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Transplantation of β -endorphin neurons into the hypothalamus promotes immune function and restricts the growth and metastasis of mammary carcinoma

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Abstract

Neurobehavioral stress has been shown to promote tumor growth and progression as well as dampen the immune system. In this study, we investigated whether inhibiting stress hormone production could inhibit the development of mammary carcinoma and metastasis in a rat model of breast carcinogenesis. To enhance β -endorphin (BEP), the endogenous opioid polypeptide that boosts immune activity and decreases stress, we generated - BEP neurons by in vitro differentiation from fetal neuronal stem cells and transplanted them into the hypothalami of rats subjected to breast carcinogenesis. BEP transplanted rats displayed a reduction in mammary tumor incidence, growth, malignancy rate, and metastasis compared to cortical cells transplanted rats. BEP neuron transplants also reduced inflammation and epithelial to mesenchymal transition (EMT) in the tumor tissues. In addition, BEP neuron transplants increased peripheral natural killer (NK) cell and macrophage activities, elevated plasma levels of anti-inflammatory cytokines and reduced plasma levels of inflammatory cytokines. Anti-metastatic effects along with stimulation of NK cells and macrophages could be reversed by treatment with the opiate antagonist naloxone, the β -receptor agonist metaproterenol, or the nicotine acetylcholine receptor antagonist methyllycaconitine. Together, our findings establish a protective role for BEP against the growth and metastasis of mammary tumor cells by altering autonomic nervous system activities that enhance innate immune function.

Introduction

Breast cancer is the most frequent malignant disease among women. The National Cancer Institute estimated that there would be 40,170 deaths due to breast cancer (1), while the American Cancer Society predicted 192,370 new cases of invasive breast cancer among American women in the year 2009 (2). Stress has been shown to be a tumor-promoting factor (3-7). Emerging evidence suggests that chronic neurobehavioral stress can promote various tumor growth and progression secondary to sustained activation of sympathetic nervous system and inhibition of parasympathetic nervous system (8-10). Stress can significantly affect many aspects of the body's immune systems. For example, higher levels of stress were shown to be associated with decrease in natural killer (NK) cell lysis activity, macrophage migration activity, decrease of T cell population, decreased lymphocyte proliferation following infection, and decrease in interferon- γ (IFN- γ) levels (reviewed in ref. 9). These factors are reported to be important components of immunity against cancer (10, 11). Therefore, manipulations to control the body's stress response may be beneficial to

increase immunity and fight against cancer. β -endorphin (BEP), an endogenous opioid polypeptide primarily produced by the hypothalamus and pituitary gland, is known to have the ability to inhibit stress hormone production, produce analgesia and a feeling of wellbeing (11, 12). BEP is a cleavage product of proopiomelanocortin (POMC), which is also the precursor hormone for adrenocorticotrophic hormone (ACTH) and α -melanocyte-stimulating hormone (α -MSH). BEP neuronal cell bodies are primarily localized in the arcuate nuclei of the hypothalamus, and its terminals are distributed throughout the CNS, including the paraventricular nucleus (PVN) of the hypothalamus (8). In the PVN these neurons innervate corticotropin releasing hormone (CRH) neurons and inhibits CRH release (13), while a μ -opioid receptor antagonist increases it (14). During stress, secretion of CRH and catecholamine stimulate secretion of hypothalamic BEP and other POMC-derived peptides, which in turn inhibit the activity of the stress system (14). BEP is known to bind to δ - and μ -opioid receptors and modulate the neurotransmission in sympathetic neurons via neuronal circuitry within the PVN to alter NK cell cytolytic functions in the spleen (15, 16). Abnormalities in BEP neuronal function are correlated with a higher incidence of cancers and infections in patients with schizophrenia, depression, and fetal alcohol syndrome and in obese patients (17-23).

