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Behavioral Stress and Tumor Progression

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Abstract. *Background: A number of laboratories have reported a possible link between behavioral stress and cancer progression. Previously published findings demonstrated a stress-induced increase in tumor growth of implanted lymphosarcoma in C3H mice. Here, two mouse models were utilized to investigate whether stress alters the growth of solid tumors. Materials and Methods: We developed a stress paradigm that involves alternating established stressors for 12 days. FVB mice implanted with melanoma were subjected to this stress protocol. We also attempted to duplicate Riley's finding. Results: Our stress paradigm markedly increased serum corticosterone levels and thymus involution. No alteration in the growth of the melanoma tumors was observed. There was also no significant effect on lymphosarcoma progression using either our own or Riley's stress protocol. Conclusion: Under the conditions used in this study, strong behavioral stress did not influence tumor progression.*

Psychosocial stress is increasingly recognized as an important public health issue. It is implicated in diseases such as irritable bowel syndrome (1, 2), coronary artery disease (3) and cancer (4). In humans, linking stress with cancer progression and outcome has proven to be difficult, with many contradictory reports in the literature. For instance, studies investigating the effects of group therapy on metastasis and survival in breast cancer patients reported conflicting results, with some studies demonstrating a protective effect and others seeing no effect (5, 6). An investigation of patient optimism prior to treatment and survival in non-small cell lung carcinoma found no correlation between the two (7). In addition, another study found no correlation between the stress of caregiving and breast cancer incidence (8). While the connection between stress and cancer has also been investigated in animals, a causal relationship has not been proven. Riley (9) reported that the increase in glucocorticoids caused by stress in mice enhanced tumor growth for tumors that are thought to be at least partially controlled by the immune response. Additional studies have indicated that behavioral stress increases tumor progression in rodents (10-14) and can also decrease the efficacy of a cytotoxic antitumor drug (15). Other studies, however, showed variable results, indicating a complex interaction between stress and cancer, including differences resulting from differential housing (16, 17).

Possible mechanisms linking stress and tumor growth have also been investigated. Behavioral stress increases corticosteroid levels, leading to lymphocytopenia, thymus involution and a decrease in spleen and peripheral lymph node mass (9). These effects may be mediated through the autonomic innervation of primary and secondary lymphoid organs, as well as through modulation of adrenergic and glucocorticoid receptors on immune cells. Nonetheless, studies on the effect of stress on the immune system have led to contradictory results. While low NK activity has been linked to decreased disease resistance, stress can decrease NK cell cytotoxicity (12, 13, 18-22) or have no effect (23-25). Another study in humans found that mirthful laughter appeared to reduce stress and increase natural killer (NK) cell activity (26).

Using two separate stress-inducing paradigms, we set out to investigate the mechanisms whereby stress regulates tumor growth, but were unable to demonstrate any effect on tumor growth or replicate an important prior finding of such regulation.

Materials and Methods

Animals. Six-week-old male FVB mice were obtained from Taconic (Germantown, NY, USA) and used in the melanoma experiments. Six-week-old female C3H/He mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and used in the lymphosarcoma experiments. The mice were housed individually to prevent differential stress levels associated with social hierarchy. All the mice were allowed to recover from transport and adjust to the Caltech animal facility for 2 weeks prior to use. Mice were stressed using a combination of 3 established stressors: rotation at 45 rpm for 45 minutes, forced cold-water swimming at 10˚C for 3 minutes, and restraint for 1 hour. Rotation occurred in a

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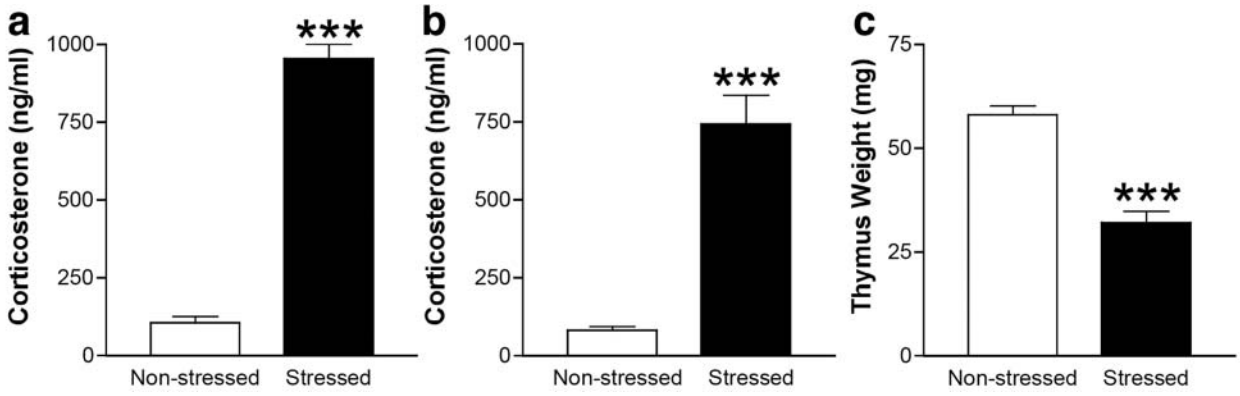

