NAT are NK1 receptor antagonists, although Emend is more lipophilic and has greater binding affinity for NK1 receptors (Bergstrom 2004; Donkin 2009). This result may indicate that autocrine SP secretion by tumour cells is required for NK1 antagonists to be consistently effective anticancer agents in vitro.

In the current study both the internal carotid artery injection and direct inoculation model of metastatic brain tumour induction showed increased SP immunoreactivity in the peritumoral area, despite being largely absent within the tumour mass. In previous cell culture studies, exogenous SP added to tumour cells has been shown to cause increased mitogenesis, migration, cytokine secretion and chemotactic behaviour (Ruff 1985; Luo 1996; Palma 1998; Drell 2003; Friess 2003; Lang 2004; Munoz 2004b; Bigioni 2005; Munoz 2005a; Munoz 2005b; Munoz 2007; Munoz 2008; Rosso 2008; Akazawa 2009; Huang 2010). Therefore the author hypothesised that the increased SP expression surrounding metastatic brain tumour growth seen in the current study would result in a similar stimulatory effect on implanted cancer cells in the striatum.

However, NK1 antagonist treatment did not alter any indicators of tumour growth, meaning that the peritumoral SP did not have a sufficient stimulatory effect on cancer cells in the direct injection model of Walker 256 brain metastases for SP blockade to

inhibit tumour growth. The current study therefore showed that the presence of NK1 receptors on tumour cell membranes is not sufficient in iteslf for NK1 antagonists to be beneficial for the treatment of cancer. It is possible that tumour cells need to be of a type that secretes SP for a treatment effect to be evident. There may, however, be a subset of cancer types that are more responsive to NK1 receptor antagonist treatment, although further investigation is required to clarify this potential.

Many human cancer specimens have been shown to express SP, although there is significant variations both between and within cancer types (Allen 1985; Zhu 1995; Khare 1998; Singh 2000; Nowicki 2002; Gonzalez Moles 2008; Brener 2009; Esteban 2009; Gonzalez-Moles 2009). Furthermore, tumours that are postitive for SP often do not express it ubiquitously and rather have subpopulations of immunoreactive cells (Munoz 2007). Thus the effectiveness of NK1 antagonist treatment may be limited, even for tumours that do express SP.

Following internal carotid artery inoculation of Walker 256 cells, there was an increase in perivascular SP immunoreactivity associated with tumour cell invasion of brain microvessels. At the same time point, there was also increased albumin immunoreactivity indicative of increased BBB permeability. It was therefore postulated that contact of tumour cells with luminal membranes of brain endothelial cells caused the release of SP, which resulted in increased BBB permeability, allowing tumour cell extravasation into the brain. However, treatment with NK1 antagonists was ineffective in preventing metastatic brain tumour growth. Therefore it was concluded that SP was not the primary mediator of Walker 256 extravasation through the BBB in this model.

The variable CNS tropism of different cancer types implies that there are inherent molecular characteristics of tumour cells that are beneficial for metastatic progression to the brain. It has been postulated that only specific cancer types, or even subpopulations of tumour cells within a neoplastic mass, have the propensity for

metastases to the brain, in particular extravasation through the BBB. Despite the largely negative results presented in this thesis, the possibility cannot be ruled out that SP may play a role in brain targeting by other cancer types, particularly if a subpopulation of their cells secrete high levels of SP, unlike the cell line used in the current study.

Increased SP immunoreactivity surrounding established brain metastases led to the hypothesis that SP caused increased permeability of blood vessels surrounding the neoplasms, which in turn allowed plasma proteins and fluid to leak into the neuropil. However, in the current study neither BBB permeability nor brain water content was affected by NK1 antagonist treatment. These findings contrast with previous studies, where NK1 antagonist treatment has been used to decrease the disruption of the BBB following traumatic brain injury and stroke (Donkin 2009; Turner 2011). This suggests that SP plays a more prominent role in the pathogenesis of these acute injury conditions as opposed to oedema formation in the metastatic brain tumour models used in the current study. The mechanism of oedema development secondary to metastatic brain tumours is therefore distinct from that driving oedema formation following stroke and traumatic brain injury, despite both being vasogenic in nature.

In contrast to NK1 antagonist treatment, dexamethasone decreased brain water content, indicative of odedma. This result was not surprising given that dexamethasone is the current standard clinical treatment for peritumoral oedema and was thus used as a positive control for the oedema study in this thesis. Interestingly, dexamethasone treatment also caused increased tumour density along with decreased tumour volume, when compared to the vehicle control group of animals. This indicates that a substantial proportion of the oedematous fluid associated with metastatic brain tumour growth is localised within the tumour mass.