We have recently shown that the neural stem cell-derived BEP neurons, when transplanted into the PVN, remained at the site of transplantation, decreased lipopolysaccharide (LPS)-induced levels of hypothalamic CRH and plasma corticosterone, increased NK cell cytolytic function and anti-inflammatory cytokines productions in response to immune challenge, and suppressed carcinogen-induced prostate cancer development in rats (16, 24). It is not known whether BEP transplants prevent mammary tumor growth. Also, the effects of BEP neural activation on cancer progression and its metastasis to distant tissues are not evaluated. In this study we examined the effect of transplantation of *in vitro* differentiated BEP neurons from fetal neuronal stem cells into the hypothalamus on tumor incidence, growth, malignancy rate, and metastasis using a rat model of breast cancer. Additionally, we determined immunological and neurochemical changes pertinent to BEP action on tumor.

Materials and Methods

Animals

Adult Sprague-Dawley (SD) and Fischer 344 male and female rats were purchased from Charles River (Wilmington, MA) and maintained in a controlled environment with a 12 h light/dark cycle at Bartlett Hall Animal Research Facility of our institute. Male and female rats of each strain were bred and their fetuses or offspring were used in this study. All the animals were housed individually, allowed free access to regular rat chow, water and maintained their normal physical activities through out the study. Animal care was performed in accordance with institutional guidelines and complied with National Institutes of Health policy.

Preparation of BEP cells from neural stem cells

We isolated neural stem cells from 17 days old fetal rat brains of Sprague Dawley rats and then differentiated these cells into BEP neurons in culture to use in this study. We used cAMP and pituitary adenylate cyclase-activating polypeptide (PACAP) to differentiate BEP neurons from rat fetal neural stem cells, as we have recently described (24). To control for transplantation, we used cortical cells prepared from 17-day-old fetal rat brains. The justification for the use of cortical neurons as control is previously described (24). Prior to transplantation, differentiated BEP cells were dissociated and resuspended at a concentration of 20,000 viable cells/ μ l in HEPES-buffered DMEM-containing serum supplement (SS; 30 nM selenium, 20 nM progesterone, 1 μ M iron-free human transferrin, 5 μ M insulin, 100 μ M

putrescine and antibiotics), cAMP (10 μ M) and PACAP (10 μ M) for the transplantation. Cells were placed on ice throughout the grafting session. Cell viability, assessed by the trypan blue exclusion assay, was routinely greater than 90%. The composition of the differentiated cultures, with respect to the absence of undifferentiated neural stem cells and the presence of mature BEP-producing cells, was verified before grafting by staining for the immature neural marker nestin and/or vimentin, and for BEP using immunocytochemistry as described previously (24).

Determining the functionality of BEP cells

We verified the functionality of the transplanted cells by doing a physiological test in the transplanted animals followed by confirming the presence of BEP neurons at the site of transplantation after the termination of the experiments. We have previously shown that animals transplanted with BEP neurons have increased expression of POMC mRNA and decreased expression of CRH mRNA in the PVN and reduced response of plasma corticosterone following a LPS challenge (16). In this study, we verified the function of transplanted BEP neurons by determining the changes in corticosterone response to LPS. We used a 100 μ g/kg dose of LPS for a period of 2 h (which was found to be an effective dose; 25) to determine the changes in the plasma corticosterone responses. After termination of the experiment, the brain was collected and processed for histochemical verification of the presence of transplanted BEP cells in the PVN of the hypothalamus using immunohistochemical methods (24). PVN nuclei do not contain BEP neuronal cell bodies in situ (13) (control transplanted PVN did not have any BEP staining; data not shown), hence the immunocytochemically detected BEP cells in this area were considered transplanted cells. All the animals included in cancer study showed transplanted BEP cells in the PVN.

