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EVALUATING THE ROLE OF SUBSTANCE P IN THE GROWTH OF BRAIN TUMORS

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Abstract—Recent research has investigated the expression and secretion of neuropeptides by tumors, and the potential of these peptides to facilitate tumor growth and spread. In particular, substance P (SP) and its receptor NK1 have been implicated in tumor cell growth and evasion of apoptosis, although few studies have examined this relationship *in vivo*. The present study used both *in vitro* and *in vivo* models to characterize the role of SP in tumor pathogenesis. Immunohistochemical assessment of human primary and secondary brain tumor tissue demonstrated a marked increase in SP and its NK1 receptor in all tumor types investigated. Of the metastatic tumors, melanoma demonstrated particularly elevated SP and NK1 receptor staining. Subsequently, A-375 human melanoma cell line was examined *in vitro* and found to express both SP and the NK1 receptor. Treatment with the NK1 receptor antagonist Emend IV resulted in decreased cell viability and an increase in cell death in this cell line *in vitro*. An animal model of brain tumors using the same cell line was employed to assess the effect of Emend IV on tumor growth *in vivo*. Administration of Emend IV was found to decrease tumor volume and decrease cellular proliferation indicating that SP may play a role in tumor pathogenesis within the brain. We conclude that SP may provide a novel therapeutic target in the treatment of certain types of brain tumors, with further research required to determine whether the role of SP in cancer is tumor-type dependent.

Key words: neuropeptides, brain tumors, NK1 antagonist, substance P, cancer therapy.

INTRODUCTION

Cancer is a leading cause of death and disability worldwide, accounting for 7.6 million deaths annually (Ferlay et al., 2010). In particular, brain tumors are inherently difficult to treat given that the unique features of the brain can complicate the use of conventional diagnostic and treatment methods. Consequently, both primary and secondary brain tumors tend to have an extremely poor prognosis, with the majority of patients succumbing to the disease within months of diagnosis. Melanoma, in particular, is a highly aggressive and invasive cancer that commonly metastasizes to the brain, with up to 75% of stage 4 melanoma patients displaying secondary brain tumors at autopsy (Budman et al., 1978; Patel et al., 1978; Shapiro and Samlowski, 2011). Given the difficulties in treatment of brain tumors, research has been increasingly directed toward finding specific mediators that can be targeted in the treatment of brain malignancies. Of particular interest are neuropeptides and their receptors, which have been implicated in many aspects of cancer growth and progression (Harford-Wright et al., 2011; Munoz et al., 2012; Harford-Wright et al., 2013).

Substance P (SP) is a neuropeptide released from the endings of sensory nerve fibers and preferentially binds to the NK1 receptor. It has a widespread distribution throughout the nervous system, where it is implicated in a variety of functions including neurogenic inflammation, nausea, depression and pain transmission (Gardner et al., 1995; Zubrzycka and Janecka, 2000; Nimmo et al., 2004; Bardelli et al., 2013), as well as in a number of neurological diseases, including CNS tumors. *In vitro* studies have confirmed that SP and the NK1 receptor are increased in numerous tumor cell lines including malignant gliomas, breast carcinoma and metastatic melanomas (Khare et al., 1998; Munoz et al., 2005a; Singh et al., 2006). The revelation that NK1 receptors may be significantly upregulated in cancer has led to the proposal that SP might facilitate tumor growth via induction of DNA synthesis and cellular proliferation (Esteban et al., 2006). However, to date the majority of studies investigating the role of SP in cancer have been performed *in vitro*, and the few *in vivo* studies have yielded conflicting results (Palma et al., 2000; Harris and Witten, 2003; Lewis et al., 2013). Additionally, no study has yet examined the effect of NK1 antagonist treatment in an *in vivo* model of melanoma brain metastases.

Only one NK1 antagonist is currently approved for human use (Hesketh et al., 2003; Herrstedt et al., 2005;

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Abbreviations: ATCC, American-Type Culture Collection; BSA, bovine serum albumin; CCM, complete culture medium; DAB, 3,3′-diaminobenzidine; ELISA, enzyme-linked immunosorbent assay; GBM, glioblastoma multiforme; HRP, horseradish peroxidase; LDH, lactate dehydrogenase; NBF, neutral-buffered formalin; NHMRC, National Health and Medical Research Council; RAH, Royal Adelaide Hospital; SP, substance P; SPC, streptavidin–peroxidase conjugate; TBS, Tris-buffered saline; TMB, 3,3′-5′5′-tetramethylbenzidine.

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Ruhlmann and Herrstedt, 2012). Fosaprepitant diglutemide (L-758,298) is the intravenous prodrug of aprepitant (L-754,030) commonly known as Emend IV. In cancer patients, Emend IV is used as an antiemetic to ameliorate the nausea and vomiting frequently associated with the use of many chemotherapeutic agents. While a number of NK1 antagonists have been identified as potential anti-cancer agents in experimental studies, Emend IV remains the only NK1 antagonist that is clinically available for any indication. Furthermore, to date, no study has examined the effect of Emend IV on the size, progression or survival of human brain tumor patients receiving this treatment. Accordingly, the current study examines the expression of SP in a variety of human primary and secondary brain tumors, as well as the effect of the NK1 antagonist, Emend IV, on tumor cell growth using both *in vitro* and *in vivo* models of melanoma brain tumor.