Figure 1. *Stress increases serum corticosterone and decreases thymus weight. a. FVB mice, without tumors, were subjected to the variable stress protocol for 12 days and blood was taken immediately following the last stressor. Mean corticosterone levels were 106.6±19.6 ng/ml (n=25) and 953.7±46.3 ng/ml (n=10) for non-stressed and stressed mice, respectively (p<0.0001). b. FVB mice were injected with B16F10 melanoma cells and subjected to the variable stress protocol for 12 days and blood was taken immediately following the last stressor. Mean corticosterone levels were 82.6±11.8 ng/ml (n=10) and 744.6±90.8 (n=10) for non-stressed and stressed mice, respectively (p<0.0001). c. FVB mice were injected with B16F10 melanoma cells and subjected to the variable stress protocol for 12 days and the thymus weight was measured following the last stressor. The mean thymus weights were 58±2 mg (n=9) and 32±3 mg (n=9) for non-stressed and stressed mice, respectively (p<0.0001).*

translucent plastic box, 3 3/8"x 4 1/4"x 2 1/2" with a ventilated lid, that was secured on a level GlasCol rotator (Fisher Scientific, Pittsburgh, PA, USA). Restraint occurred in a ventilated 50-ml conical centrifuge tube. The animals experienced each stressor once per day for a total of 3 stresses a day, and rested for 3-4 hours between each stress. The order of stressors was rotated each day to minimize habituation. In the melanoma experiments, mice were stressed for 12 days following tumor cell implantation, and sacrificed after the first stress on the 12th day. In the lymphosarcoma experiments, mice were stressed from days 4-6 after tumor implantation and sacrificed on the 23rd day. Tumor size was measured at the end of the experiment using calipers in 3-dimensions to calculate volume.

Cell culture. Mouse melanoma B16F10 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured in RPMI 1640 (ATCC) supplemented with 10% fetal bovine serum (Gemini Biological Products, Calabasas, CA, USA) in a humidified incubator with 5% CO_2 at 37˚C. Mouse lymphosarcoma cells, 6C3HED, were obtained from the NCI-Frederick Cancer DCT Tumor Repository, USA, and cultured as above. In each case, $5x10^5$ cells were injected subcutaneously into the right flank.

Corticosterone assay. Blood was collected immediately following the last stress by cardiac puncture in a serum separation tube (Becton, Dickson & Company, Franklin Lakes, NJ, USA), allowed to clot at room temperature and centrifuged at 1200 xg for 10 minutes. Serum samples were stored at –80˚C until analysis. The corticosterone concentration (ng/ml) was determined using a radioimmunoassay kit following the manufacturer's instructions (MP Biomedicals, Costa Mesa, CA, USA).

Statistics. Data is represented in all cases as mean ± standard error (SEM). Statistical significance was determined in all cases by an unpaired *t*-test.

Results

Behavioral stress increases serum corticosterone and causes thymus involution. To maximize the stress response and limit habituation, a rotating schedule of 3 modes of behavioral stress to activate the hypothalamic-pituitary-adrenal axis were used. These stressors included rotation, forced cold-water swimming and restraint. The efficacy of this paradigm was illustrated by the dramatic increase in serum corticosterone levels assessed immediately following the last stressor (Figure 1a). In fact, many of the stressed mice displayed levels that surpassed the maximal sensitivity of the assay (1000 ng/ml). When this stress paradigm was applied to mice injected subcutaneously with melanoma cells, a similar corticosterone response was seen (Figure 1b). The stress response was also illustrated by a striking decrease in thymus weight (Figure 1c).

Behavioral stress does not alter melanoma progression in vivo. The mice were injected with melanoma cells on day 0, stressed on days 1-12 and sacrificed immediately following the first stress on day 12. Despite a few animals with increased tumor size, the mean tumor volume of the stressed group did not significantly differ from the non-stressed group (Figure 2). There were also no visible metastases in any of the mice.

Behavioral stress does not alter lymphosarcoma progression in vivo. We also attempted to duplicate the reported stress-induced increase in 6C3HED lymphosarcoma tumor growth in C3H mice cells (9). As previously published, tumor cells