Despite the consistency of dexamethasone effects on tumour-associated oedema, the exact mechanism of its action remains controversial. It is thought that dexamethasone

predominantly acts by decreasing the permeability of the BBB (Nakagawa 1987; Guerin 1992; Gu 2009b). Previous studies have shown that dexamethasone modulates vasoactive molecules and substances involved in tight junction structure between cerebral endothelial cells of the BBB. For example, dexamethasone treatment has been shown to decrease tumour cell secretion of VEGF in vitro and reduce the action of conditioned media from the tumour cells on vascular permeability in vivo (Heiss 1996; Das 2008). Furthermore, dexamethasone treatment of brain tumours has resulted in increased expression of the tight junction protein occluden within the tumour (Gu 2009a).

Despite previous studies showing the modulatory effect of dexamethasone on the BBB, only a small BBB response was evident in the current study, with Evans blue concentration in the neuropil still elevated in the dexamethasone treated group compared to control level. Despite this, dexamethasone caused a reduction in brain water content to a level equivalent to brains that were not exposed to tumour growth. Therefore it is possible that modulation of BBB permeability is only partially responsible for the actions of dexamethasone on cerebral oedema. Also suggesting that permeability to water and Evans blue bound to albumin are driven by different mechanisms.

An alternative mechanism of dexamethasone action is modulation of water channels in the tumour cells or the surrounding neuropil. Under normal conditions, AQP-4 is expressed by brain astrocytes and AQP-1 is present in the choroid plexus and is involved with CSF production (Lukaszewicz 2011). Previous studies have shown that dexamethasone modulates brain AQP-4 expression following intracerebral haemorrhage and that these alterations in AQP-4 were correlated with oedema clearance achieved by dexamethasone treatment (Gu 2007b). A similar mechanism may be involved in the action of dexamethasone treatment of peritumoral oedema following direct injection of Walker 256 cells into the striatum. In conjunction, dexamethasone has demonstrated ability to induce AQP-1 expression in gliosarcoma cells in vitro (Hayashi 2007), although AQP-1 has been linked to increased migration

and metastatic potential of cancer (Hu 2006). Thus, in the current study, dexamethasone treatment may have acted by modulation of water channel proteins in both the tumour cells and the peritumoral environment to effect the clearance of cerebral oedema and tumour invasiveness. This requires further characterisation.

NK1 antagonist treatment did not alter the permeability of the BBB in either the internal carotid artery or direct inoculation models used in these studies. This suggests that NK1 antagonists are not suitable as preventative agents against BBB permeability or to treat the complications associated with metastatic brain tumours. However, it is possible that SP had a small contribution as one of many factors involved in the pathogenesis of tumour associated oedema in the direct inoculation model. Similarly, prevention of cancer cell extravasation across the BBB may still involve, but not require, the activity of SP. Therefore agents that act via multiple pathways, like dexamethasone, may be more suitable to target the BBB in metastatic brain tumour progression and subsequent complications.

Along with other possible pathways already discussed, there is some evidence of SP modulation by glucocorticoids, like dexamethasone. For example, topical glucocorticoid treatment inhibits oedema caused by intradermal SP injection (Ahluwalia 1995). Similarly, glucocorticoids inhibit plasma extravasation following tachykinin release from sensory neurones in tracheal mucosa, presumably by upregulation of proteins involved in the cleavage of SP (Piedimonte 1991). Furthermore, glucocorticoid treatment reduces capsaicin induced plasma extravasation in the trachea (Piedimonte 1990). Therefore if SP does have a minor role in the increased BBB permeability evident in the studies described in this thesis, its inhibition may be one of the multiple pathways by which dexamethasone successfully reduces cerebral oedema. However, the high brain water content evident in the NK1 antagonist treated group clearly shows that this is not the sole mechanism.

IBA1and GFAP immunoreactivity were used as indicators of brain tumour interaction with microglia and astrocytes, respectively. The number of astrocytes and microglia were substantially increased surrounding metastatic brain tumour growth in both models used. Furthermore, this study showed that microglia infiltrated the entirety of the tumour mass, but that astrocytes were only evident at the tumour border. This pattern of IBA1 and GFAP immunoreactivity has been observed in numerous studies where immunostained human specimens were analysed. These clinical studies demonstrated substantial labelling of microglia and astrocytes in the peritumoral area, whereas only microglia were evident within the human brain metastases, and astrocytes were apparent in septa between tumour cells at the border of the neoplasm (Zhang 1995; Berghoff 2012b). This pattern of staining occurs regardless of tumour malignancy or type (Strik 2004). However, the extent of microglial infiltration has been reported to vary from a few microglial cells to a 1:1 ratio of microglial cells to tumour cells (Pukrop 2010).

