Activation of neuroimmune pathways increases therapeutic effects of radiotherapy on poorly differentiated breast carcinoma

Nuray Erin a,*, Aylin F. Korcum b, Gamze Tannöver c, Şule Kale a, Necdet Demir c, Sadi Köksoy d

a Department of Medical Pharmacology, Akdeniz University, School of Medicine, Antalya, Turkey
b Radiation Oncology, Akdeniz University, School of Medicine, Antalya, Turkey
c Histology and Embryology, Akdeniz University, School of Medicine, Antalya, Turkey
d Medical Microbiology and Immunology, Akdeniz University, School of Medicine, Antalya, Turkey

ABSTRACT

Recent studies document the importance of neuronal dysfunction in cancer development and metastasis. We reported previously that both depletion of neuropeptides in capsaicin-sensitive sensory nerve endings and vagotomy increases metastasis of triple negative breast carcinoma. Of the sensory neuropeptides, Substance P (SP) is distributed widely for regulation of immune functions. We therefore examined the affects of continuous exposure to low doses of SP on brain metastatic cells of the mouse breast carcinoma (4TBM) in the presence of radiotherapy (RT) thought to increase antigenicity of cancer cells. 4TBM cells have a cancer stem cell phenotype and induce extensive visceral metastasis after orthotopic inoculation into the mammary pad. Results demonstrated that SP treatment decreases the number of tumor-infiltrating myeloid-derived suppressor cells as well as the TNF-α response to LPS challenge. SP also increased CD4+CD25+bright cells in draining lymph nodes of tumor-bearing animals and IFN-γ secretion from leukocyte culture prepared from lymph nodes and spleens of tumor-bearing animals. SP also prevented tumor-induced degeneration of sensory nerve endings and altered release of angiogenic factors from cancer-associated fibroblasts (CAF) and tumor explants. In accordance with these observed immunological effects, combination treatment of continuous SP with a single dose of RT induced complete tumor regression and significantly reduced or prevented metastasis in 50% of the animals while suppressing primary tumor growth and metastasis in the remaining mice. These original findings demonstrate that SP through neuroimmune modulation can prevent formation of immune suppression in the tumor microenvironment, enhance cytotoxic immunity in the presence of RT and prevent metastatic growth.

1. Introduction

The triple negative breast cancer sub-type (TNBC) denotes tumors which are negative for estrogen and progesterone receptors without over expression of human epidermal growth factor receptor 2 (Bauer et al., 2007). Patients with TNBC are at high risk of early relapse and visceral metastasis, particularly to the lung and brain as compared with other breast cancer sub-types (Blows et al., 2010; Heitz et al., 2009). TNBCs constitute 10–20% of all breast cancers, affecting younger patients more frequently (Morris et al., 2007). Survival rate for 5 years is less than 30% in women with metastatic TNBC despite adjuvant chemotherapy, the mainstay of treatment. Significant heterogeneity exists within the TNBC class which may contribute to differences in effectiveness of chemotherapy (Carey et al., 2007; Lehmann et al., 2011) high immune module scores, assessed by gene expression arrays, have been reported to increase the likelihood of a pathologic-complete response to neoadjuvant chemotherapy in a subgroup of patients with TNBC, whereas similarly high levels of tumor-infiltrating lymphocytes associated with a good prognosis in patients with TNBC reported by others (Ignatiadis et al., 2012). Collectively, these results suggest that patients with the TNBC subtype having high immune score and elevated levels of tumor-infiltrating lymphocytes respond well to immunotherapy.

Radiotherapy (RT) is also used commonly in breast cancer treatment, especially in early stages (Elsamany and Abdullah, 2014). Ionizing radiation increases the effectiveness of antitumor immune responses even at distant sites from radiation exposure in patients.
with prostate or colorectal cancer (Nesslinger et al., 2007; Schau et al., 2008; Formenti and Demaria, 2009). Hence, cancer cell death not only results from direct cytotoxic effects of radiation, but also enhances immunogenic death, which appears largely dependent on the immune status of the host (Stone et al., 1979). In accordance, immunomodulatory antibodies, targeting both co-stimulatory molecules and immunosuppressive receptors such as CTLA-4 (cytotoxic T-lymphocyte antigen-4), have been shown to enhance antitumor immune responses and abscopal effects of radiotherapy treatment of melanoma and breast carcinoma (Postow et al., 2012; Verbrugge et al., 2012; Lee and Harris, 2009; Cruz-Merino et al., 2014).