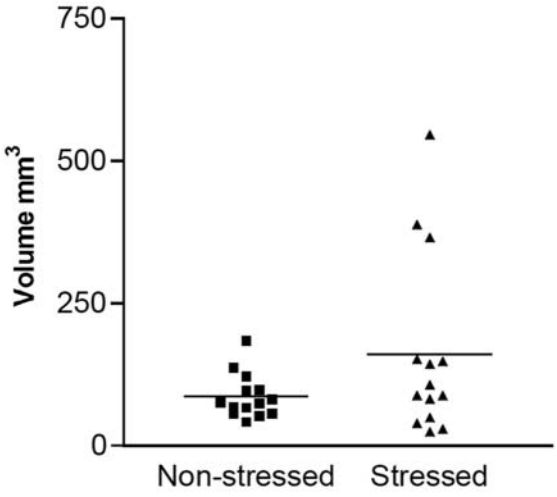

Figure 2. *Stress did not alter melanoma tumor growth in vivo. FVB mice were injected with B16F10 melanoma cells and subjected to the variable stress protocol for 12 days. Tumor volume was determined following the last stressor. Mean tumor volumes were 86.5±10.4 mm3 (n=14) and 160.7±42.2 (n=14) for non-stressed and stressed mice, respectively (p=0.1).*

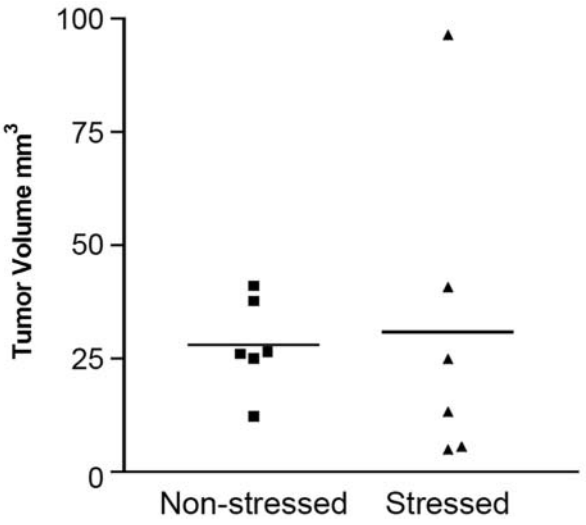

Figure 3. *Stress did not alter lymphosarcoma progression in vivo. C3H mice were injected with 6C3HED lymphosarcoma cells and subjected to rotational stress for 10 minutes of every hour on days 4-6. Tumor volume was determined on day 23. Mean tumor volumes were 28.1±4.2 (n=6) and 30.9±14.2 (n=6) for non-stressed mice and stressed mice, respectively (p=0.85).*

were injected subcutaneously on day 0 and the mice stressed on days 4-6. In this case, the stress was solely rotational. The tumors were then allowed to continue growing until day 23, when the animals were sacrificed. Riley's prior work (9) showed similar tumor growth curves for stressed and non-stressed mice until day 11 or 12, at which time the tumors in the non-stressed mice plateaued in size, while tumors in the stressed mice continued to increase. Thus, by day 23, there was a significant difference in the tumor size between the groups. Our replication of this work did not duplicate this effect of stress (Figure 3). We also repeated this experiment using our stronger, alternating stress paradigm during days 4-6. This approach also did not lead to increased tumor growth in the stressed mice (data not shown). In both cases, the serum corticosterone had returned to control levels by day 23. Riley did not report, nor did we see, any evidence of metastasis.

Discussion

Our experiments did not demonstrate any effect of behavioral stress on tumor growth. The stress paradigm that we developed, consisting of alternating rotation, restraint and cold-water swimming, caused striking increases in serum corticosterone levels and thymus involution, indicating very high levels of behavioral stress. Applied to two models of solid tumors, melanoma and lymphosarcoma, this method did not significantly alter on tumor progression. While three mice in the melanoma experiment displayed increased tumor size, there was no significant difference between the stressed and control groups. Moreover, the mice with the largest tumors did not display higher levels of corticosterone compared to the mice with smaller tumors (data not shown). In addition, we failed to duplicate the differential tumor growth findings of Riley (9) using his stress paradigm of rotation alone.

Our conditions differed from those of Riley's in one respect. While we housed our animals in a conventional animal facility, Riley employed a low-stress environment involving enclosed, individually ventilated shelves that reduced sound and odor transfer between the cages. This low-stress housing reduced the baseline serum corticosterone to 0-35 ng/ml (9). Our control mice had a higher basal level of stress that could have masked any changes similar to those seen by Riley. If this were the case, it would suggest that stress only affects tumor progression when the baseline stress approaches zero. Such a baseline stress level is unrealistic when translating Riley's results to humans. In support of our results, another investigation into the effect of stress on primary tumor growth and metastasis also concluded that mice in conventional animal housing did not display a stress-induced immunomodulation of tumor progression (27).

That we were not able to replicate Riley's finding could alternatively be explained by a possible genetic drift in the mouse strain or an alteration in the tumor cell line. On the other hand, we also found no effect of stress on melanoma growth.

In summary, no association was found between behavioral stress and tumor progression using two mouse models. While our stress paradigm significantly increased serum corticosterone levels and caused thymus involution, it did not influence the growth of either melanoma or lymphosarcoma tumors. Additionally, we could not replicate a previously published finding (9) that demonstrated increased tumor growth following behavioral stress.

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