EXPERIMENTAL PROCEDURES

Human tissue

A total of 63 de-identified human surgical cases were used to investigate SP and NK1 receptor expression in a variety of primary and secondary brain tumor specimens, with all studies performed according to the National Health and Medical Research Council (NHMRC) guidelines and approved by the Royal Adelaide Hospital (RAH) and University of Adelaide human ethics committees. Following surgical excision, all human tissue was immediately immersion fixed in formalin, processed and embedded in paraffin wax. The mean age was 52 years, with an even split of females ($n = 31$) and males ($n = 32$). Tumors were categorized into primary brain tumors including grade 1 and 2 astrocytoma ($n = 9$), grade 3 astrocytoma ($n = 5$), and glioblastoma multiforme (GBM) ($n = 10$), or as secondary tumors arising from primary tumors of the lung ($n = 6$), breast ($n = 10$), colon ($n = 10$) and skin (melanoma, $n = 10$). Age-matched non-pathological brain tissue ($n = 3$) served as control. All human tissue was classified according to the pathological diagnosis performed by the RAH pathologists at the time of surgery.

Cell culture

A-375 human melanoma cells were obtained from American-Type Culture Collection (ATCC, CRL-1619) and passaged immediately upon receipt. ATCC authenticates cell lines routinely with short tandem repeat profiling to establish a DNA fingerprint of human cell lines, monitoring of cell morphology and karyotyping to identify the species and any variation within the cell line.

For all experiments, A-375 cells were placed in a 12-well plate at a cell density of ~105 cells per well with 2 mL of complete culture medium (CCM) consisting of Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 5% L-glutamine and 1% penicillin and streptomycin. Cells were allowed to grow for 48 h and subsequently treated with the NK1 antagonist fosaprepitant dimeglumine (Emend IV®, MERCK & CO, USA) or saline. The NK1 antagonist Emend IV was prepared by dissolving 0.6 mg/mL of Emend IV in normal saline, with triplicate wells being treated with each of the following concentrations: 10, 100 and 1000 μg/mL (equivalent to 9.995, 99.52 and 995.22 μmol/l) for 24 h.

Cell viability assay

A trypan blue exclusion assay was employed to assess the response of A-375 human melanoma cells to differing doses of the NK1 antagonist. Trypan blue is a vital dye that is only taken up by cells thought to be undergoing end stage cell death. Cultures were grown up and treated as described above before the supernatant from each well was removed and frozen for separate enzyme-linked immunosorbent assay (ELISA). Cell viability counts were then performed with the addition of 0.4% trypan blue at a ratio of 1:1 to cell suspension. Both clear and blue cells were counted using a hemocytometer and results expressed as a percentage of viable cells.

Lactate dehydrogenase (LDH) ELISA

An ELISA was used to determine the level of LDH, a stable cytoplasmic enzyme that is rapidly released upon damage to the plasma membrane (Giordano et al., 2011). The amount of LDH detected in the culture supernatant is thought to correlate with the number of lysed cells. Following treatment of A-375 melanoma cells *in vitro*, samples of media from cell culture (20 μL of media/80 μL Tris-buffered saline (TBS)) were loaded into each well of a 96-well Maxisorp plate in triplicate, with 400 ng/100 μL of bovine serum albumin (BSA) serving as controls. The protein was allowed to coat the wells overnight at 4 °C. Samples were tipped off and the blocking agent applied (3% BSA solution) to each well and gently agitated for 1 h. Samples were incubated with 100 μL LDH primary antibody (Abcam, UK; 1:1000) at 37 °C in a humid container for at least 1 h and subsequently incubated with secondary anti-goat horseradish peroxidase (HRP) (Vector Laboratories, USA; 1:1000) for 1 h in a 37 °C oven. Finally, 100 μL of 3,3′-5′5′-tetramethylbenzidine (TMB) was used to reveal protein expression in each well and the reaction stopped with 50 μL of 0.5 M H_2SO_4 at the same time for each well. The level of LDH expression was determined by reading the absorbance at 450 nm on a Synergy Mx plate reader. To demonstrate reproducibility of results, the LDH ELISA was performed three times.

Cell culture for *in vivo* inoculation

Prior to tumor cell inoculation, A-375 cells growing in 150-cm^2 culture flasks were passaged until >90% confluence was reached. The CCM was removed from the flask and 3.5 mL of trypsin was added to detach cells. Following this 7.5 mL of CCM was added and the cells spun down in a centrifuge (5 min at 1500 rpm). Cells were re-suspended in serum-free media and

counted using a hemocytometer and subsequently diluted, so that $\sim 10^5$ cells/5 μL were available for tumor cell inoculation.

Animals

Animal procedures were performed in accordance with the NHMRC guidelines and were approved by the University of Adelaide and Institute of Medical and Veterinary Science animal ethics committees. All *in vivo* experimental work was performed using male Balb/C nude mice aged 8–10 weeks, weighing between 17 and 22 g. Animals were randomly assigned to each group and group housed in specific pathogen-free (SPF) conditions at 24 °C on a 12-h day–night cycle. Animals were allowed access to standard rodent pellets and water *ad libitum.*

Implantation of tumor cells

Tumor cell inoculation was performed using stereotactic implantation of $\sim 10^5$/5 μL A-375 human melanoma cells. Briefly, mice were anesthetized with 3% Isoflurane and placed in a stereotactic frame, with anesthesia maintained at 1.5% Isoflurane using a nose cone. A midline incision was performed and the skull exposed to enable visualization of the bregma. Using a hand-held drill, a burr hole was drilled 2.5 mm to the right of bregma, and a Hamilton syringe held in a micromanipulator was lowered, with the tip of the needle being inserted to a depth of 3 mm allowing injection of the tumor cell suspension or culture medium slowly over 10 min. Following injection, the needle remained in the brain parenchyma for an additional 5 min to allow tumor cells to disperse. The needle was subsequently removed, the burr hole closed using bone wax, the wound sutured closed and the animal allowed to recover. Control animals were subjected to the identical procedure but injected with culture medium only.