Tumor induction and characterization

In order to determine the effect of BEP neuronal transplants on mammary tumor growth and progression, 50 days old ovary intact virgin Sprague Dawley rats were injected i.p. with a dose of NMU (50 mg/kg body weight). Six weeks after the NMU injection, animals were anesthetized and injected with cortical neurons (Control) or BEP neurons in both sides of PVN of the hypothalamus using stereotactic procedures described by us previously (24). No tumors were detected at this time. Beginning 1 week, when the animals recovered from the brain surgery, animals were weighed and palpated every week to check for tumor growth. Tumor length, width and depth were measured with a calibrator as previously described (23). Sixteen weeks after the NMU injection, animals were sacrificed, tumors were collected, and slices of tumors were immersed in formalin and processed for histology staining. Fixed tissue was dehydrated, cleared and paraffin infiltrated overnight using a tissue processor. The tissues were paraffin-embedded, sectioned into 5 μ m thick slices and placed on slides. One slide from each tissue was stained with hematoxylin and eosin (H&E) to evaluate tissue histology and tumor pathology. Slides were evaluated by a pathologist blinded to treatment. Ductal/cystic hyperplasia was defined by increased proliferation of benign glandular structures, with predominantly regular cells and nuclei. Adenomas were defined by a more solid phase glandular structure with regular cells and nuclei predominating. Adenocarcinomas presented primarily as solid-phase lesions containing many atypical and anaplastic cells, a high mitotic rate (including numerous atypical mitoses) and observable zones of tumor necrosis (apoptotic) and some show significant invasiveness.

Immunohistochemical localization of various proteins

Thin paraffin sections (5 μ m) of mammary tumors were stained using the ABC Elite Vectastain kit (Vector Labs, Burlingame, CA) according to manufacturer's instructions using various primary antibodies. Primary antibodies for immunohistochemistry were used as follows: polyclonal rabbit antibodies against TNF- α (1:250), NF- κ B (1:100), E-cadherin

(1:250) and N-cadherin (1:250) (all from Abcam, Cambridge, MA), as well as Snail (1:200), Slug (1:200), and Twist (all from Santa Cruz Biotechnology, Santa Cruz, CA). After the primary antibody incubation and PBS wash, sections were incubated with peroxidase-coupled anti-rabbit Ig ImmPRESS reagent (Vector Laboratories, Inc., Burlingame, CA). Antigen localization was achieved by using the 3,3'-diaminobenzidine-peroxidase reaction and sections were dehydrated, and coverslipped. To evaluate the immunohistochemical staining, sections were photographed using Nikon-TE 2000 inverted microscope. Intensity of staining was categorized as negative (-) and strongly positive (+++).

Western blotting

For Western blotting, tumor tissue extracts equivalent to 50 µg total protein were separated by 4-20% SDS/PAGE and transferred overnight to immobilon-P PVDF membranes. Membranes were incubated with primary antibody for 18 h at 4°C in blocking buffer. Membranes were then washed and incubated with peroxidase conjugated secondary antibody (1: 5000) for 1 h. Afterwards membranes were washed and then incubated with ECL Western blot chemiluminescence reagent (Pierce, IL, U.S.A.). Membranes were exposed to X-ray films and developed using X-Ray developer. Actin served as an internal control for loading and transfer of equal amounts protein samples. Details of all primary antibodies used are described above.

Metastasis study

We used the MADB106 cells, a Fischer 344 rat mammary adenocarcinoma cell line maintained in DMEM containing 10% FCS. Female Fischer-344 rats were transplanted with BEP cell transplants or control cell transplants at 50 days of age. After about 2 weeks, these PVN cell transplanted rats were inoculated with MADB106 tumor cells (100,000 cells/0.2 ml/rat) into jugular vein under sodium Nembutal solution (50 mg/kg) anesthesia. Wounds were closed with a surgical clip, and rats were left on heating pad till recover. Rats were sacrificed at 4 weeks, after the tumor cells inoculation, whole body of each animal was inspected for the presence of visible tumors. Tumors were only located in lungs in most animals, and at the inoculation site in a few animals. Lungs were collected, fixed in formalin, embedded with paraffin, and sectioned for H&E staining. Stained slides were examined under the microscope for the verification of the presence of tumor. Brain tissues were processed for verification of the site and viability of BEP cell transplantation.

