Sympathetic Nervous System Innervation and Function in Breast Cancer Models

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INTRODUCTION

Cancer patients often experience chronic emotional stress and/or other negative psychological factors, such as depression or lack of social support, with diagnosis and successive treatment[1]. Exposure to stress has been shown to influence tumor growth in a number of animal models in association with greater tumor angiogenesis and elevated vascular endothelial growth factor (VEGF), an important initiator of angiogenesis and tumor progression[2]. The effects of stress were blocked by β-adrenergic receptor (β-AR) antagonists. Nasopharyngeal and ovarian carcinoma and melanoma cell lines *in vitro* are similarly susceptible to β-AR-mediated elevation of VEGF and other proangiogenic and prometastatic factors, such as interleukin-6 (IL-6) and matrix metalloproteinases (MMP)[3-6]. Together, these results suggest that stress-induced activation of the sympathetic nervous system and norepinephrine (NE) release promotes tumor growth through β-AR-induced tumor angiogenesis.

We have demonstrated considerable heterogeneity of β-AR expression and β-AR-induced alterations in VEGF production in a panel of human and murine breast cancer cell lines[6]. We previously determined that MDA-MB-231 (MB-231), a human breast cancer cell line that represents the more aggressive, 'triple negative' type of breast cancer, expresses many β-AR sites per tumor cell, by far the highest of the breast adenocarcinoma cell lines that we tested (Table 1). In comparison, the 4T1, a murine mammary adenocarcinoma cell line that readily grows and metastasizes in BALB/c mice, expresses no detectable β-AR. Hence, 4T1 is a good model for examining NE effects on β-AR-expresssing tumor stromal cells in the absence of involvement of β-AR-expressing tumor cells.

To investigate a role for sympathetic nervous system involvement in breast cancer pathogenesis *in vivo*, we have: 1) assessed sympathetic tyrosine-hydroxylase-positive (TH+) innervation of MB-231 and 4T1 tumors grown orthotopically (in the mammary fat pad), and 2) investigated the impact of sympathetic activation on *in vivo* tumor growth of of MB-231 and 4T1 tumors. Two approaches were used to activate the sympathetic nervous system: 1) social isolation, a chronic stressor that has been shown to facilitate ovarian tumor growth[2], and 2) chronic desipramine treatment (a tricyclic antidepressant used clinically) that inhibits NE reuptake by nerve fibers, thereby increasing NE availability to target cells.

Cell Line	Species	Cells Isolated from:	β-AR sites per cell (SEM)	Hormone Receptor Expression
MB-231	Human	Pleural Effusion	13,798 (4858)	ER-, HER2-
4T1	Mouse	Spontaneous Mammary Tumor (BALB/c)	None detectable	-

Table 1. Breast adenocarcinoma cell line characterization. Descriptions of the MB-231 and 4T1 cell lines including β-AR expression as determined by radioligand binding.

METHODS

Cell Lines. The breast adenocarcinoma cell lines MB-231 and 4T1 were acquired from American Type Culture Collection. MB-231 and 4T1 were maintained in high glucose DMEM and RPMI, respectively, containing penicillin/streptomycin and 10% fetal calf serum.

Immunocytochemistry (ICC). Noradrenergic nerves and blood vessels were visualized in parafomaldehyde-fixed 20 μM sections from orthotopic MB-231 and 4T1 tumors. Sections were stained with primary antibodies for TH+ (Millipore) noradrenergic nerve fibers and CD31+ (Abcam) endothelial cells, followed by fluorescent secondary antibodies (Invitrogen) using standard techniques.

Social Isolation and Tumor Growth. Female SCID mice (The Jackson Laboratories) were housed 5 per cage upon arrival. After a two-week adaption period, the mice were divided into two groups: group-housed (mice remain housed 5/cage) and singly-housed. Seven days after re-housing, all mice were implanted with $3.5x10^6$ MB-231 tumor cells into one mammary fat pad (MFP). Tumors were measured with calipers every 4-7 days, and volume was determined by length*width^2/2. At sacrifice, tumor and spleen were harvested for further analyses.

