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Psychological Stress is Associated with Altered Levels of Myeloid-Derived Suppressor cells in Breast Cancer Patients

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Abstract

Our group has shown in a randomized clinical trial that psychological intervention to reduce stress in patients with stage II and III breast cancer led to enhanced immune function, fewer recurrences and improved overall survival. We hypothesized that patients with high levels of stress would have alterations in myeloid-derived suppressor cells (MDSC) compared to patients with lower stress. PBMC from 16 patients with high stress (n = 8) or with low stress (n = 8) after surgery as measured by the Impact of Event Scale (IES) questionnaire were evaluated for the presence of MDSC. Patients with higher IES scores had significantly elevated salivary cortisol levels (P = 0.013; 13 µg/dl vs. 9.74 µg/dl). Levels of IL-1Rα were also significantly elevated in the higher IES group (45.09 pg/mL vs. 97.16 pg/mL; P = 0.010). IP 10, G-CSF, and IL-6 were all higher in the high stress group although not to a significant degree. Flow cytometric analysis for CD33+/HLA-DR-neg/CD15+/CD11b+ MDSC revealed increased MDSC in patients with lower IES scores (P = 0.009). CD11b+/CD15+ cells constituted 9.4% of the CD33+/HLA DR-neg cell population in patients with high IES, versus 27.3% in patients with low IES scores. Additional analyses of the number of stressful events that affected the patients in addition to their cancer diagnosis revealed that this type of stress measure correlated with elevated levels of MDSC (P = .064). These data indicate the existence of a complex relationship between stress and immune function in breast cancer patients.

Keywords

myeloid derived suppressor cell; stress

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Introduction

The negative impact of stress on immune function is well established. Multiple studies have demonstrated that stressful events activate the hypothalamic-pituitary-adrenal axis which increases the production of hormones such as epinephrine, norepinephrine, and cortisol. Immune cells express receptors for these hormones and their function can be markedly altered by chronic exposure to these factors [1–3]. T cell cytokine production is shifted from Th1 towards a Th2 profile, and T cell proliferation and NK cell lysis is inhibited after chronic exposure of the organism to stress [1, 4]. Stress can also lead to alterations in systemic levels of interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and interleukin-1 (IL 1), cytokines which are thought to be involved with tumor progression [5–9]. Additionally, these pro-inflammatory cytokines have been associated with the induction of suppressor cell populations that can further inhibit immune responses [10].

Myeloid-derived suppressor cells (MDSC) are a class of immune suppressor cells that are comprised of a heterogeneous population of early myeloid cells. They are produced in the bone marrow from hematopoietic stem cells and represent less than 1% of circulating cells in normal individuals. MDSC are generally defined phenotypically as being positive for CD33+/CD11b+ (the common myeloid lineage and macrophage markers, respectively) and negative for other lineage markers [10]. Some investigators have further characterized subsets of MDSC as being negative for CD14, and positive for CD15 and IL 4R α [11–13]. The apparent variety of MDSC subsets now being described could represent various stages of differentiation or functionally distinct cellular compartments.

MDSC are known to accumulate in patients with cancer and their numbers seem to correlate with tumor burden [14–17]. The generation of MDSC in the bone marrow is believed to be enhanced in response to tumor-derived factors and these substances also promote the migration of MDSC from the bone marrow to the tumor site [10]. The available evidence strongly suggests that immune suppressor cells are able to blunt the ability of the innate and adaptive immune system to eliminate the developing tumor. The depletion of MDSC in murine models leads to a reduction in tumor growth and enhances the anti-tumor effects of immunotherapeutic regimens [18–21]. MDSC use multiple different mechanisms to induce immune suppression. They produce nitric oxide which can inhibit immune cell signal transduction and they release arginase which depletes L arginine from the tumor microenvironment, thus crippling T cell function [10, 13, 14, 22, 23]. In addition, they are known to produce reactive nitrogen and oxygen species that can further impair immune cell function [10, 13, 23–28].