Preclinical studies focusing on direct immune stimulation using multiple specific targets in conjunction with RT can provide complete regression of breast cancer (Verbrugge et al., 2012) and its metastatic lesions; immune toxic effects of these methods, however are likely to hamper the clinical response as recent studies document severe toxic effects of immune stimulation in cancer patients. Specifically ipilimumab, which increases cytotoxic T cell responses by blocking CTLA-4, was reported to induce potentially fatal autoimmune diseases in 64% of patients with metastatic melanoma (Voskens et al., 2013). Moreover, the clinical results of immune-based strategies for treating human cancers also have been disappointing (Murala et al., 2010). Hence new approaches to induce additional anti-tumoral immune responses offering greater safety combined with RT are needed.

The nervous system plays a fundamental role in regulating the immune response in a wide variety of diseases (Besedovsky and Rey, 2007; Elenkov et al., 2000). The role of neuroimmune regulation in cancer development and progression however, only recently has been appreciated (Lissoni and Rovelli, 2012). We observed previously that neuropeptides released from capsaicin-sensitive sensory nerve endings decrease the metastatic growth of mouse triple-negative breast carcinoma (4T1 cells) Erin et al. (2004, 2006). Similarly modulation of vagal nerve activity alters metastasis of murine breast carcinoma (Erin et al., 2008, 2012), demonstrating more directly that the nervous system contributes significantly to metastatic growth as in other systemic pathologies (De and Gidron, 2013; Ohira et al., 2013).

Substance P (SP), one of the peptides found widely in sensory nerve endings, and the vagus nerve (Szolcsanyi, 2004), regulate immune functions in different ways. Specifically, SP increases T-cell proliferation, immunoglobulin biosynthesis by B cells and cytokine production by monocytes (Kavelaars et al., 1994). While enhancing lymphokine-activated killer cell cytotoxicity as well as NK cell cytotoxicity and augmenting IL-12, IL-10 and TNF production by murine and human macrophages (Croitoru et al., 1990), SP acting through Neurokinin 1 receptors (NK1R), found on Dendritic cells (DC) promote potent type 1 immunity, IL-12 secretion and DC maturation that collectively enhance the efficiency of DC vaccines (Janelins et al., 2013).

These findings demonstrate that SP could be used as an adjuvant to enhance the RT-induced immunogenic death. It is, however, not known how neuroimmune modulation using SP alters the growth and metastasis of TNBC. Because neuroimmune pathways may not induce a direct immune response directed to tumor cells, we here used radiotherapy to correspondingly increase antigenicity of the tumor cells, postulating a synergistic enhanced anti-tumoral immune response of TNBC to SP in animals treated with RT. Experiments were designed to determine therapeutic effects of systemic SP treatment alone, and in conjunction with RT on the viability of 4TBM cells originally from brain metastasis of a mouse model of TNBC cells (Erin et al., 2013).

SP was reported to have tumor promoting effects on breast carcinoma expressing truncated from of the NK1R (Zhou et al., 2013). Hence expression of NK1R subtypes, SP secretion from primary tumors as well as possible mitogenic effects of SP on 4TBM cells were also examined.

Myeloid derived suppressor cells (MDSCs) are heterogeneous groups of immature myeloid cells which are recognized to inhibit innate and adaptive immunity and are broadly defined as Gr-1\(^+\)CD11b\(^+\) in mice (Youn et al., 2008). More selectively, Gr-1\(^+\)CD11b\(^+\)F4/80\(^+\) cells in tumor-bearing mice also have been defined as myeloid-derived suppressor cells (Movahedi et al., 2008). MDSCs were examined in our studies because their recognized functional impairment of both innate and adaptive immune systems, that potentially could limit the effectiveness of immune-based therapies (Movahedi et al., 2008; Banyash et al., 2014). It is not known how SP alters the level of MDSC. Furthermore, MDSCs increase in breast cancer patients, with the highest levels present in patients with metastatic disease (Diaz-Montero et al., 2009). CD4\(^+\)CD25\(^{bright}\) cells, considered to be T regulatory cells (Treg), may have anti-inflammatory and anti-tumoral effects in mouse model of TNBC (Erin et al., 2014).

We here also examined changes in the level of the inflammatory cytokines IL-6 and TNF-\(\alpha\). IL-6 is one of the main mediators of inflammation-induced stemness in breast cancer (Sansone et al., 2007; Iliopoulos et al., 2009) and increases the aggressiveness of the tumor cells (Tamm et al., 1989; Studebaker et al., 2008). TNF-\(\alpha\), a major pro-inflammatory cytokine mediating interactions between tumor and stromal cells, contributes to an up regulation of genes implicated in tumor cell growth, survival, invasion, metastasis and neoangiogenesis (Wu and Zhou, 2010). A tumor-promoting effect of TNF-\(\alpha\) has been demonstrated in triple negative breast carcinoma (Geng et al., 2013), and clinical studies have also documented an important role for TNF\(\alpha\) in advanced breast carcinoma resistant to conventional treatments (Montagut et al., 2006). The changes in IFN-\(\gamma\) and IL-10 were also examined for their role in regulating the anti-tumoral immune response (Street et al., 2002; Mosser and Zhang, 2008).