SP ELISA

For the SP ELISA, at 4 weeks following tumor cell inoculation or control surgery ($n = 5$ per group), mice were re-anesthetized with pentobarbitone (60 mg/kg, IP) and rapidly decapitated. The brain was removed, the olfactory bulbs and cerebellum discarded, separated into left and right hemispheres and rapidly frozen in liquid nitrogen. Samples were subsequently homogenized, and the amount of protein determined using the Biorad protein assay, with each sampled diluted with TBS to 400 ng of protein per 100 μL of TBS. Tissue samples of 400 ng of protein were loaded into each well of a Maxisorp plate in triplicate. Blank wells with no loaded protein served as controls. The protein was allowed to coat the wells overnight at 4 °C. Samples were tipped off and the blocking agent applied (0.5% gelatine solution) to each well and gently agitated for 1 h. Wells were incubated with 100 μL SP primary antibody (Chemicon, USA; 1:1000) at 37 °C in a humid container for at least 1 h. Following this, samples were incubated with secondary anti-rabbit HRP for 1 h in a 37 °C oven. Finally, 100 μL of TMB was added to each well to reveal protein expression. Using 50 μL of 0.5 M H_2SO_4 the reaction was stopped at the same time for each sample. The level of SP expression was determined by reading the absorbance at 450 nm on a Synergy Mx plate reader. The ELISA was repeated three times to ensure reproducibility of results.

Treatment

At 3 weeks following tumor cell inoculation, animals were randomly assigned to vehicle ($n = 14$) or Emend IV ($n = 14$) (3 mg/kg/day; 2.99 mM/kg) treatment groups. Animals received a daily intraperitoneal injection of their assigned treatment for 7 days prior to euthanasia. Emend IV was prepared by dissolving the drug in 0.9% sodium chloride solution, with the vehicle control animals administered saline only. The dose of Emend IV was determined from previous studies, which have demonstrated this administration exerts central effects (Watanabe et al., 2008).

Tissue processing

For histological analysis animals were transcardially perfused at 4 weeks following tumor cell inoculation ($n = 8$ per group for tumor volume, $n = 6$ per group for immunohistochemistry). Mice were terminally anesthetized with pentobarbitone (60 mg/kg, IP) and perfused with 10% neutral-buffered formalin (NBF). Brains were subsequently removed, the whole brain processed and embedded in paraffin.

Immunohistochemistry

Both human and experimental tissue was examined using immunohistochemistry. For the animal study, assessment of immunohistochemistry was performed on all sections containing tumors. Briefly, 5-μm coronal sections were cut and stained with standard H&E, SP (Santa Cruz, USA; 1:2000), NK1 receptor (ATS, USA; 1:8000), caspase-3 (Bio Vision, USA; 1:400) and Ki67 (Abcam, USA; 1:2000). Following overnight incubation with the primary antibodies, the appropriate secondary antibody was applied (1:250), followed by streptavidin–peroxidase conjugate (SPC, 1:1000) with bound antibody then detected with 3,3′-diaminobenzidine (DAB) and sections counterstained with hematoxylin.

SP and NK1 receptor immunohistochemistry was also performed in the A-375 cell line *in vitro*. Cultures were fixed by adding 1 mL of 10% NBF to each well for 10 min following removal of media. Cells were then incubated with primary antibody for SP (1:100) and NK1 receptor (1:500) overnight at 4 °C. The following day, cultures were incubated with the secondary antibody (1:250) followed by application of SPC (1:1000). Cells were stained with DAB and counterstained with half-strength hematoxylin.

For analysis, all slides were digitally scanned using the Nanozoomer (Hamamatsu, Japan) and images exported as jpeg files using the NDP viewer software. To assess proliferative and apoptotic activity following

treatment, the number of Ki67 and caspase-3-positive cells were counted at 40× in four sections from each level containing tumor using ImageJ and the mean determined as count per animal. The non-positive cells within the area were also counted, and the cells were subsequently expressed as a percentage of total tumor cells within the observed area. Levels of SP and NK1 immunohistochemistry were evaluated qualitatively.

Assessment of tumor volume

To determine the effect of treatment on tumor size, coronal sections were taken 200-μm apart and stained with H&E. To ensure inclusion of the entire tumor, 5-μm sections were taken from brain regions from bregma 0.5 to −2.7. The area of tumor in each of these sections was then calculated, with tumor volume determined as described previously (Semple et al., 2010).

Statistical analysis

All data are expressed as mean ± SEM and were assessed using a one- or two-way analysis of variance (ANOVA), followed by Bonferonni post-tests or unpaired *t*-tests, as appropriate. A p value of less than 0.05 was deemed significant in all experiments.