Determination of pharmacological modification of autonomic influence on immune system on lung metastasis

We hypothesize that the beneficial effect of BEP neuron transplants on immune function is regulated through autonomic nervous system, and that central BEP modulate peripheral immune function by inhibiting the sympathetic activity and activating the parasympathetic activity. Metaproterenol (MET) is an agonist for β -receptors, which are a class of G protein-coupled receptors that are targets of the catecholamines, especially norepinephrine and epinephrine (26, 27). Methilycaconitine (MLA) is a selective antagonist of $\alpha 7$ nicotine acetylcholine receptor ($\alpha 7$ nAChR) (28). By injecting MET and MLA i.p., we mimicked the stimulation of sympathetic nerves and inhibition of parasympathetic nerves. We also used naloxone, an opiate receptor antagonist to test whether BEP neuronal effects is acted via opiodergic receptor (15). Forty-eight BEP neuron transplanted Fischer rats and 48 control rats were divided into 8 groups and treated with saline, naloxone (10 mg/kg body weight), metaproterenol (MET; 0.8 mg/kg body weight), or methilycaconitine (MLA; 2.5 mg/kg body weight) for 8 days (the doses used for these drugs are recognized as effective biological doses). A day after the first injection of these agents, animals were inoculated with MADB106 cells (100,000/0.2 ml/rat). Twenty-four hours after tumor cell inoculation, blood was collected by orbital puncture. Plasma was collected, and PBMCs were separated

for migration assay and NK cytolytic assay. These animals continued receiving the blocker treatments for 7 days. After 4 weeks they were sacrificed, their lungs were fixed in formalin and processed to H&E staining for determination of cancer pathology. Brain tissues were processed for verification of the site and viability of BEP cell transplantation.

Immune reaction after tumor cell inoculation

We checked immune reaction to tumor cell inoculation in animals after they received BEP neuron transplants or cortical neuron transplants for 4 weeks. Animals were anesthetized with Nembutal, 1 ml of blood was drawn from jugular vein, and MADB106 cells (100,000/0.2 ml/rat) were inoculated into the jugular vein. After 24 hours, animals were anesthetized again. 1 ml of blood was drawn from the jugular vein, and then animals were sacrificed by decapitation. Vein blood before and after tumor inoculation was used for flow cytometry to determine cell populations in PBMC as described previously (26). Trunk blood peripheral blood mononuclear cells (PBMCs) and splenocytes were used for NK cell cytolytic assay (24) and macrophage migration assay (CytoSelect 96-well cell migration assay, 5 μ m, Cell BioLab). Trunk plasma was used for multi-cytokine assay. After termination of experiment, spleen were obtained and splenocytes were prepared and used for measuring mRNA levels of various cytokines and cytotoxic factors using real-time RT PCR methods as described by us previously (26).

Plasma analysis of hormones and cytokines

Plasma was analyzed for corticosterone levels by a competitive enzyme-linked immunosorbent assay (ELISA) (Immunodiagnostic Systems, Fountain Hills, AZ) according to manufacturer's recommendations. All samples were run on one 96-well plate for each variable. Plasma cytokine levels were measured by AssayGate, Inc (Ijmasville, MD) using their multiplex platform which measures multiple cytokines in a single plasma sample. Each sample was measured in triplicate. Immune related cytokines data are presented in figures.

Statistics

Differences in average tumor incidence, tumor number and tumor volume were assessed using two-way ANOVA with a Bonferroni post-test at the level of $\alpha = 0.05$. To evaluate tumor type, a Chi-square test was performed. Differences in tumor incidence and immune after various drugs were assessed using one way ANOVA with a Newman-Keuls post-hoc analysis at the level of $\alpha = 0.05$. T-tests were used to evaluate the differences in various protein and cytokine levels.