Angiogenesis is the process of blood vessel formation essential for tumor growth and development of metastases (Bakker et al., 2013). SP is also recognized as an angiogenic factor (Kohara et al., 2010) which may counteract its possible anti-tumor immune response. Hence the effects of SP treatment on the release of angiogenic factors such as VEGF, MIP-2 (Scapini et al., 2004) and SDF-1 (CXCL12) Cojoc et al., 2013; Brown, 2014 from tumor and tumor associated fibroblasts were also determined.

2. Materials and methods

2.1. Animals

Female Balb/c mice were obtained from Kobay (Ankara-Turkey) and kept under a 12 h light–dark cycle and a controlled diet. All protocols were approved and performed under the supervision of Akdeniz University Institutional Animal Care and Use Committee.

2.2. Cell lines

4TBM cells were originally obtained from brain metastasis of 4T1 originated heart metastatic cells (4THM) Erin et al., 2013. 4TBM cells were grown in DMEM-F12 supplemented with 5% FBS (fetal bovine serum), 2 mM l-glutamine, 1 mM sodium pyruvate, and 0.02 mM nonessential amino acids.

2.3. Metastasis assay

4TBM cells (10\(^5\) cells per mouse) were injected orthotopically into the right upper mammary gland of 8–10-week-old Balb-c mice. Depending on the experimental procedures, necropsies were
performed 12–45 days after injection of 4TBM cells and tissues were processed as described before (Erin et al., 2009, 2013). Eight mice per group were used for experiments in which metastatic indexes were determined. Microscopic metastatic lesions in the liver were selected and measured using Spot advanced 4.6 programme.

2.4. Radiotherapy

Briefly, for irradiation of subcutaneous tumors, mice were placed with supine position on 4 cm thick solid water phantom (RW3) for backscattering purposes. They were taped from legs and tail and a 0.5 cm thick bolus material was placed over the skin of mice to achieve maximum dose (Supplementary Fig. 1). A single dose radiation of 18 Gy was applied with an Elekta Synergy LINAC using 4 MV photons and a dose rate of 30 Gy/min (300 MU/min). 3 mice were irradiated per batch by configuring the X–Y collimators and MLC to have 2 × 2 cm² openings at 100 cm SSD (Skin Source Distance).

2.5. SP and docetaxel treatment

100 µl of 0.1 and 1 mM SP was placed into the Alzet pumps model 1004 as described by the company and pumps were placed into the back of the mice under the skin toward the left side 2 days before the injection of 4TBM breast carcinoma cells in the first set of experiments (n = 13 for each treatment group, 5 of the mice were sacrificed 12–13 days after for immunological studies, 8 of the animals were used for determination of metastatic index). Pumping rate was 0.11 µl/hr for 28 days, which started two days after insertion as the manufacturer states. Larger pumps and an intermediate concentration of SP were used in the second set of experiments. Specifically, 200 µl of 0.15 mM SP was placed into the Alzet pumps, model 2004, with a pumping rate of 0.22 µl/hr for 28 days. An NK1R antagonist L-733060, 1 mg/kg, was injected ip two days after injection of tumor cells given five days a week for four weeks. L-733060 is a potent non-peptide antagonist of NK1R (Seabrook et al., 1996).

In a separate group docetaxel (5 mg/kg/ip) was administered once a week 5 days after injection of 4TBM cells in SP-treated animals (n = 8). In this group a separate control group (untreated n = 8) was also included.

2.6. Measurement of vascular endothelial growth factor (VEGF), stromal cell-derived factor 1 (SDF-1 or CXCL12) and MIP2 levels

Primary cell cultures were begun from primary tumors isolated 25 days after injection. Briefly, tumor tissues with adjacent tissues containing fibroblasts were cut into 1–2 mm pieces and plated as described before (Erin 2013). Conditioned mediums (CM) from primary tumor explants as well as CAF were obtained when the cells reached 60 and 40% confluence, respectively.

2.7. Mix leucocyte culture

Spleen and draining lymph nodes of animals (n = 5) were removed aseptically 12–13 days after injection of 4TBM cells and single cell suspensions prepared for mixed leucocyte cultures (MLCs). Cells (4 × 10⁶/well in 48-well tissue culture plate) were cultured alone (control for basal cytokine release) or stimulated with LPS 3 µg/ml or irradiated (20 Gray) 4TBM cells (5 × 10⁶). TNF-α levels were measured 20 h after stimulation, while measurements of IFNγ, IL-6 and IL-10 were performed in supernatants obtained 40 h after stimulation. Standard ELISA kits were used for cytokine measurements of IL-6, TNF-α and IL-10 (BD Biosciences) and IFN-γ (eBioscience).