Desipramine (DMI) Treatment and Tumor Growth. Female BALB/c mice (The Jackson Laboratories) were housed 3-4 per cage upon arrival. After a two-week adaption period, the mice were implanted subcutaneously with either placebo, 5 mg DMI, or 10 mg DMI 21-day continuous release pellets (Innovative Research of America). Two days later, all mice were injected with $1x10^5$ 4T1 tumor cells into one MFP. Tumors were measured every 2-3 days, and mice were sacrificed at varying days. Tumors, spleens, and lungs were harvested for further analyses.

Norepinephrine, VEGF, IL-6 and MMP-9 Analyses. For NE, tumors and spleens were homogenized in 0.01M HCl. NE was determined using the NE ELISA from Rocky Mountain Diagnostics per manufacturer's instructions. For proteins, tumor homogenates were prepared in RIPA buffer with protease inhibitor (Pierce). VEGF, IL-6, and MMP-9 in tumor homogenates were determined using ELISA kits from R&D Systems per manufacturer's instructions. Total protein in homogenates was determined with the BCA protein assay (Pierce). Results are presented per μg protein.

Statistics. Tumor growth over time was analyzed using a two-way repeated measures ANOVA. Significant main effects or interactions were analyzed using Bonferroni's post-hoc analysis. For comparing two groups, Student's t-test was used. For all analyses, $p<0.05$ is considered a statistically significant.

RESULTS

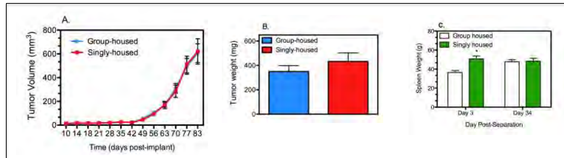

Figure 2. Social isolation prior to tumor cell injection did not alter MB-231 tumor growth. Seven days after single housing, all SCID mice were injected with $3.5x10^6$ MB-231 tumor cells in the MFP. A) Tumors were measured over time, and mice were sacrificed after 83 days. Social isolation did not significantly alter tumor volume. B) Final tumor weight did not differ between groups. Results are shown as mean ± SEM (n=8-9). These results are representative of 3 experiments using similar experimental design, using different cell concentrations for injection. No consistent changes in tumor NE, VEGF, or IL-6 were detected in these experiments (data not shown). C) Spleen weight increased 3 days after transfer to single housing relative to group-housed mice (p=0.02). By 34 days post-separation, this effect was no longer present. Results are shown as mean ± SEM (n=5).

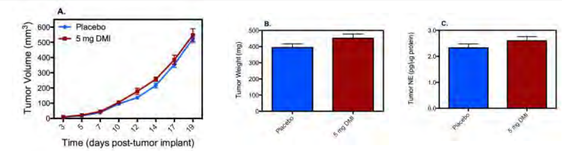

Figure 3. Low dose DMI treatment did not alter 4T1 tumor growth. A) Two days after placebo or 5 mg DMI implantation, mice were injected with $1x10^5$ 4T1 tumor cells in the MFP. Tumor volume was measured over time, and mice were sacrificed 19 days after tumor cell injection. 5 mg DMI treatment did not significantly alter tumor volume. B) Final tumor weight and tumor NE (C) did not differ between groups. Results are shown as mean ± SEM (n=9-10).

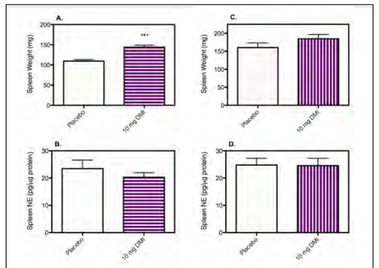

Figure 7. Kinetics of DMI treatment: Early, transient increase in spleen weight is not associated with altered splenic NE concentration. Two days after placebo or 10 mg DMI implantation, mice were injected with $1x10^5$ 4T1 tumor cells in the MFP. Mice were sacrificed after 7 (A-B) or 12 (C-D) days after tumor cell injections. A) On day 7, there was a significant increase in spleen weight ($p<0.0001$) but not spleen NE concentration (B) in the DMI-treated group. C) On day 12, there was no difference in spleen weight or NE concentration (D). Results are shown as mean ± SEM (n=6-9).

