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

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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

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with prostate or colorectal cancer (Nesslinger et al., 2007; Schaue 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 Dentritic cells (DC) promote potent type 1 immunity, IL-12 secretion and DC maturation that collectively enhance the efficiency of DC vaccines (Janelsins 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; Baniyash 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-α. 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-α, 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-α has been demonstrated in triple negative breast carcinoma (Geng et al., 2013), and clinical studies have also documented an important role for TNFα in advanced breast carcinoma resistant to conventional treatments (Montagut et al., 2006). The changes in IFN-γ 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

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