2.8. Antibodies and cell staining

All anti-mouse monoclonal antibodies used for cell-surface phenotype characterization (CD45-FITC, GR1-PeCy.7, CD3-APC, CD8-PE, CD8-FITC, F4/80-PE, CD11b-FITC, CD4-PE, CD25-PeCy.5.5) were purchased from BioLegend. Multicolor flow cytometric analyses were conducted to characterize draining lymph node cells, splenocytes, and tumor infiltrating cells. Tumors, draining lymph nodes, and spleens were digested as described previously (Gorczyński et al., 2010). Single color controls were included in each experiment for compensation purposes, as well as fluorescence-minus-one (FMO) controls. Cells were analyzed using a BD FACScalibur Flow Cytometer and FACSDiva software. Samples from 2–3 mice were pooled per staining group and staining was performed in triplicate.

Western blot and immunohistochemistry was performed as described before (Erin et al., 2013). Antibodies used for western blot were anti-mouse NK1R (PA1-32229, Thermo Fisher Scientific), anti-mouse GAPDH (Meridian Cat. No.: H86504M).

2.9. Extraction of neuronal SP

SP was extracted from freshly frozen urinary bladders obtained at the time of necropsy. Extraction of Substance P was performed, as described previously, without column extraction (Erin and Clawson, 2004; Erin and Ulusoy, 2009). Briefly, 20–25 mg tissues were cut into small pieces and kept in 1 ml of 2% acetic acid at 95 °C for 15 min which reveals predominantly neuronal SP (Erin and Ulusoy, 2009). Supernatants dried out completely in a speed-vacuum, were reconstituted in 300 µl of sample buffer of SP EIA kit. From each sample 10 and 25 µl were used for immunobssay, which mostly gave results within confidence interval 95%). If necessary, further dilutions were tested. The SP EIA kit was obtained from Cayman Chemical (Catalog No. 583751).

2.10. Statistics

Student’s t-test was used to compare metastatic indexes and secretion of MIP-2, SDF-1 and VEGF. Changes in neuronal SP were also determined using Student’s t-test. When the variance expressed as s.d. between the groups differed, the Welch t-test or non-parametric (Mann–Whitney test) was used. Analysis of variance with Dunnett’s post test was used for the analysis of cytokine results. P-values < 0.05 were considered biologically significant. Statistical analyses were performed using GraphPad InStat 3 software.

3. Results

3.1. SP does not enhance proliferation of 4TBM cells which express both full length and truncated forms of the NK1R

Expression of different isoforms of NK1R was determined in 4TBM cells using western blot with brain tissue used as a positive control. As seen in Fig. 1, 4TBM cells expressed both truncated and full-length NK1R forms, with the former, however, dominating slightly. SP levels in conditioned mediums and cell extracts were undetectable, however, low concentrations, possibly originating from supporting cells or infiltrating immune cells, were quantified in extracts of primary tumors. Furthermore treatment of 4TBM cells with SP did not alter their proliferation rate.

3.2. SP treatment in addition to RT induces tumor regression and decreases metastatic growth

The dose of RT determined after a preliminary experiment in which 12 (n = 3) and 18 (n = 3) Gray were applied locally on the
Because single exposure to 18 Gray ionizing radiation was well tolerated, 18 Gray was given through all the experiments. Initially, SP was given in two different concentrations to tumor-bearing animals. SP decreased lung metastasis at relatively low doses (0.1 mM 100 μl). SP exerted a hormetic dose–response such that ten-fold increase in SP dose did not alter the extent of lung metastasis but increased the extent of liver metastasis (Supplementary Fig. 1) without altering growth of primary tumor and extent of lung metastasis. Hence, an intermediate dose was chosen for further experiments. SP did not alter growth rate of 4TBM cells under in vitro conditions (Supplementary Fig. 1).

Twelve days after injection of 4TBM cells, microscopic visceral metastasis are seen, hence RT was given alone or in combination with SP (0.15 mM 200 μl) 6 days after 4TBM injection when visible primary tumors were found (6–10 mm²). Mice were sacrificed when co-morbidities occurred, such as decreased mobility and breathing difficulties. RT alone delayed tumor growth, decreased metastasis and increased survival; control (sham-irradiated): 31.8+/–0.7 days; RT group: 41.33+/–2.1 days. Mice were sacrificed when co-morbidities occurred, such as decreased mobility and breathing difficulties. RT alone delayed tumor growth, decreased metastasis and increased survival; control (sham-irradiated): 31.8+/–0.73 days; RT group: 41.33+/–2.11 days. Combination of RT with Substance P treatment induced both complete regression of tumors and metastasis in 50% of the animals (Fig. 1). Specifically, 8 animals received RT + SP treatment; two of the animals lost the pump within the first week, half of the remaining animals (3 of 6) were tumor free (Supplementary Fig. 2) after 6 months. Those animals were sacrificed and visceral metastasis were evaluated with no macroscopic and microscopic tumors observable. Treatment with NK1R antagonist could only partly reversed the anti-tumoral effects of SP such that two of the eight animals treated with SP, RT and NK1R antagonist L-733060 at 1 mg/kg were tumor free and remained tumor free for 6 months. Overall changes in primary tumor growth, lung and liver metastasis are presented in Fig. 2A–D of animals receiving RT at day 6 alone, or in combination with SP treatment.

