Syrmpathetic Nerves in Breast Cancer: Function and In Vivo Imaging in an Orthotopic Animal Model

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INTRODUCTION

Evidence from breast cancer patients and animal models of breast cancer suggests that stress can augment breast tumor growth and metastasis. Activation of the sympathetic nervous system (SNS) and release of norepinephrine (NE) from sympathetic nerves is an important stress pathway. The functional interactions between sympathetic nerves and nearby target cells have not been investigated in breast cancer. One objective of this work is to better understand how breast tumor sympathetic nerves and NE influence tumor growth, angiogenesis, and metastasis. We use pharmacological means to reduce or elevate NE availability in 4T1 breast tumors. 4T1 is a murine breast cancer tumor model that readily grows and metastasizes in BALB/c mice. A second objective of this work is to explore the dynamic effect of catecholamine signaling on tumor vasculature using in vivo imaging with multiphoton laser scanning microscopy (MPLSM). To observe the relationship between sympathetic nerves and blood vessels, we have employed a transgenic mouse line in which enhanced green fluorescent protein (EGFP) expression is expressed in sympathetic tyrosine hydroxylase-positive (TH) nerve fibers (TH-EGFP mice). Here, we use MPLSM to visualize green fluorescent nerves and blood vessels labeled with a red fluorescent molecule deep within a tumor. Ultimately, we will examine the relationship between sympathetic nerves and blood vessels as the tumor grows and in established tumors treated with antiangiogenic therapy to determine if sympathetic nerves and NE can be targeted to improve the efficacy of antiangiogenic therapy.

MATERIALS & METHODS

Cell line: The mouse mammary adenocarcinoma 4T1 was purchased from American Type Tissue Culture Collection (Veroia, VA), and maintained in α-MEM containing 10% FCS and penicillin/streptomycin.

Mice: Transgenic GFP-Tg mice (Swiss Webster background, Mouse Repository Resource Center, St. Charles, IL) and TH-EGFP mice were back-crossed to the BALB/c (The Jackson Laboratory) background (expressing EGFP)

6-Hydroxydopamine treatment: BALB/c female mice were injected intraperitoneally (IP) with 100 µg 6-OHDA dissolved in 10 µl saline at a concentration of 10 mg/ml (at 4 and 5 days) to test tumor cell survival or partial sympathetic nerve reinnervation. EGFP+ mice do not cross the blood-brain barrier, leaving sympathetic and dopaminergic nerve fibers in the central nervous system intact.

Dissection (immuno) staining: BALB/c female mice were injected subcutaneously with a 3 µl 333 µg/ml palladium peroxide (resonance energy from palladium to enhance endothelial staining) 2 days prior to 4T1 tumor cell line forcing injected (day 0). Animals were euthanized the next day, and tumors were extracted and frozen in the presence of 10% neutral buffered formalin. The skin, fat, muscle, and subcutaneous tumor were placed in a single piece and embedded in paraffin. Sections were cut in 6 µm and used for immunohistochemistry analysis.

Immunohistochemistry: Tumor sections were transferred to an antibody dilution in formalin prior to paraffin embedding. This allowed for the tissues to be sectioned in a thin slice to view each 100 µm for both immunohistochemistry and in vivo imaging. Sections were stained by microwave antigen retrieval with pH 7.6 retrieval buffer (pH 7.6), followed by incubation in a blocking solution (5% normal horse) and primary antibodies antinerves (anti-TH, 1:1000) and blood (CD31, red). Secondary antibodies were used with Alexa Fluor 488 or Alexa Fluor 568 and coverslips with a 50% glycerin in PBS solution.

Multiphoton imaging: For imaging EGFP-TH+ nerve fibers, a custom built two-photon laser-scanning microscope (Olympus MX50w) fitted with a 100x objective and mounted on an upright microscope (Olympus IX81) was used. The laser was centered at 870 nm, and the laser intensity was adjusted using a half-wave plate (Newport). The field of view was 100 × 100 µm. A single z-stack through the whole tumor volume was acquired every 400 s. A final stack of images was collected every 200 s for a total imaging time of 10 minutes.

RESULTS

4T1 Tumor Sympathetic innervation (anti-TH, green) versus Blood vessels (anti-CD31, red)

In Vivo Imaging by MPLSM in Dorsal Skinfold Chamber

SUMMARY

1. Sympathetic TH+ nerves are detected in the peripheral regions of breast tumors surrounding blood vessels and in the parenchyma of the tissue.
2. 6-OHDA, a drug that selectively abrogates sympathetic TH+ nerves, markedly depleted NE in orthotopic breast tumors.
3. Chronic NE depletion with 6-OHDA reduced tumor weight trend) and significantly reduced spleen weight. Tumor MMMP-9, an important antiangiogenic protein, was also significantly reduced by 6-OHDA treatment. However, no significant changes in lung metastases were detected.
4. Chronic DMI treatment (low dose) to increase NE availability transiently increased tumor volume. However, at the time of sacrifice, neither tumor weight or tumor volume were altered by DMI treatment. (DMI-induced increase in tumor growth has been confirmed with higher doses of DMI. See poster P53-26.)

5. Using MPLSM, we detected EGFP-expressing cells in transgenic EGFP mice in orthotopic breast tumors and spleen ex vivo and in tumors growing in vivo in a dorsal skinfold chamber. The morphology of the EGFP-expressing cells corresponded to the long TH+ nerves observed using standard immunohistochemical detection.

FUTURE PLANS

• Continue to investigate the relationship between sympathetic activation and tumor growth and metastasis.

• Use MPLSM to dynamically characterize the relationship between breast tumor blood vessels and sympathetic innervation in vivo developing tumors and under a variety of treatment conditions, for example, antiangiogenic therapy.

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