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Sympathetic innervation, norepinephrine content, and norepinephrine turnover in orthotopic and spontaneous models of breast cancer

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ABSTRACT

Activation of the sympathetic nervous system (SNS) drives breast cancer progression in preclinical breast cancer models, but it has yet to be established if neoplastic and stromal cells residing in the tumor are directly targeted by locally released norepinephrine (NE). In murine orthotopic and spontaneous mammary tumors, tyrosine hydroxylase (TH)+ sympathetic nerves were limited to the periphery of the tumor. No TH+ staining was detected deeper within these tumors, even in regions with a high density of blood vessels. NE concentration was much lower in tumors compared to the more densely innervated spleen, reflecting the relative paucity of tumor TH+ innervation. Tumor and spleen NE concentration decreased with increased tissue mass. In mice treated with the neurotoxin 6-hydroxydopamine (6-OHDA) to selectively destroy sympathetic nerves, tumor NE concentration was reduced approximately 50%, suggesting that the majority of tumor NE is derived from local sympathetic nerves. To evaluate NE utilization, NE turnover in orthotopic 4T1 mammary tumors was compared to spleen under baseline and stress conditions. In non-stressed mice, NE turnover was equivalent between tumor and spleen. In mice exposed to a stressor, tumor NE turnover was increased compared to spleen NE turnover, and compared to non-stressed tumor NE turnover. Together, these results demonstrate that NE in mammary tumors is derived from local sympathetic nerves that synthesize and metabolize NE. However, differences between spleen and tumor NE turnover with stressor exposure suggest that sympathetic NE release is regulated differently within the tumor microenvironment compared to the spleen. Local mammary tumor sympathetic innervation, despite its limited distribution, is responsive to stressor exposure and therefore can contribute to stress-induced tumor progression.

1. Introduction

Activation of the sympathetic nervous system (SNS), release of norepinephrine (NE), and adrenergic receptor (AR) signaling regulates solid tumor growth and metastasis (reviewed in (Cole et al., 2015)). AR-expressing cells reside within tumors and within extra-tumoral organs that receive abundant sympathetic innervation, such as spleen and bone marrow (Campbell et al., 2012; Katayama et al., 2006; Mendez-Ferrer et al., 2008). In ovarian and pancreatic tumors, psychosocial stressors and environmental conditions that activate the SNS elevate tumor NE concentration (Eng et al., 2015; Lutgendorf et al., 2011; Thaker et al., 2006). In prostate cancer, the density of tumor sympathetic nerves correlates with poor clinical outcomes (Magnon et al., 2013). These reports provide evidence for local release of NE within the microenvironment of solid tumors, but in breast cancer, local sympathetic innervation and release of NE have yet to be evaluated.

Several experimental approaches have been used to characterize sympathetic innervation and NE release in peripheral tissues. Immunohistochemical detection of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis, provides insight into the abundance and anatomical location of sympathetic nerves (Felten and Olschowka, 1987; Lorton et al., 2009). The neurotoxin 6-hydroxydopamine (6-OHDA) selectively ablates sympathetic nerves and markedly depletes NE in innervated organs (Lorton

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et al., 1990; Madden et al., 1994). 6-OHDA treatment has been used in experimental cancer models to investigate the impact of the SNS on solid tumor progression (Magnon et al., 2013; Raju et al., 2007). Tissue NE concentration is an indicator of the density of sympathetic innervation (e.g., see (Bellinger et al., 2008)), but the majority of tissue NE is located within the neuron and cannot directly be distinguished from released NE (Eisenhofer et al., 2004). With SNS activation, tissue NE concentration can increase, decrease, or not change due to the dynamic balance between processes that regulate tissue NE concentration: NE synthesis, release, reuptake, and metabolism (Eisenhofer et al., 2004). Therefore, NE turnover has been used as an index of sympathetic activation under a variety of conditions (Bellinger et al., 2008; Jones and Musacchia, 1976; Migliorini et al., 1997).

NE turnover is measured by treating mice with α-methyl-*p*-tyrosine (AMPT), an inhibitor of TH. Inhibition of NE synthesis produces a decline in tissue NE. The rate of NE decline is a function of the rate of released NE and its subsequent metabolism. Quantitative measures of NE turnover include (1) turnover rate, the amount of NE synthesized and degraded per gram of tissue per hour, and (2) turnover time, the time required to synthesize the steady-state tissue pool of NE (Bellinger et al., 2008; Jones and Musacchia, 1976). Under conditions that activate the SNS, a higher rate of NE turnover indicates greater NE utilization as defined by the processes of synthesis, release, reuptake, and metabolism. Thus, NE turnover measured in mammary tumors is an indicator of NE synthesis and release from sympathetic nerve fibers within the tumor microenvironment.