Timing of the RT seems to be critical for the observed effect because SP did not further enhanced tumor suppressor effects of RT applied when primary tumor diameters were approximately 15–20 mm² (12 days after injection of 4TBM cells), significantly suppressed primary tumor growth and the number of metastatic lesions without markedly affecting survival (Supplementary Fig. 1).

### 3.3. SP decreases myeloid-derived suppressor cells in draining lymph nodes and in primary tumor tissue

Similar to its effects on metastasis, lower dose of SP treatment decreased GR1+CD11b+ cells in draining lymph nodes (Supplementary Fig. 3) in both irradiated and mock-irradiated group at day 12. At this point very few F4/80+ cells were found in all groups whereas numbers of Gr-1+CD11b+F4/80+ cells increased in both draining lymph nodes and primary tumors at the final stage of the disease. SP at low dose markedly decreased Gr-1+CD11b+F4/80+ cells in draining lymph nodes as well as in primary tumor tissue of mice receiving RT or mock-irradiated 25 days after inoculation of tumor cells. Hormetic effects of SP was also observed here such that ten times higher dose of SP did not decrease MDSCs. Timing of the RT alters the phenotype of tumor infiltrating cells. Specifically, RT applied on day 6, but not day 12, decreased the level of Gr-1+CD11b+F4/80+ tumor infiltrating cells compared to the mock-irradiated group. SP treatment in this group further decreased the level of MDSCs (Fig. 3, panels A–E).

### 3.4. SP increases CD4+CD25bright cells in draining lymph nodes

CD4+CD25bright cells considered to be T regulatory cells (Treg) which may have anti-inflammatory and anti-tumoral effects in inflammation-driven carcinogenesis. 4TBM cells induced a systemic inflammatory response characterized by excessive neutrophilic infiltration into metastatic sites (Supplementary Fig. 4) as did the parental 4T1 and 4THM cells (Erin et al., 2004, 2014). SP at low dose markedly increased the number of CD4+CD25bright cells in draining lymph nodes in both irradiated and mock-irradiated mice while higher dose of SP was ineffective (Fig. 4).
3.5. **SP increases IL-6 and IFN-γ and decreases IL-10 secretion from stimulated mix leukocyte culture (MLC)**

MLC were prepared 12–13 days after injection of tumor cells to determine changes in immune response at the time of metastasis. SP, prominently at lower doses, increased LPS and irradiated 4TBM-induced IL-6 secretion (Fig. 5A). On the other hand SP treatment decreased TNF-α and IL-10 response to LPS stimulation (Fig. 5B and C). Furthermore, 4TBM-induced IL-10 secretion significantly decreased in the MLC of animals treated with 0.1 mM of SP. LPS-induced IL-6 secretion was significantly higher in MLC of mice treated with 18 gray RT on day 6. Although RT was ineffective, SP at 0.15 mM in combination with RT increased 4TBM-induced IL-10 secretion in a NK1R-dependent manner (Fig. 5D). The IFN-γ response to 4TBM cells was enhanced in mice treated with RT which increased further in the presence of SP treatment. The NK1R antagonist completely prevented the IFN-γ response to 4TBM and LPS indicating these effects of SP is mediated by NK1R.

3.6. **Substance P alters secretion of angiogenic factors from primary tumors and cancer-associated fibroblasts (CAF)**

Differential effects of SP on secretion of angiogenic factors were observed. Specifically, exogenous exposure to SP decreased MIP-2 and VEGF secretion from primary tumor explants in vitro and MIP-2 secretion from CAF. SDF-1 secretion however increased in CAF obtained from SP treated mice (Fig. 6, panels A–D).

3.7. **Radiotherapy inhibits secretion of angiogenic factors from primary tumors and cancer-associated fibroblasts (CAF)**

VEGF, SDF-1 and MIP-2 secretion from CAF was markedly suppressed by RT demonstrating that CAF are phenotypically altered (Fig. 6, panels E–G). Similarly VEGF and SDF-1 secretion of tumor-explants of mice treated with RT was significantly lower compared to the untreated group (Fig. 6, panels H and I). Effects of RT on CAF were unchanged by SP treatment whereas SP increased SDF-1 and VEGF levels in tumor explants of RT-treated mice in a NK1R-dependent manner. Similar effects of SP on SDF-1 was observed in otherwise untreated mice (Fig. 6, panel D and I).