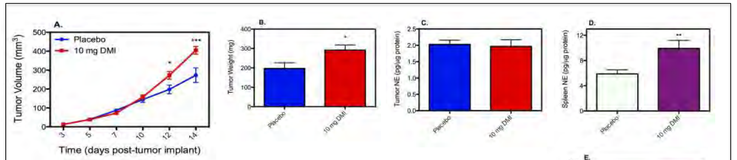

Figure 4. High dose DMI treatment augmented 4T1 tumor growth in association with elevated splenic NE concentration. A) Two days after placebo or 10 mg DMI implantation, mice were injected with $1x10^5$ 4T1 tumor cells in the MFP, and tumors were measured over time. DMI treatment significantly increased tumor volume on days 12 ($p<0.05$) and 14 ($p<0.001$) after tumor cell injection. B) Final tumor weight was significantly greater in the DMI-treated group ($p=.031$), but tumor NE was not significantly altered (C). D) A significant increase in spleen NE concentration ($p=0.0099$) in the DMI-treated group was detected in the absence of altered spleen weight (E). Results are shown as mean ± SEM (n=9-10).

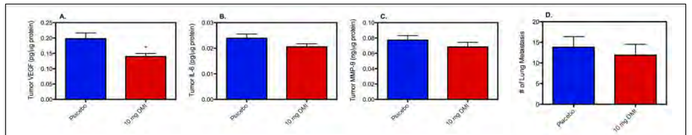

Figure 5. DMI-induced tumor growth is not associated with increased tumor proangiogenic or prometastatic proteins. Two days after placebo or 10 mg DMI implantation, mice were injected with $1x10^5$ 4T1 tumor cells in the MFP, and mice were sacrificed 14 days after tumor cell injection. A) There was a decrease in tumor VEGF in the DMI-treated group ($p=0.019$). There was no significant difference in tumor IL-6 (B), total MMP-9 (C), or lung metastasis (D). Results are shown as mean ± SEM (n=9-10).

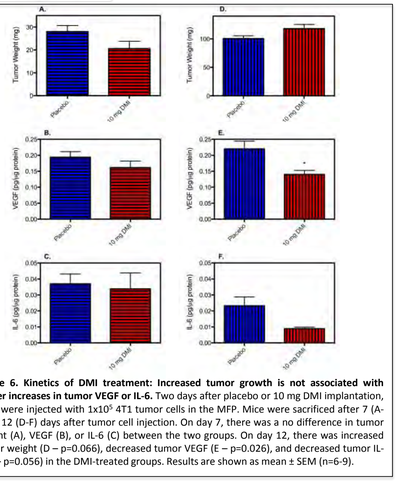

Figure 6. Kinetics of DMI treatment: Increased tumor growth is not associated with earlier increases in tumor VEGF or IL-6. Two days after placebo or 10 mg DMI implantation, mice were injected with $1x10^5$ 4T1 tumor cells in the MFP. Mice were sacrificed after 7 (A-C) or 12 (D-F) days after tumor cell injection. On day 7, there was a no difference in tumor weight (A), VEGF (B), or IL-6 (C) between the two groups. On day 12, there was increased tumor weight (D – $p=0.066$), decreased tumor VEGF (E – $p=0.026$), and decreased tumor IL-6 (F – $p=0.056$) in the DMI-treated groups. Results are shown as mean ± SEM (n=6-9).

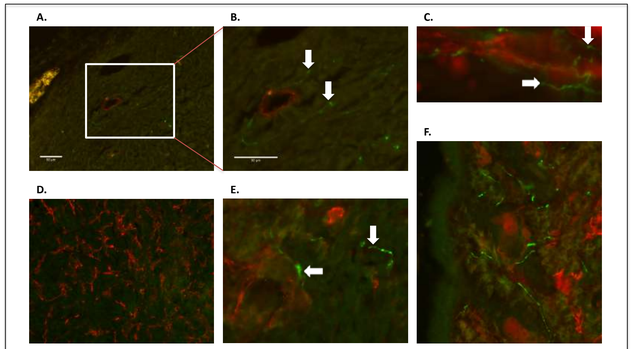

Figure 1. Sympathetic innervation and blood vessels in MB-231 and 4T1 tumors. TH+ noradrenergic nerve fibers (green) and CD31+ endothelial cells (red) in MB-231 (A-C) or 4T1 (D-F) tumors grown in the MFP. TH+ nerves (white arrows) are more prevalent in the periphery of the tumor, where TH+ nerves are often associated with blood vessels (C, F). Few TH+ nerve fibers are detected in the well-vascularized areas in the center of the tumor (D). Images magnified 20X (A, D, F) and 40X (B, C, E).