It is well established that the presence of tumour growth within the brain results in microglial and astrocytic activation through factors released from the tumour cells. In turn, the glial cells also release factors that act upon the neoplastic tissue. Therefore the relationship between neoplastic growth and the brain microenvironment is bidirectional. Previous studies have shown that co-culture of breast cancer cells with microglia caused microglial activation, whilst the activated microglia simultaneously enhanced tumour cell invasiveness (Hagemann 2004; Pukrop 2006; Pukrop 2010).

Glial activation by tumour cells is achieved through release of pro-inflammatory factors, as evident when human lung cancer cells activate astrocytes in vitro through secretion of macrophage migration inhibitory factor, interleukin-8 and plasminogen activator inhibitor-1 (Seike 2011). Several studies have also shown that serum albumin activates microglia, functioning to clear albumin through phagocytosis (Hooper 2005; Hooper 2009; Alonso 2011). This is pertinent, as it is widely accepted that within and surrounding metastatic brain tumours, blood vessel permeability is substantially increased. This was also demonstrated in the current thesis through the

increased albumin immunoreactivity in the neuropil, associated with metastatic brain tumour growth in both models used. Therefore, this leakage of albumin from the vasculature may have caused glial activation and the accumulation of these cells evident in the peritumoral area.

The function of tumour infiltration of microglia and peritumoral accumulation of glia is debatable. Both factors that promote growth and tumoricidal factors may be released from glia to act on neoplastic tissue. However, glial cell manipulation of tumour growth properties is likely dependant on activation of the glia by the tumour cells themselves. This was evident in previous studies when control microglia did not have any effect on tumour cell viability, whereas once activated, caused tumour cell lysis in vitro (Brantley 2010). Furthermore, activated microglia potently act on cancer cells but non-neoplastic cells remained unaffected (Brantley 2010). Moreover, one study showed that the supernatant from lipopolysaccharide activated microglia caused dose dependant apoptosis of human non-small cell lung cancer cells (He 2006). Thus, factors with tumoricidal properties are secreted by the microglia and do not require cell-to-cell contact.

In contrast, co-culture of MDA-MB-231 human breast cells with mixed glial cells caused tumour cell mitogenesis to increase 5 fold (Fitzgerald 2008). More specifically, activation of astrocytes by tumour cells causes production of inflammatory mediators, such as interleukin-6, tumour necrosis factor-alpha and interleukin-1 beta, which induce tumour cell proliferation (Langley 2009; Seike 2011). Furthermore, co-culture of human melanoma, lung cancer and breast cancer cells with murine astrocytes protected cancer cells from chemotherapy induced apoptosis, a process that was dependent on gap junctions (Lin 2010; Kim 2011).

These studies, along with the glial staining patterns evident in the current thesis, suggest that targeting specific activation pathways of microglia may be a useful therapeutic target for metastatic brain tumour treatment. In contrast, astrocyte

activation promotes tumour growth. Therefore, development of treatment options that enhance microglial activation whilst inhibiting activation of astrocytes may be beneficial for patients suffering from neoplastic growth in the CNS.

Treatment with Emend largely reduced the glial reaction evident with neoplastic growth in the brain, although NAT was not as effective in this capacity. This is likely the result of superior BBB penetration and NK1 receptor binding affinity of Emend when compared to NAT (Bergstrom 2004; Donkin 2009). Previous studies have shown that NK1 antagonist treatment partially reverses the increase in GFAP and IBA1 immunoreactive cells evident after 6-OHDA induced Parkinson's disease in rodents (Thornton 2012). Furthermore, NAT treatment has been found to inhibit microglial proliferation following traumatic brain injury (Carthew 2012). Therefore in the current study the mechanism behind the decrease in microglia with NK1 antagonist treatment is likely through reduced proliferation of these cells rather than drug toxicity. Similar pathways may also be responsible for the reduction in astrocytes surrounding metastatic brain tumours as a result of NK1 antagonist treatment.