RESULTS

SP and NK1 receptor expression in primary and secondary human brain tumors

All examined tumor types exhibited an increase in positive staining for SP when compared to controls (Fig. 1). The elevation in SP was particularly evident in the high-grade astrocytomas, where a marked increase in positive staining was observed within the cytoplasm of neoplastic astrocytes. Secondary brain tumors exhibited

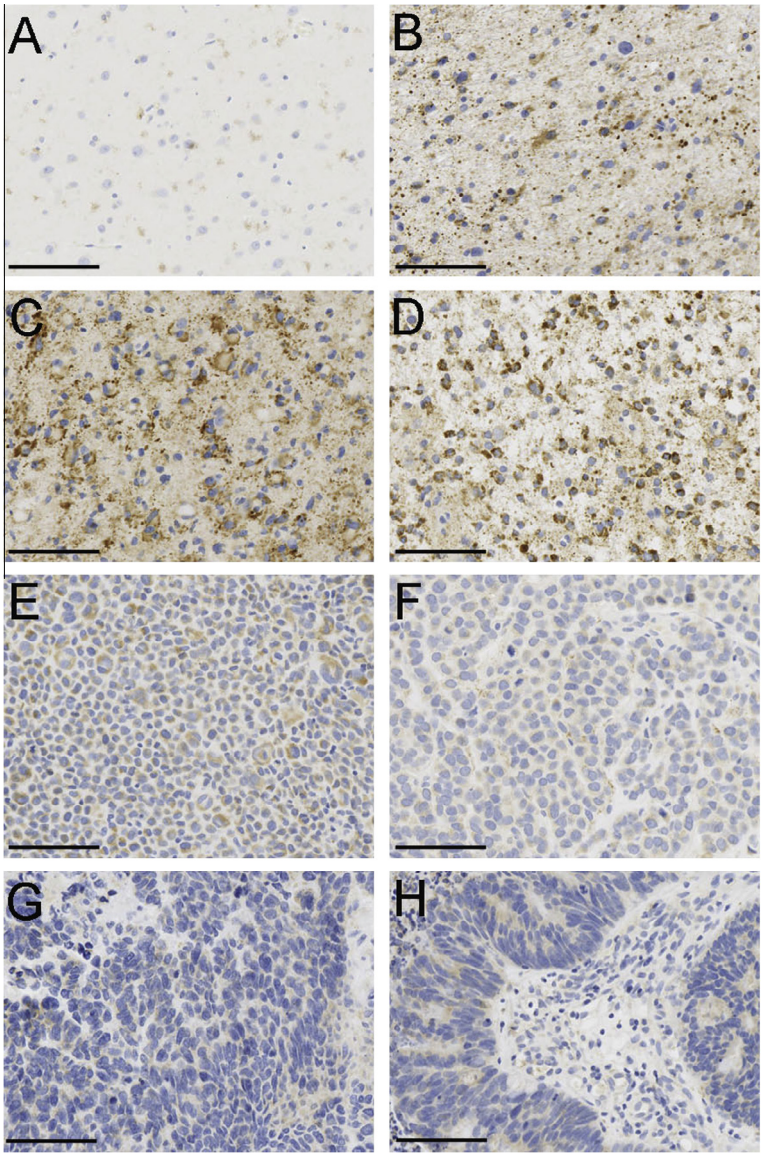

Fig. 1. SP immunoreactivity in human brain tumors. Compared to control tissue (A), a marked increase in SP immunoreactivity is evident within the cytoplasm of primary brain tumor cells (B–D). Moderate positive staining is seen in tumors arising from melanoma (E) and breast (F). However both lung (G) and colon (H) metastases demonstrated minimal SP immunoreactivity compared to the other tumor types (Scale bars = 100 μm).

only a moderate increase in SP immunoreactivity in the cytoplasm of tumor cells, with melanoma and breast cancer cells demonstrating more intense staining than the tumors of lung or colon origin.

Similarly, levels of the NK1 receptor within tumors were determined by immunohistochemistry (Fig. 2). In the gray matter of non-pathological controls, NK1 receptor staining was noted predominately around the neuronal cell bodies and their processes, but also faintly around the vessels and glia. As with SP, a clear increase in NK1 receptor staining was noted within the cytoplasm of cells of all tumor types, but was particularly evident in populations of cells within the grade 3 astrocytomas, GBM and melanoma brain metastasis.

SP and NK1 receptor expression *in vitro*

Given that of all the secondary brain tumors investigated melanoma and demonstrated particularly elevated levels of SP and NK1 receptor immunoreactivity, a human melanoma cell line was chosen for our experimental models. Morphologically, A-375 human melanoma cells were assessed using routine H&E staining, appearing as epithelial, multinucleated cells (Fig. 3A). Granular SP immunoreactivity was evident predominately within the cytoplasm of tumor cells, however some positive staining was also noted in the nuclei (Fig. 3B). Similarly, positive NK1 receptor immunoreactivity was observed within the cytoplasm tumor cells. This pattern of staining was consistent with what was observed in the human tissue.

Cell viability assay

Assessment of A-375 cell viability following treatment with Emend IV was determined using a trypan blue assay. NK1 antagonist treatment demonstrated effects *in vitro* on A-375 cell viability in a dose-dependent manner