Results

BEP neuronal transplantation into the hypothalamus suppresses mammary tumor growth and progression

We used the *in vitro* produced BEP neuron in this study (Figure. 1A). Prior to testing BEP neuronal effects on mammary cancer growth, we evaluated the functionality of BEP-neuron transplantation into the hypothalamus following long-term transplants. For this, female rats at age of 50 days were implanted with BEP neuronal cells or control cells in both sides of PVN. After two to three months of cell transplants, these rats were inspected for changes in reproductive cyclicity and body growth. Like the untreated rats, both control and BEP cell transplanted rats showed regular 4-5 day estrous cycle. Body weights of untreated control, control cell transplanted rats and BEP cell transplanted rats were similar (Untreated, 243 ± 6 ; control cell transplanted, 251 ± 5 ; BEP cell transplanted, 253 ± 6 ; $N = 8-10$). After 6 months, they were used to determine their plasma corticosterone response to LPS, as a functional test of BEP neuronal activity (16), and their brains were employed for

immunohistochemical verification of transplanted neurons in the PVN. Transplanted BEP cells remained at the site of transplantation, as they were detected in the PVN by immunostaining (Fig. 1A & B). As reported by us previously (16), BEP transplanted (BEP) animals showed lower plasma corticosterone response to LPS (Fig. 1C).

We used N-methyl-N-nitrosourea (NMU) to induce mammary cancer in rats (23). Six weeks after the administration of NMU, animals were anesthetized and injected with *in vitro*-differentiated BEP neurons or *in situ* cortical neurons, which served as the controls, in both sides of the PVN of the hypothalamus (24). Weekly body weight gain were similar between rats with control transplant or BEP transplants (Fig. 1D). Weekly measurement of tumor number, length and width for a period of sixteen weeks revealed that BEP neurons implanted animals had lower tumor incidence, tumor number and tumor volume (Fig. 1E-G). At the termination of the experiment, whole body inspection revealed that tumors were localized only in mammary glands. Tumors from the study were classified by histological analysis (Fig. 1H-L). Histopathological evaluation of tumors showed that, unlike control animals, which had mostly adenocarcinoma (both invasive and non-invasive), most of the BEP-treated animals had benign adenoma with glandular hypertrophy.

Numerous studies have demonstrated that the inflammatory tumor microenvironment potentiates not only tumor development, but also the progression of adenoma to carcinoma and induction, stabilization of epithelial-mesenchymal transition (EMT) in tumor tissues (29-31). Loss of E-cadherin and aberrant N-cadherin expression associates with the acquisition of invasiveness and more advanced tumor stage for many cancers including breast cancer (32). Therefore, we determined the expression of pro-inflammatory cytokine TNF- α and pro-inflammatory NF κ B, transcription factors linked to the morphogenetic processes causing EMT (Snail, Slug and Twist) expression, and expression of mesenchymal adhesion factor (N-cadherin) and epithelial adhesion factor (E-cadherin). Immunohistochemical and Western blot determination of various cytokines in tumor tissues obtained from rats with BEP cell transplants showed reduced levels of TNF- α and NF- κ B, as well as Snail, Slug and Twist with concomitant decreased in the level of N-cadherin, and increased the level of E-cadherin in mammary gland as compared to control transplanted animals (Fig. 2A-G). In summary, the cellular and morphological data of tumors suggest the possibility that BEP neuron transplants have prevented the development of advanced stage carcinoma possibly by suppressing the inflammatory response and repressing EMT factors in tumor tissues.

BEP neuron transplantation into the hypothalamus prevents mammary tumor metastasis to lung

In order to determine the effects of BEP neuron transplants on tumor metastasis, we used non-immunogenic syngeneic MADB106 mammary cancer cells, which are widely used for lung metastasis studies in rats (33). Four weeks after inoculation of MADB106 cells through jugular vein, 70~80% of inoculated control cell-transplanted animals showed visible multiple tumor foci in lungs (Fig. 3A). Some of these animals also had a single visible tumor at the site where the tumor cells were inoculated (data not shown). None of the BEP-transplanted animals showed visible tumors either in the lung or any other body sites. Histological examinations of lung tissues identified focal and invasive tumors in control-transplanted rats while no tumors in BEP transplanted rats (Fig. 3C). These data suggest that the BEP transplantation completely eliminated retention of MADB106 tumor cells in the lungs.