3.8. **Radiotherapy alone or in combination with Substance P prevents leukocytosis**

Number of neutrophils increase in peripheral bloods of mice 25–30 days after injection of 4TBM cells that was prevented by treatment with ionizing radiation at day 6 (Supplementary Fig. 5). RT applied at day 12 and SP treatment did not alter blood leukocyte count (data not shown).

3.9. **Docetaxel inhibits anti-tumoral effects of Substance P**

Experiments were also designed to determine whether SP can augment the anti-tumoral effects of other cytotoxic therapies with immune modulatory effects. Docetaxel, a widely used chemotherapeutic, inhibited proliferation of 4TBM cells, in vitro (Fig. 7A). Hence docetaxel was combined with SP using in vivo experiments. Surprisingly, docetaxel treatment (5 mg/kg ip, once in a week 5 days after injection of 4TBM cells) significantly reversed the protective effects of SP as illustrated in Fig. 7B. Docetaxel is toxic to sensory neurons hence anti-tumoral effects of SP might be due to activation/sensitization of sensory neurons. In order to examine this possibility, changes in neuronal SP content of the urinary bladder, a organ highly innervated with capsaicin-sensitive sensory neurons (Ercan et al., 2001) were examined in low dose SP-treated animals.
Fig. 3. Flow cytometric analysis of immune cells within the draining lymph nodes and the primary tumor tissue. Panels A and B: expression of F4/80 and CD11b on GR1+ cells within the draining lymph nodes of 4TBM breast carcinoma bearing mice. Lymph nodes were removed 26–30 days after injection of tumor cells ($n=8$, tissues from 2 or 3 animals were pooled together). Panel C: expression of F4/80 and CD11b on GR1+ cells within the 4TBM primary tumors. Panels D and E: expression of F4/80 and CD11b on GR1+ cells within the primary tumors of animals treated with radiotherapy (RT). Vehicle - mice received pumps containing 1% BSA. SP 0.1 and SP 1 - mice received 0.1 mM and 1 mM SP containing pumps respectively. RT12-18 gray radiotherapy applied 12 days after injection of tumor cells. RT6-18 gray radiotherapy applied 6 days after injection of tumor cells.
Urinary bladder was also chosen because 4TBM breast carcinoma cells do not metastasize to this organ. As seen in Fig. 7C, neuronal SP levels decreased markedly in tumor-bearing animals and continuous low dose SP treatment (0.1 mM) prevented this decrease in neuronal SP content of urinary bladder.

4. Discussion

Our findings demonstrate that SP, an important neuro-immune mediator, augments the anti-tumoral effects of RT, most effectively at a relatively low dose. Host defenses may mediate or have a role
in this action of SP, since administration of the peptide did not have direct cytotoxic effects. Neuro-immune mechanisms also contribute to the anti-tumoral effects in that SP alone, or in-combination with RT, decreased MDSC, increased IFN-γ and decreased TNF-α response to 4TBM. SP also attenuated the TNF-α response to an LPS challenge, while augmenting numbers of CD4+CD25bright cells in draining lymph nodes of tumor-bearing animals. Furthermore, degenerative effects of 4TBM breast carcinoma

**Fig. 6.** Changes in MIP-2, VEGF and SDF-1 secretion from primary tumor explants and cancer-associated fibroblasts (CAF) of mice treated with vehicle, Substance P (SP), Radiotherapy (RT) or 1 mg/kg NK1R antagonist (NK1RA) L-733060 (n = 8 for each group). Panel A: microscopic appearance of primary tumor explants and CAF at the time of the experiments. Panels B-D: changes in MIP-2, VEGF, SDF-1 secretion from primary tumor explants and cancer-associated fibroblasts (CAF) of mice treated with vehicle or SP. *p < 0.05 compared to corresponding vehicle-treated group. Panels E-G: the effects of RT on MIP-2, VEGF and SDF-1 secretion from CAF. *p < 0.05 compared to control group. Panels H and I: the effects of RT on VEGF and SDF-1 secretion from primary tumor explants. *p < 0.05 compared to control group, **p < 0.05 compared to SP+NK1RA treated group.

**Fig. 7.** Panel A: the effects of docetaxel alone or in combination with SP on proliferation of 4TBM cells in vitro. T0 denotes the cell number just before the treatment. *p < 0.05 compared to vehicle group. Panel B: effects of SP and docetaxel treatment on lung metastasis. *p < 0.05 compared to SP 0.1 mM group. SP-Substance P (n = 8 for each group). Panel C: changes in neuronal SP content of urinary bladder of tumor-bearing mice. Control-mice were not injected with 4TBM cells; 4TBM-4TBM-injected mice; 4TBM+SP- Mice injected with 4TBM cells and treated with Substance P 0.1 mM.
on sensory nerve endings were prevented by SP treatment. We here observed a marked decrease in the ability of tumor cells and CAF to secrete angiogenic factors following RT.