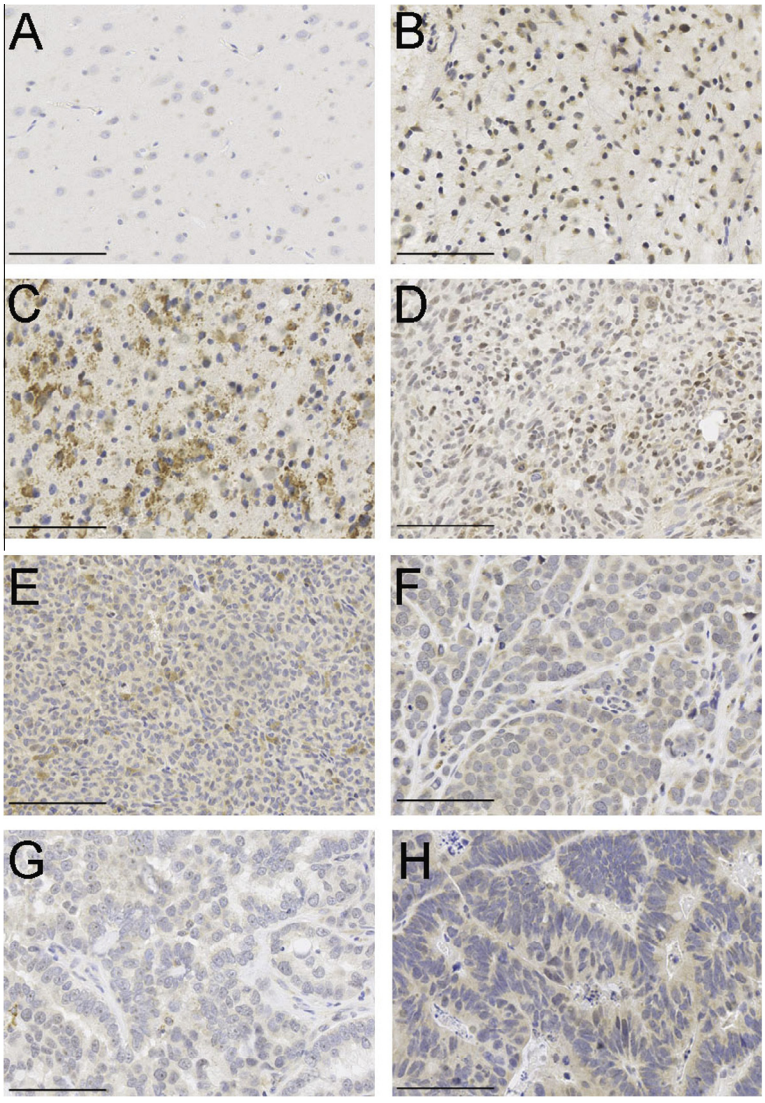

Fig. 2. NK1 immunoreactivity in human brain tumors. Compared to controls (A) an increase in NK1 receptor staining is evident in all tumor types. Low-grade astrocytomas (B), grade 3 astrocytomas (C) and GBM (D) demonstrate a more profound increase in cytoplasmic staining than secondary tumors arising from melanoma (E), breast (F), lung (G) and colon (H). Scale bars = 100 μm.

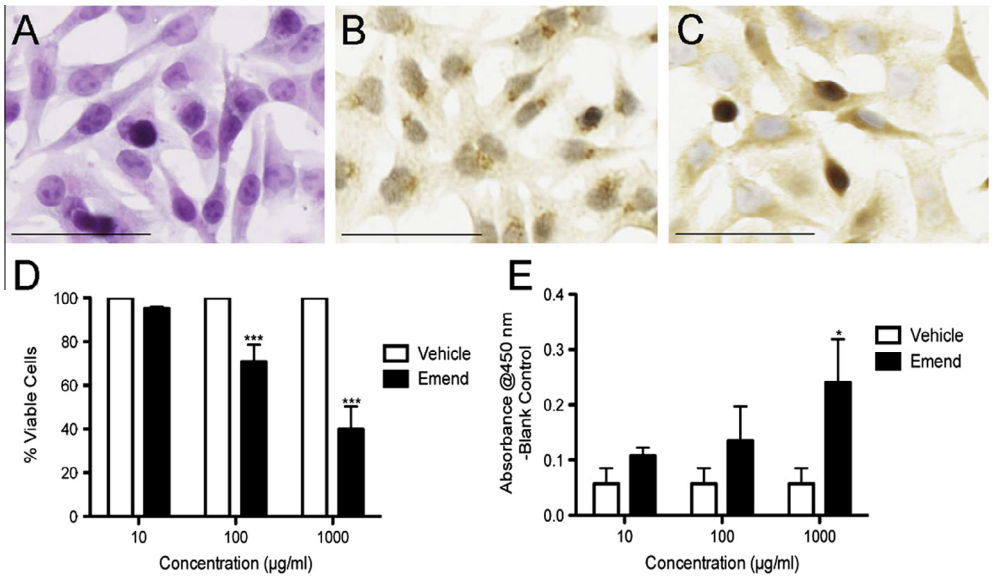

Fig. 3. *In vitro* assessment of A-375 human melanoma cells (A) H&E staining showing the morphological features of the A-375 melanoma cell line, (B) SP immunostaining revealing positive granular staining within the cytoplasm and nuclei of tumor cells, (C) NK1 receptor immunoreactivity is observed predominately within the cytoplasm of melanoma cells. (D) Cells treated with Emend IV demonstrated a significant decrease in cell viability as indicated by a trypan blue assay. (E) Effect of NK1 antagonist treatment on cell death as assessed using a LDH ELISA (n = 4/group) ($^{*}p < 0.05$, $^{***}p < 0.001$). Scale bars = 50 μm.

(Fig. 3D). At the 100 and 1000 μg/mL concentrations of Emend IV, there was a highly significant reduction in the number of viable tumor cells ($p < 0.001$).