Activation of hypothalamic BEP neurons increases innate immune functions

Since BEP transplantation increases immune activity in normal animals and since the innate immune system is critical for tumor cell clearance (reviewed in ref. 34, 35), we hypothesize that the elimination of tumor cells from lungs of BEP transplanted animals may have been caused by increased innate immune function. Using the MADB106 cancer model, we checked immune response (36) 24 hours following tumor cell inoculation. Several studies have demonstrated that MADB 106 tumor cells metastasize only to the lungs and subjected to NK cell cytotoxic activity when injected intravenously (33). In this study, we have not quantified the cytokine profile for longer observation, because it may be possible that there may be some changes in the cytokines profile by adaptive antitumor immune response only in control cell transplanted rats that may not reflect the innate immune responses as like the early stage of tumor development. We found that the BEP-transplanted rats had greater NK cell cytolytic activity in PBMCs and in splenocytes as well as greater macrophage migration and cell proliferation activity than those in control cell transplanted rats (Fig. 4A). Consistent with these data, we found that splenocytes of BEP-transplanted rats had higher mRNA and protein levels of NK cell cytolytic activity regulatory factors (e.g., granzyme B; 37), NK cell activator receptor (e.g., NKG2; 38), cytokines that is produced after NK activation (e.g., IFN- γ but lower TNF α ; 39), a chemokine that recruits and activates macrophages (MCP1; 40), and a protein that is produced in an increased amount from activated macrophages (IL-1b;41) (Fig. 4B). Furthermore, measurement of cytokines and protein levels in PBMCs of tumor-inoculated rats showed that the levels of macrophage-regulatory/derived factors (GM-CSF, MIP-1 α , IL-18; 42-44) and an NK cell-regulatory/derived factor (IFN- γ ; 39) are higher in BEP-transplanted rats than those in control-transplanted rats (Fig. 4C). Also, lower levels of several inflammatory cytokines (IL-1 α , IL-12, TNF- α ; 45) were observed in plasma of BEP-transplanted rats as compared to PBMCs of control-transplanted rats. In addition, BEP transplants increased NK and macrophage cell numbers in PBMCs, but decreased their numbers in splenocytes after tumor cell inoculations (Fig. 4D,E), suggesting that the transplants also promoted migration of these immune cells out of the spleen to the blood and/or inhibited emigration from the blood into the spleen to promote defense against tumor cells. These data suggest that the BEP neuronal supplement promotes innate immunity and produces an anti-inflammatory environment in recipient animals.

Antagonists of opiate receptor and $\alpha 7$ nicotine acetylcholine receptors and agonists of β -adrenergic receptors prevent BEP neuronal ability to enhance immunity and suppress tumor metastasis

BEP neurons produce opioid peptides and other products of POMC (46). To address the question whether BEP alone and/or other peptide products from the transplanted neurons are responsible for the observed actions on the immune system and cancer, we tested the ability of a general opiate antagonist naloxone to block the effects of BEP neuron transplants on immune activation and metastasis prevention. Additionally, we tested the effects of norepinephrine agonist MET and a $\alpha 7$ nicotine acetylcholine receptor ($\alpha 7$ nAChR), antagonist MLA. We found that 24 h after tumor inoculation, PBMC NK cell activity (Fig. 5A) and macrophage migration activity (Fig. 5B) were higher in saline-treated BEP-transplanted animals than in saline-treated cortical cells-transplanted controls. Naloxone, MET, and MLA all had moderate or strong inhibitory effect on basal and BEP-stimulated NK cell activity and macrophage migration activity. Consistent with these findings, we observed that naloxone, MET, and MLA prevented, at various degrees, the beneficial effect of BEP in eliminating tumor cell lung retention (Fig. 5C). These data suggest that BEP neurons activate innate immunity for cancer cell clearance via altering the function of the autonomic nervous system.