Our results, however, do not correspond with the reported tumor-promoting effects of SP and the proposed anti-tumoral effects of an NK1R antagonist (Munoz et al., 2014). It was recently shown that expression of both NK-1R and SP are significantly elevated in tumors of the HER2* subtype of breast carcinoma, compared to the TNBC, and that SP contributes to the persistent activation of the HER2 in tumor cells, increased aggressiveness and therapeutic resistance (Garcia-Reco et al., 2013). This discrepancy may relate to differential effects of SP on tumor cells versus on the host's response. Furthermore, effects of SP are expected to differ depending on the NK1R form expressed in the tumor cells. Specifically, the two naturally occurring forms of NK1R (full-length and truncated) have counteracting effects on tumorogenesis (Zhou et al., 2013). Expression levels of the truncated NK1R form is recognized to dominate in metastatic breast carcinoma; for example MDA-MB-231, a human TNBC cell line expresses only truncated NK1R whereas non-tumorigenic HBL-100 breast cells that are non-tumorigenic express predominantly the full-length NK1R. Induction of full length NK1R expression in MDA-MB-231 decreases aggressiveness and the colony forming ability of tumor cells in soft agar. Furthermore, SP-induced colony forming ability correlated with a decreased expression of full length NK1R as demonstrated by Zhou and colleagues (Zhou et al., 2013). In our studies, the breast carcinoma cells used (4TBM) expressed both full-length and truncated forms and proliferation of 4TBM cells was not enhanced by SP treatment. Thus, full-length NK1R may neutralize the mitogenic effects observed following SP-induced activation of truncated NK1R (Zhou et al., 2013). Differing effects depending on cellular compartmentalization likely contribute since NK1R expression in primary tumors is both cytoplasmic and membranous. Cytoplasmic localization presumably represents inactive receptor recycling to the membrane where binding to SP elicits the tumorogenic effects of SP. Hence, it is proposed that the observed effects of SP in our model are mediated predominately via the host's response to metastatic breast carcinoma.

SP has an establish role in augmenting inflammatory responses (Bost, 2004). We herein observed a bi-directional effect such that whereas SP increased IL-6 and IFN-γ secretion, it also decreased TNF-α levels and increased CD4+CD25bright cell number. Other studies also suggest SP effects during an immunological response are bidirectional and dependant on the local immunological milieu. As an example, SP released during repeated stress exposure suppresses inflammation by increasing levels of IL-2 and T regulatory cell numbers in local lymph nodes and associated inflamed tissue in a NK1R-dependent manner (Pavlovic et al., 2011). As reported herein, 4TBM tumors induce an extensive systemic inflammatory response as leukocytosis occurs with neutrophil infiltration in affected tissue. Accordingly, SP treatment at anti-tumor doses decreased TNF-α release from MLC challenged with LPS, while increasing CD4+CD25bright cells in draining lymph nodes, both of which may suppress excessive inflammation (Erin et al., 2014).

Tumors associated with inflammatory micro- and macro-environments are recognized to recruit MDSCs which functionally impairs both the innate and adaptive immune systems, and limits the effectiveness of immune-based therapies (Movahedi et al., 2008; Baniyash et al., 2014). It was observed for the first time that SP decreased MDSCs in the tumor microenvironment, as well as, in draining lymph nodes. IL-6 has been shown to enhance the effectiveness of DC-mediated cancer vaccines by stimulating cytotoxic T-lymphocyte responses and antitumor immunity (Bhanumathy et al., 2014). Similarly, IFN-γ decreases survival of tumor-induced MDSC (Medina-Echeverz et al., 2014). Hence SP-induced increases in IL-6 and IFN-γ may likewise contribute significantly to the anti-tumoral effects observed following radiotherapy and SP treatment. Moreover, SP promotes DC maturation and a potent type 1 immunity via NK1R activation, responses required to eradicate cancer cells (Wright, 2012) and counterbalance the predominating Th2 cytokine release (Janesins et al., 2013; Mathers et al., 2007). Substance P has also been shown to increase both lymphokine-activated killer cell cytotoxicity and NK cell cytotoxicity (Kavelas et al., 1994; Croitou et al., 1990; Flageole et al., 1992). Collectively, our finding with previously published data demonstrate that success of co-treatment of RT with SP relates to suppression of suppressor cells and enhancement of the cytotoxic immune response by SP, combined with an RT-induced increased immunogenicity of cancer cells.