LDH ELISA

LDH content of untreated melanoma cells was significantly elevated compared to BSA controls (0.021 ± 0.012 vs. 0.004 ± 0.002, $p < 0.01$, data not shown) demonstrating production of LDH by this cell line *in vitro*. NK1 antagonist treatment resulted in increasing LDH levels, which corresponded with increasing drug concentration. At the 1000 μg dose of Emend IV, blockage of the NK1 receptor produced a significant increase ($p < 0.05$) in LDH expression within A-375 cells when compared to controls (Fig. 3E).

SP and NK1 receptor expression *in vivo*

Levels of SP were assessed within the tumor mass using immunohistochemistry for SP (Fig. 4A). Histological examination of the tumor mass itself revealed positive

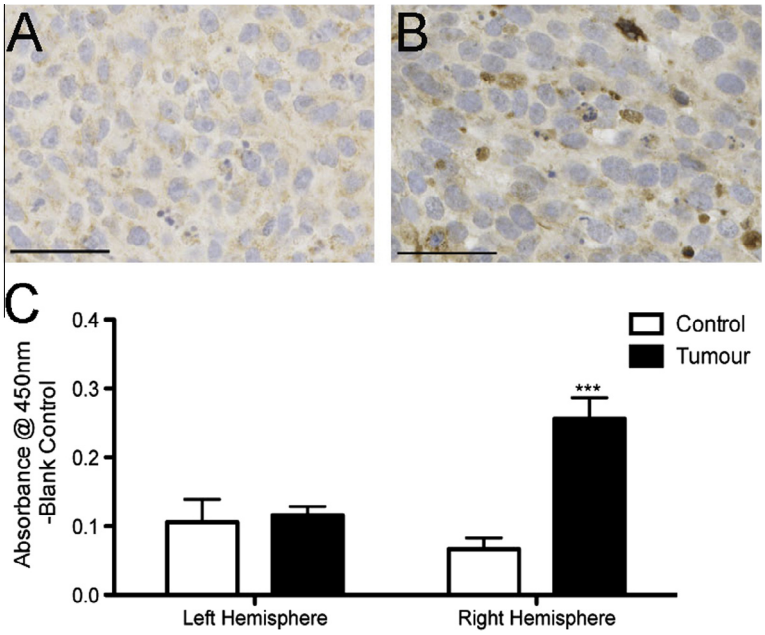

Fig. 4. Substance P expression *in vivo*. (A) Positive SP immunoreactivity is observed in the cytoplasm of tumor cells *in vivo*, and corresponds with NK1 receptor staining evident within the tumor cells (B). The increase in SP is corroborated using an ELISA (C) (n = 6/group) ($^{***}p < 0.001$). Scale bars = 50 μm.

SP staining in the cytoplasm of A-375 melanoma cells. As with SP, NK1 receptor staining was present within the cytoplasm of tumor cells (Fig. 4B). The elevation of SP was corroborated using an ELISA (Fig. 4C). At 4 weeks following tumor cell inoculation, a highly significant increase ($p < 0.001$) in SP content was evident within the right hemisphere of tumor-injected animals when compared to the same location in control mice. Furthermore, SP content in the right hemisphere of tumor-inoculated brains was significantly elevated ($p < 0.05$) when compared to the contralateral hemisphere of the same animals, indicating a potential role for SP within and surrounding the tumor area. There was no significant difference between the left hemispheres of control animals and the left hemisphere of tumor-injected animals.

Effect of NK1 antagonist treatment on tumor growth *in vivo*

To assess the role of SP in tumor growth, lesion volume following NK1 antagonist treatment was examined by H&E staining. Assessment of H&E sections revealed considerable variability in tumor size in this model, which we subsequently sub-categorized into groups of $< 1\ mm^3$, 1–3 mm^3 and $> 3\ mm^3$ (Table 1) for analysis. Vehicle-treated animals had a higher percentage of larger tumors than those animals treated with Emend IV. Moreover, a greater number of Emend IV-treated animals had no tumors as compared to vehicle-treated control animals. Given that most of the tumors in both treatment groups were in the $< 1\ mm^3$ group, this group was further analyzed for any significant differences with treatment (Fig. 5). Lesion volume determinations showed a significant reduction ($p < 0.05$) in tumor volume following Emend IV treatment when compared to vehicle-treated controls (Fig. 5).

Table 1. The effect of Emend IV treatment on tumor size

Treatment	$> 3\ mm^3$	1–3 mm^3	$< 1\ mm^3$	No tumor
Vehicle $n = 14$	3 (21.4%)	2 (14.3%)	8 (57.1%)	1 (7.2%)
Emend $n = 14$	2 (14.3%)	1 (7.2%)	8 (57.1%)	3 (21.4%)

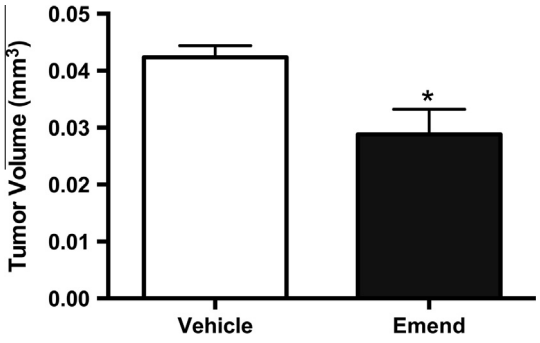

Fig. 5. The effect of NK1 antagonist treatment on tumor growth. Calculation of tumor volume revealed a significant reduction in tumor volume following Emend IV treatment compared to vehicle controls in tumors $< 1\ mm^3$ ($n = 8$/group, $^*p < 0.05$).