Discussion

In situ, BEP neurons originating from the arcuate nucleus terminate in the PVN and are known to regulate both stress axis and immune functions. The data presented here show that the neural stem cell–derived BEP neurons, when transplanted in the PVN, remained viable and increased endogenous opioid inhibitory tone to the HPA axis so that plasma corticosterone levels responded lower during stressful conditions like immune challenge. BEP cell transplantation does not appear to affect general body growth or reproductive hormone profiles as the body weight and reproductive cyclicity of these rats were similar to those in controls. Within the context of immune-related function, *in situ* BEP neurons in the hypothalamus are known to increase NK cell function via inhibition of sympathetic neurons to the spleen (16); our present data are consistent with this (Fig. 5D). Furthermore, we observed for the first time that BEP neurons were able to stimulate parasympathetic neurons to activate both NK cells and macrophages. Recently a role for parasympathetic neurons in immune activation has been revealed (44). Studies have shown that decreased NK cells activity is associated with growth and progression of variety of cancers in animals (47, 48) and humans (49-51), because NK-cells appear to represent a first line of defense against the metastatic spread of tumor cells (47). In breast cancer patients, low NK cell activity seems to be heavily related with larger tumor growth (50) and also a predictive parameter of advanced disease (stages II, III, and IV) than in women with limited disease (stage I) (51). At present, modulation of immune function especially enhancing NK cell activities, appears to be the most promising and new approach to cancer treatment. In this study, we showed here that supplementation of BEP neurons, through transplants, prevents mammary tumor growth, progression, and metastasis. Importantly, when the BEP transplants were given at the early stage of tumor development, many tumors were destroyed possibly due to increased innate immune activity, and the surviving tumors lost their ability to progress to high-grade cancer due to BEP cells' suppressive effects on inflammation-induced EMT regulators.

It is well known that inflammatory tumor microenvironment propel the migration and invasion of tumor cells through induction of EMT (30). Hence, regulating inflammation and EMT may be a potential novel approach to reverse the progression of tumor. Another remarkable effect of the BEP transplantation was that it promoted the activation of the innate immune activity following tumor cell invasion to such an extent that tumor cell migration to another site was completely halted. The NK cells and macrophages are critical components of the innate immune system and play a vital role in host defense against tumor cells (52). Hence, the increased level of innate immunity may have caused unfavorable conditions for cancer cell survival. In the BEP cell–treated animals the lower inflammatory milieu that was achieved by the higher level of anti-inflammatory cytokines and the lower level of inflammatory cytokines may have also been involved in inhibiting cancer growth and transformation. Several studies have addressed the involvement and roles of the inflammatory chemokines and cytokines in breast malignance (29, 30). Additionally, pharmacological modification of autonomic function significantly blocked the innate immune response and enhances the tumor cell metastasis and thus provides a plausible molecular mechanism for the protective role of BEP neurons against the cancer progression and metastatic diffusion of mammary tumor cells. Our study not only identified the importance of stress maintenance in regulating immune function in cancer patients but also provided support for a potential therapeutic use of BEP cell therapy for controlling breast cancer and possibly other cancers.

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Figure 1.

BEP neuronal transplant in the hypothalamus suppresses mammary tumor growth and progression. Animals were administered with a single I.P. injection of NMU at 50 mg/kg at 50 days of age. After six weeks of NMU, rats were administered with 20,000 cells/ μ l of BEP cells or cortical cells (control) into the PVN bilaterally. Rats were palpated for tumors once a week following injections. **A.** A representative photomicrograph showing BEP cells in primary cultures after stained for the BEP peptide by immunofluorescence technique. **B.** A representative immunofluorescence pictures of BEP cells in the PVN area of the hypothalamus after 6 months of transplants. **C.** Corticosterone response to LPS. **D.** Body weight changes. **E.** Graph represents percent (tumor incidence) of rats presenting with tumors each week post-injection. **F.** Graph represents average number of tumors per treatment group each week post injection. **G.** Average volume of tumor per animal in each group. **H.** Tumors malignancy rate as determined by histological evaluation. Percentage of each histological tumor type that developed per treatment group. Data of panel C were analyzed using one-way analysis of variance followed by Newman-Keuls post-hoc analysis. Data in panels D-G were analyzed using a two-way ANOVA and only the data shown in E-G were found to have significant treatment and time interactions at $P < 0.001$ ($n = 12/\text{group}$), and panel F was analyzed using a Chi-square test with a $P < 0.0001$. **G-J.** Representative images of different histological tumor types developed in our model.