Dexamethasone also reduced glial reaction to metastatic brain tumour growth, similar to Emend. This effect was potentially due to the anti-inflammatory immune suppression properties of dexamethasone or alternatively may have been a result of widespread cytotoxicity due to the high dose used in this study. In vitro, dexamethasone has previously been shown to inhibit astrocyte proliferation, and a similar phenomenon was observed in vivo when excess adrenocorticotropic hormone decreased astrocyte numbers in the frontal cortex, although this was evident under non-pathological conditions (Unemura 2012).

When gram-negative bacteria derived lipopolysaccharide is applied to mixed glial cells in culture, its effects to decrease functionality of astrocytes, increase microglial proliferation, nitric oxide and reactive oxygen species production are ameliorated with

dexaemthasone treatment (Hinkerohe 2010; Huo 2011). Similarly, dexamethasone reduced microglial activation in a cell culture system made up of astrocytes with 30% microglia, representing a pathological condition of microglial overrepresentation (Hinkerohe 2011). Dexamethasone has also been shown to decrease inflammatory mediator mRNA expression by microglia in culture (Graber 2012). Therefore, dexamethasone has repeatedly been shown to reduce the pathological alterations in astrocytes and microglia commonly evident with many neurological diseases as was evident in the current study following dexamethasone treatment of metastatic brain tumours. However the exact mechanism and possible implications of these actions are yet to be fully elucidated.

8.4 Further research

The current thesis has demonstrated that NK1 antagonist treatment was ineffective for prevention of tumour cell extravasation, tumour growth inhibition and resolution of peritumoral oedema. SP therefore does not play a prominent role in the progression, growth or complications of the Walker 256 internal carotid artery injection or direct inoculation models of metastatic brain tumour. These results were apparent despite the NK1 receptor expression on the Walker 256 breast carcinoma cells. However, it is possible that SP secretion by tumour cells is required for NK1 antagonist treatment to modulate the BBB in animal models of metastatic brain tumours. In further studies it would be pertinent to investigate the effect of NK1 antagonist treatment on the BBB in models of metastatic brain tumours that employ tumour cells that secrete high levels of SP. Thus, the additional effect of the combination of tumour-secreted and peritumoral SP could be investigated, rather than only the tumour initiated release of SP from the brain microenvironment, as was the case in the current thesis.

Similarly, future research should employ both human and murine tumour cell lines. Moreover, more than one species should be utilised for animal models of brain metasatatic disease. Human tumour cells develop more complex mutations over a longer period of time, although their use in animal models of cancer requires immune compromised animals (Rangarajan 2004). Therefore, employing human tumour cells

more closely replicates the human disease process, but not the interaction with host microenvironment evident in human pathology.

Interaction with the host immune system is particularly important for determining the role of SP in metastatic brain tumour pathogenesis. This is because SP is an inflammatory mediator that has a modulatory effect on the immune system and has previously been shown to inhibit cancer growth, but only in the presence of natural killer and T cells (Manske 2005). Therefore experiments investigating NK1 receptor antagonist effects on cancer that are performed in immune compromised animals may show positive results that may not be replicated when used in the human condition.

In the current thesis, dexamethasone effectively resolved tumour induced cerebral oedema, although the exact mechanism of its action is yet to be elucidated. Dexamethasone has been found to modulate many different pathways including, but not limited to, aquaporin water channels, VEGF, SP, bradykinin and tight junction proteins. The multiple mechanisms of dexamethasone action may be the reason for its effectiveness in treating peritumoral oedema clinically, as it is likely that tumour progression and complications also occurs via a combination of multiple pathways. Unfortunately, its use is associated with many harmful side effects, which limit its use.

Subsequent studies should aim to elucidate the pathways of dexamethasone action on oedema and determine those actions which cause the unwanted side effects. Therefore, if the beneficial and detrimental pathways do not overlap, scientists may endeavour to develop more targeted therapies that are able to replicate the effects of dexamethasone on cerebral oedema without the side effects currently experienced by patients. This may allow for more prolonged high dose treatment of oedema, which remains a serious complication of metastatic brain tumours.

8.5 Conclusion

Despite elevated SP levels surrounding tumour-invaded blood vessels and in well established metastatic brain tumours, NK1 antagonist treatment did not alter tumour incidence, tumour volume or brain water content. This was in spite of NK1 receptor expression by the Walker 256 cells used and the effective reduction of tumour cell viability with Emend treatment in vitro. The results in this thesis suggest that tumour cell secretion of high levels of SP are required for NK1 to be an effective anticancer agent or to resolve tumour induced increases in BBB permeability.

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