SUMMARY

1. The cellular targets of TH+ sympathetic nerve fibers in orthotopic MB-231 and 4T1 breast tumors are located in the periphery of the tumor and include blood vessels and cells in the tumor parenchyma.
2. Using the MB-231 breast cancer model, tumor growth in mice exposed to social isolation prior to tumor cell injection did not differ compared to group-housed mice. By measuring spleen weight, we observed that social isolation elicits early, transient effects, but subsequent adaptation to the stressor minimized the impact of social isolation on tumor growth.
3. In the 4T1 breast cancer model, treatment with DMI, a NE reuptake inhibitor that increases NE availability, significantly augmented 4T1 tumor growth. The DMI-induced increase in tumor growth was not associated with increased tumor VEGF and IL-6, suggesting that the DMI-induced increase in tumor growth may not be elicited though increased angiogenesis.
5. A doubling in splenic NE concentration occurred at the same time point in which tumor growth was significantly increased by DMI treatment. However, tumor NE was not affected by DMI treatment at this time point.
6. Splenic NE concentration was not altered early post-DMI implantation, at a time when spleen mass was increased in the DMI-treated mice. It is possible that the lack of an increase in tumor NE may have been due to the increase in tumor mass in the absence of a corresponding increase in the density or distribution of TH+ nerve fibers.
7. In DMI treated mice, no alterations in lung metastasis were detected.

CURRENT AND FUTURE GOALS

- Determine the temporal relationship between sympathetic nervous system activation and tumor growth in the context of both breast tumor models.
- Determine the mechanism underlying DMI-induced increase in tumor growth in the 4T1 model. Compare the effect of DMI in immunodeficient SCID mice to immunocompetent BALB/c mice.
- Design experimental models to manipulate sympathetic nervous system activation or responsiveness to activation solely within the tumor versus other peripheral organs.

CONCLUSIONS

We had predicted that *in vivo* growth of MB-231, a high β-AR-expressing breast cancer cell line, would be sensitive to sympathetic nervous system activation via social isolation, a stressor known to activate the sympathetic nervous system. We have been unable to establish a consistent effect of social isolation on MB-231 growth. The results presented here suggest that 1) the timing of stressor exposure relative breast tumor growth may be an important determinant of the impact of stressor exposure on breast tumor growth, and 2) that adaptation to stressor exposure may minimize the impact of a stressor such as social isolation, particularly when a tumor is relatively slow-growing, such as MB-231 in SCID mice.

The murine breast cancer model 4T1 is useful for characterizing sympathetic nervous system-induced alterations in breast tumor growth in the absence of tumor β-AR signaling. Chronic DMI treatment elevated NE and increased 4T1 tumor growth, but we found no evidence that increased tumor growth was mediated through increased tumor angiogenesis, as has been observed with high β-AR-expressing cancer cells. Instead, we propose that DMI-induced elevation in NE elicits an influx of leukocytes from peripheral immune organs (bone marrow being a prime candidate) to increase tumor and spleen mass. The functional implication of sympathetically-mediated influx of cells derived from other peripheral tissues into breast tumors has yet to be determined.

Our results imply that the impact of sympathetic nervous system activation and β-AR stimulation on breast cancer pathogenesis varies with tumor cell β-AR expression and signaling capability. Furthermore, the impact of sympathetic activation and subsequent response to stressor exposure is modulated by the ability of the host to adapt to the activation and elevated NE. Understanding the implications of heterogeneity of tumor cell β-AR expression in the context of stressor exposure is critical to predict efficacy of therapeutics that block or activate the sympathetic nervous system, including drugs commonly used clinically for other conditions, including cardiovascular disease and anti-depression therapies.

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