Effect of NK1 antagonist treatment of cellular proliferation and apoptosis *in vivo*

The effect of NK1 antagonist treatment on cellular proliferation was investigated using immunohistochemistry for Ki67 within the tumor mass (Fig. 6A–C). Untreated animals demonstrated high levels of Ki67-positive cells within the tumor mass, which was reduced with Emend IV treatment. This was confirmed by counting the percentage of Ki67-positive cells within the tumor, which indicated that blocking the NK1 receptor resulted in a significant reduction of Ki67-positive cells ($p < 0.05$) when compared to the control-treated animals. Caspase-3 activity was subsequently assessed as a marker of apoptotic cell death, with vehicle-treated animals displaying minimal numbers of caspase-3-positive cells. However, following Emend IV treatment a moderate increase in apoptotic cells was evident. This was verified using a cell count, which indicated NK1 receptor antagonist treatment, resulted in a non-significant increase in the percentage of caspase-3-positive cells within the tumor (Fig. 6D–F).

DISCUSSION

This study investigated the role of SP in promoting the development of brain tumors. An increase in the levels of SP was noted in human brain tumors, with the NK1 antagonist Emend IV, decreasing the viability of A-375 melanoma cells *in vitro* and *in vivo*, following direct injection of the cells into the brain. Our results suggest that SP and its NK1 receptor may play a major role in the progression of certain types of brain tumors.

The human study produced results consistent with the hypothesis that SP may play a role in the pathogenesis of cancer, with human tissue demonstrating increased SP and NK1 receptor immunoreactivity in both primary and secondary brain tumors. This increase in SP varied with the type of tumor. Specifically, primary brain tumors had the highest levels of SP, while metastatic tumors of melanoma and breast origin demonstrated increased SP immunoreactivity when compared to colon or lung metastasis. Similarly, NK1 receptor expression was increased in all tumor types, but most notably in grade 3 astrocytomas and metastatic melanoma. Our findings are consistent with previous reports indicating an abundant expression of SP within the CNS, and high levels of expression of the NK1 receptor on astrocytoma cell lines (Lee et al., 1989; Palma et al., 2000; Meshki et al., 2011). Likewise, high levels of the NK1 receptor have previously been reported in malignant melanoma, as well as in the cytoplasm and nuclei of a number of human tumor cell lines (Palma et al., 2000; Esteban et al., 2009; Munoz et al., 2011).

The differences observed in SP expression between tumor types suggest that SP may play a role in cancer that is dependent on the specific tumor type. Given the enhanced SP and NK1 expression within melanoma metastases, a human melanoma cell line (A-375) was

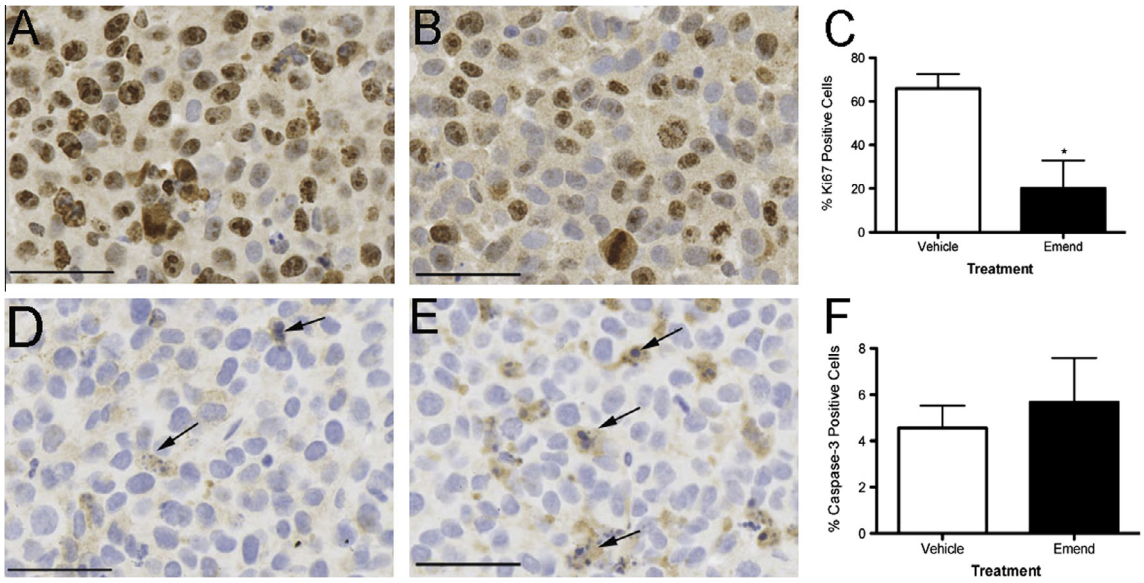

Fig. 6. The effect of NK1 antagonist treatment on cellular proliferation and apoptosis. A vehicle-treated animal (A) demonstrates increased number of Ki67-positive cells when compared to an animal from the Emend IV-treated group (B). This was confirmed by cell counts demonstrating a significant ($p < 0.05$) reduction in proliferating cells following Emend IV administration (C). Emend IV-treated animal (E) shows a small increase in the number of caspase-3-positive cells compared to a vehicle-treated animals (D), with a trend toward an increase in the number of caspase-3-positive cells with treatment (F). Images are representative of $n = 6$ per group. Scale bars = 50 μm, $^{*}p < 0.05$ compared to vehicle.

employed for the *in vivo* experimental part of this study. Indeed, *in vitro* staining confirmed that this cell line expressed SP and NK1, supporting a potential role for SP in the development and progression of melanoma-induced brain tumors.