Figure 2.

Effects of BEP neuron transplants on the expression of inflammatory markers (TNF- α and NF- κ B), epithelial-mesenchymal transition factors (Snail, Slug, Twist), mesenchymal marker (N-cadherin), and epithelial marker (E-cadherin) in tumor tissues. Cortical cells were used as control transplants. Tumors were removed from animals treated with MNU followed by BEP neuron or control neuron transplants as described in Figure 1 legends. Cellular levels of inflammatory and EMT factors were determined by immunohistochemistry (shown on the left of each panel) and Western blotting (shown on the right in each panel). **A.** TNF- α expression. **B.** NF- κ B expression. **C.** Snail expression. **D.** Slug expression. **E.** Twist expression. **F.** N-cadherin expression. **(G)** E-cadherin expression. Actin expression served as an internal control. $n = 5$ rats. ***, $P < 0.001$ vs. control.

Figure 3.

Evaluation of effects of BEP-cell transplantation on mammary cancer metastasis. Rats were transplanted with 20,000 viable cells/ μ l of BEP cells or cortical cells (control) into two PVN, and then after 4 week they were inoculated with 100,000/0.2 ml/rat of MADB106 cells via jugular vein. **A.** At sacrifice, 70~80% of inoculated animals showed tumor at the position of lung in control but the BEP inoculated animals did not (representative lung figures). Showing multiple tumor foci in lungs of control cell transplanted rats. **B.** Showing absence of tumor foci in lungs of BEP cell transplanted rats. **C.** Representative photomicrographs of H&E stained normal and metastatic lung tumor.

Figure 4.

Evaluation of effects of BEP-cell transplantation on cancer cell retention and immune functions. Rats were transplanted with 20,000 viable cells/ μ l of BEP cells or cortical cells (control) into two PVN, and then after 4 week they were inoculated with 100,000/0.2 ml/rat of MADB106 cells via jugular vein. **A.** NK cell cytolytic activity and macrophage migration activity in splenocytes or in PBMC at 24 hours after tumor inoculation. **B.** Splenocytes mRNA levels of cytotoxic factors and cytokines genes known to regulate NK cell and macrophage functions at 24 hours after tumor inoculation. **C.** Plasma levels of various cytokines at 24 hours after tumor inoculation. **D.** NK cell populations in PBMC before and after tumor inoculation, and in splenocytes 24h after tumor inoculation. **E.** Macrophage cell populations in PBMC before and after tumor inoculation, and in splenocytes 24 hours after tumor inoculation. $n = 8-12$ rats. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. control.

Figure. 5.

Autonomic mediation of the beneficial effect of BEP transplants on immune activation and tumor cell metastasis in lung. Animals were treated with the opiate blocker naloxone (NAL; 10 mg/kg body weight), β -receptors agonist metaproterenol (MET; 0.8 mg/kg body weight), nicotine acetylcholine receptor antagonist methyllycaconitine (MLA; 2.5 mg/kg body weight), or vehicle, were inoculated with MADB106 cells for determining immune and tumor clearance responses. **A.** PBMC-derived NK cells' cytolytic activity. **B.** PBMC-derived macrophage migration activity. **C.** Percentage of tumor incident as compared to control. $n = 12$ rats. **, $P < 0.01$ vs. control of the similarly drug treated group. **D.** A hypothetical model showing how autonomic nervous system may mediate BEP neuronal control of immune system.