Here we have characterized tumor sympathetic innervation, NE content, and NE turnover at baseline and with stress exposure in preclinical models of breast cancer. We have focused on an orthotopic mammary model commonly used to study highly metastatic breast cancer preclinically, the mammary adenocarcinoma 4T1. We have also investigated innervation and NE content in spontaneous mammary tumors from the MMTV-PyMT transgenic mouse. In this mouse line, over-expression of the polyoma middle T antigen in the mammary epithelium produces mammary tumors that progress from premalignant hyperplastic lesions to late stage metastatic disease, histopathologically mimicking human breast cancer (Lin et al., 2003). The results demonstrate limited distribution of TH+ sympathetic nerves in orthotopic and spontaneous mammary tumors, but the capacity for NE turnover under baseline and stress conditions indicates that AR-expressing tumor cells and/or host stromal cells within mammary tumors can be targeted by local NE release.

2. Materials and methods

2.1. Mice

Female BALB/cByJ mice (6–8 weeks of age), NOD.SCID (6–8 weeks of age), and MMTV-PyMT mice (5–6 weeks of age) were purchased from The Jackson Laboratories (Bar Harbor, ME) and housed 3–4 per cage with food and water *ad libitum* on a 12:12 h light:dark cycle. Mice were adapted to the vivarium for 2 weeks prior to use. Mice were euthanized by pentobarbital overdose (200 mg/kg, intraperitoneally (IP)) followed by cervical dislocation. All procedures were approved by the University of Rochester Committee on Animal Resources.

2.2. Cell lines

4T1 tumor cells (ATCC CRL-2539) and MDA-MB-231 cells (ATCC CRM-HTB-26; referred to as MB-231) were purchased from American Tissue Type Collection (Manassas, VA). MDA-MB-231BR, a brain-seeking variant of MB-231 was obtained from Dr. T. Yoneda (University of Texas Health Science Center, San Antonio, TX). 4T1 was grown in RPMI containing L-glutamine and supplemented with 10% fetal calf serum (FCS) and penicillin and streptomycin (P/S). MB-231 and MB-231BR were maintained in DMEM containing L-glutamine and supplemented with 10% FCS and P/S. All cell lines were tested monthly for mycoplasma contamination and new cells were obtained from frozen stock after 12 weeks in culture.

2.3. Tumor Implantation

BALB/c mice were injected with 2×10^5 4T1 tumor cells in sterile saline into the right inguinal mammary fat pad (orthotopic injection) under isofluorane gas anesthesia. NOD.SCID mice were injected orthotopically with 1×10^6 MB-231 or MB-231BR cells under ketamine/xylazine (90/9 mg/kg IP) anesthesia.

2.4. Chemical sympathetic ablation

6-hydroxydopamine (6-OHDA; Sigma–Aldrich, St. Louis, MO) was dissolved in sterile saline containing 0.01% ascorbate (Sigma–Aldrich) immediately prior to injection. In BALB/c mice, 6-OHDA was administered IP 4 and 2 days prior to orthotopic 4T1 tumor implantation and thereafter every 5 days to prevent reinnervation and maintain a long-term sympathectomy (Lorton et al., 1990). Vehicle controls were injected IP with 0.01% ascorbate in sterile saline. MMTV-PyMT mice were injected 2 times with vehicle or 100 mg/kg 6-OHDA (2 days apart) at 9 weeks of age and sacrificed 1 week after the second injection.

2.5. Tissue harvest

Spleen and tumors were dissected free of fat and weighed. Larger spleens and tumors were divided into 100–200 mg pieces. In the NE turnover experiments, the entire spleen or tumor was homogenized for catecholamine determination. Tissue was frozen on dry ice and stored at −80 °C until further processing.

2.6. NE determination

Tissue was homogenized 10% w/v in ice-cold 0.01 N hydrochloric acid. Homogenates were kept on ice at all times to minimize catecholamine degradation. NE concentration was determined by ELISA (Rocky Mountain Diagnostics; Colorado Springs, CO) following the manufacturer's instructions. Appropriate homogenate dilutions were pre-determined. Absorption was measured at 450 nm using a multiwell plate reader (Synergy HT, Biotek Instruments Inc, Winooski, VT). Curve fitting and sample concentration calculations were conducted with Gen5 software (Biotek).

In experiments evaluating the relationship between tissue NE concentration and tissue weights from MMTV-PyMT mice (Fig. 3G and H), NE concentration data was pooled from 3 different experiments using 3 different groups of mice ranging in age from 9 to 12 weeks old and 3 ELISAs. For the same comparison in 4T1 tumor and spleen (Fig. 3E and F), NE concentrations were determined in the same group of mice using a single ELISA.

2.7. Dual stressor exposure

See Fig. 6a for diagram. BALB/c mice were housed 3 per cage upon arrival. Two weeks later, mice in the stressed group were transferred to single housing (social isolation). Group-housed controls remained in their original housing. Six days after initiating social isolation, all mice were injected with 1×10^5 4T1 tumor cells orthotopically. Eight days later, singly-housed mice were subjected