Treatment of the human melanoma cell line, A-375, with the SP antagonist Emend IV, decreased the number of viable cells both *in vivo* and *in vitro*, and increased LDH production in the *in vitro* model. These results are consistent with previous reports that SP may play multiple stimulatory roles in cancer, such as in increased cellular proliferation and potentiation of angiogenesis (Munoz et al., 2012), with NK1 receptor antagonists previously reported to elicit anti-tumor action in several human cancer cell lines (Palma et al., 2000; Munoz et al., 2004, 2005a,b). However, while the role of SP in human cancer lines *in vitro* has been extensively studied, only few studies have examined the roles of SP and NK1 antagonist treatment on tumor growth *in vivo*. The experiments detailed in this manuscript demonstrate that following direct injection of A-375 melanoma cells; treatment with a NK1 antagonist significantly reduced tumor volume and tumor cell proliferation, suggesting that Emend IV may be inhibiting further tumor growth. Thus, these *in vivo* results in combination with the *in vitro* findings support a potential role for SP in the growth and progression of brain tumors.

It is not yet known how blockade of the NK1 receptor may induce cell death in tumor cells. It has been proposed that SP is required to promote DNA synthesis and tumor cell proliferation through the mitogen-activated protein kinase (MAPK) pathway, with NK1 receptor antagonism found to promote apoptotic cell death. In addition, SP release has been found to induce recruitment and activation of granulocytes, and thus enhance angiogenesis (Kohara et al., 2010), upon which tumor growth is dependent (Ribatti et al., 2007). Of note NK1 receptor antagonists have been found to block the SP-mediated endothelial cell mitogenesis, thus reducing angiogenesis (Ziche et al., 1990), and hence potentially enhancing cell death by depriving tumor cells of the nutrients they require to survive.

Another mechanism by which SP may aid tumor cell growth is through promotion of an inflammatory response. SP induces and augments many aspects of the inflammatory response, including leukocyte activation, endothelial cell adhesion molecule expression, cytokine production and mast cell activation (Quinlan et al., 1999). In particular, inflammatory mediators such as cytokines are thought to contribute directly to malignant progression (Naylor et al., 1993; Nakashima et al., 1998; Ueno et al., 2000; Lech-Maranda et al., 2012). Of potential interest is the role of SP in promoting the release of IL-1 and IL-6 (Veronesi et al., 1999; Yamaguchi et al., 2008), which have both been shown to exacerbate metastasis. Treatment with an IL-1 antagonist in a mouse model of hepatic melanoma metastasis significantly decreased tumor development, implicating local production of this cytokine in the development of metastases (Vidal-Vanaclocha et al., 2000). Furthermore, IL-6 deficient mice demonstrated defective recruitment of macrophages and reduced incidence of myeloma (Tricot, 2000). Thus, there are a number of potential mechanisms whereby SP may act to enhance tumor cell growth, with further investigation necessary to identify which specific pathways may be playing a role in this model. Irrespective of the mechanisms, it is evident from the present study that preventing the actions of SP

via an NK1 antagonist decreases tumor cell growth and enhances tumor cell death.

Interestingly, the results of the present study directly conflict with those performed concurrently in our laboratory (Lewis et al., 2013) which did not report any effect of Emend IV on tumor growth following direct implantation of Walker 256 rat breast carcinoma cells. However, this apparent contradiction may be a reflection of the differing tumor cell lines, given the findings observed in the current study of human tumors that SP expression can differ between different tumor types. Indeed, the SP immunoreactivity observed in the Lewis et al. study was less profound than that evident in the present study, indicating that tumor growth in the cell line used by Lewis et al. may not be SP-dependent (Lewis et al., 2013). In addition, the use of rodent and murine cancers experimentally is unable to fully replicate the complexity of human cancer lines. The histological characteristics of non-human cell lines differ significantly, with, for example, an allograft model of GBM failing to show single cell migration to the contralateral hemisphere and microvascular abnormalities typically associated with this tumor type (Mariani et al., 2006; He et al., 2009). Alternatively, it has been suggested that the role of SP in cancer may be dependant on its interaction with the immune system. Previous studies in immunocompetent animals have demonstrated that treatment with SP reduced tumor development via its immune stimulatory properties (Harris and Witten, 2003; Manske and Hanson, 2005). However, treatment in these studies was prior to tumor development, suggesting that SP may be beneficial in the early stages of DNA damage, with no studies yet to examine the effect of SP treatment in established tumors.

Nonetheless, investigation of other cell types and the role of the immune system will be required to further elucidate the role of SP in cancer progression.

CONCLUSION

This study has demonstrated that SP via the NK1 receptor may play a major role in the progression in certain types of brain tumors. A clear increase in SP and NK1 receptor immunoreactivity was evident in human brain tumor tissue, as well as in the A-375 human melanoma cell line *in vitro* and *in vivo*. Correspondingly, administration of the NK1 antagonist Emend IV resulted in reduced tumor cell viability and increased cell death in both the *in vitro* and *in vivo* models used in the present study, indicating that SP is acting as an anti-cancer agent in these models. SP is thought to mediate a number of features of cancer growth, namely cellular proliferation and angiogenesis, and accordingly NK1 antagonists may potentially act in multiple ways to reduce tumor growth. Thus, SP may provide a novel therapeutic target in the treatment of certain types of brain tumors, with further research required to determine whether the role of SP in cancer is tumor-type dependent.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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