Timing of the RT also was shown to affect the phenotype of tumor infiltrating cells. Specifically, RT applied on day 6 but not day 12, decreased the level of tumor infiltrating MDSCs compared to the mock-irradiated group. SP treatment in this group (RT-day 6) further decreased the level of MDSCs demonstrating an additive effect. To our knowledge there is only one report in which RT decreased MDSC in the peripheral blood of a melanoma patient (Postow et al., 2012). Lack of effects of RT on MDSC during advanced disease likely reflects the presence of counteracting factors. SP is also recognized as an angiogenic factor (Kohara et al., 2010) which would be expected to increase primary tumor growth and metastasis. In our studies, however, tumor explants and CAF obtained from SP-treated animals secreted significantly lower levels of angiogenic factors such as VEGF and MIP-2 (Scapini et al., 2004), with presumably attenuated actions mediated by MIP-2 in certain aspects of carcinogenesis and metastasis such as neutrophil infiltration and inflammation (De et al., 2013). Collectively, these effects correlate with the anti-tumorigenic effects of SP and the decreased MIP-2 and VEGF release from CAF mediated by RT alone.

Unexpectedly, SP co-treatment reversed the effects of RT to decrease VEGF secretion both from tumor explants and CAF, but in a NK1R-dependent manner. Bidirectional effects of SP on VEGF levels have been observed during wound healing (Kant et al., 2013) making it likely that the angiogenic factors affected by SP are influenced by environmental factors. These findings suggest that inhibitors of VEGF may be expected to increase the anti-tumoral effects of combined SP and RT treatments. On the other hand, there has been limited clinical success with anti-VEGF therapy and anti-angiogenic therapy at doses which induce vascular normalization is suggested. Tumor tissue has disordered vascularization and ischemia precedes a hypothermic environment, all of which have important roles in local immune suppression (Du et al., 2013). Similarly, vascular normalization was shown to improve the hypothermia, promote tumor immune destruction and enhance immunotherapy (Hamzah et al., 2008; Huang et al., 2012). Hence, anti-angiogenic treatment is a double-edge sword with the ultimate outcome likely determined by the concentration of angiogenic factors and the degree of inhibition.

Differently RT also decreased secretion of SDF-1 from CAF and tumor explants. Similar to VEGF, SP reversed the effects of RT on SDF-1 secretion from tumor explants in a NK1R dependent manner. SDF-1 (CXCL12) is involved in cancer cell proliferation, invasion, dissemination as well as angiogenesis (Cisic et al., 2013; Brown, 2014). SDF-1 is not only an angiogenic factor but also is involved in invasion and metastasis. Hence, SP-induced increases in SDF-1 may attenuate anti-tumoral responses during RT and an SDF-1 antagonist would be predicted to enhance therapeutic potential of SP. Further studies are warranted to examine this hypothesis.

Docetaxel, a commonly used chemotherapeutic, increases anti-tumor immune responses (Hodge et al., 2013) similar to RT,
suggestive that Doctaxel may likewise enhance the anti-tumoral effects of SP. But, unexpectedly, Doctaxel reversed the protective effects of SP. Sensory neurotoxicity associated with Doctaxel (Hagiwara and Sunada, 2004) may explain this result, since SP-induced anti-tumoral immune responses likely require sensory nerve ending activation via NK1 receptors (Lindberg and Dolata, 1993). In our studies, neuronal SP content increased in SP-treated 4TMH-injected animals, suggestive that sensitization of nerve endings may be involved. Herein, the urinary bladder was chosen to reflect systemic changes in sensory neurons since it is highly innervated with capsaicin-sensitive sensory nerve endings (Ercan et al., 2001; Aftuntas et al., 2014). These findings have clinical relevance such that a combination of Doctaxel with anti-tumor immune stimulation (Naidoo et al., 2014) may aggravate the inflammatory response and systemic toxicities of immunotherapy.

We herein observed that lower concentrations of SP have anti-tumoral effects whereas high doses act in an opposite manner. This yin–yang response may result from antagonistic effects of high doses of SP which induce prolonged internalization of the SP receptor (NK1R) resulting in desensitization (Roosterman et al., 2004).

4TM cells were originally derived from brain metastasis of 4T1-originated breast carcinoma cells which are a model for TNBC (Erin et al., 2006, 2009, 2013). 4TM cells also induce systemic and local inflammatory responses which may also mimic the human TNBC phenotype (Hartman et al., 2013). Our results demonstrated that SP which, at low doses is devoid of the human TNBC phenotype (Hartman et al., 2013; Schmidt et al., 2003). This yin–yan response may result from antagonistic effects of high doses of SP which induce prolonged internalization of the SP receptor (NK1R) resulting in desensitization (Roosterman et al., 2004).

Conflict of interest
None declared.

Acknowledgment
This work was supported by funds from TÜBİTAK (The scientific and technological research council of Turkey; project no.: 109S449).

Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2015.02.024.

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