Neuroscience 261 (2014) 85–94

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.

0306-4522/13 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2013.12.027

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