MDSC may represent a link between stress and cancer progression. It is known that stress leads to elevations in pro-inflammatory cytokines, and many of these stress induced factors are associated with the generation and/or expansion of MDSC [4]. However, the relationship between MDSC and stress has not been previously explored. A randomized clinical trial conducted by investigators at The Ohio State University found that a psychological intervention to reduce life stressors in surgically-treated breast cancer patients led to a significant reduction in recurrence, and an increase in overall survival [29]. Further, these positive outcomes were associated with improved function of immune cells as measured by NK cell cytotoxicity and T cell proliferation [30]. Based on these observations, we hypothesized that high levels of chronic stress would be associated with increased levels of stress hormones and cytokines that in turn would stimulate the generation and accumulation of MDSC. These suppressor cells are known to negatively impact immune function and could theoretically have led to the decrease in breast cancer survival as was seen in the clinical trial.

To evaluate this hypothesis, we examined patients with high or low stress as measured by the Impact of Event Scale (IES) to determine the relationship between cancer stress, stress hormones, pro inflammatory cytokines, and MDSC levels in stage II and III breast cancer patients who were post-surgery and awaiting the start of adjuvant therapy.

Materials and Methods

Patients

Patients with stage II or III breast cancer were recruited for a randomized trial studying the effects of a structured psychological intervention on breast cancer recurrence and overall survival. Blood and saliva samples were taken from patients post surgery and prior to the initiation of adjuvant therapy. This study describes baseline data, prior to any psychological intervention for a subset of trial participants (N=16). All samples were obtained under an Institutional Review Board-approved protocol (IRB No. 1992C0350).

Stress Groups

Two groups were defined based on the Impact of Event Scale (IES), which is a self-report measure of symptoms of traumatic stress (intrusive thoughts and avoidance behaviors) related to a specific event [32]. In this study, questions were worded to reflect the cancer diagnosis specifically. Women reported the frequency of these symptoms during the previous week using a 4-point Likert scale (not at all = 0, rarely = 1, sometimes = 3, and often = 5). Items were summed for a total score which can range from 0 to 75 with higher scores reflecting greater cancer related stress. Scores of 30 and higher indicate clinically significant stress symptoms suggestive of post-traumatic stress disorder [33]. We have previously shown that higher scores on the IES questionnaire correlate with decreased immune function in newly diagnosed breast cancer patients [30, 31]. The majority of patients on the trial reported an IES score of 20-30, therefore patients with scores outside of this normal range were chosen. We identified a High Stress group of 8 patients who had IES ≥ 30 at approximately one month after surgery. IES scores for the High Stress group ranged from 30 to 46 (mean=37.1, SD=6.3). For comparison, we selected 8 patients who had low stress at the same time period post surgery and had sufficient samples for analysis. IES scores for the Low Stress group ranged from 0 to 21 (mean=13.5, SD=8.8). These two groups were significantly different with respect to IES scores ($p<.001$).

Stressful Life Events

Patients also filled out a Life Event Scale that was adapted from the Women's Health Initiative study [34, 35]. Patients were asked whether they had experienced each of 5 different significant life events in the past year (death of a friend or family member, financial difficulty, divorce or separation from a family member or friend, major conflict with children or grandchildren, robberies or accidents). The total number of different events reported (range 0–5) was analyzed. This scale was used to objectively measure stressful events that occurred in the patient's life in addition to a cancer diagnosis.

Plasma Cytokine Analysis

Plasma was procured via centrifugation of peripheral blood samples of study patients and stored at -80° until analysis. For cytokine profiling, plasma samples were thawed at room temperature and then analyzed in duplicate with the premixed Bio Plex Pro Human Cytokine Array (Bio-Rad) according to manufacturer's instructions. Plasma samples were available for n=14 patients.

Immune Function Studies

Natural killer cell cytotoxicity assays (NKCC) were performed using a standard chromium release assay as described [35]. Briefly, K562 target cells were labeled with chromium and incubated with NK cells at effector: target (E:T) cell ratios of 100:1, 50:1, 25:1, 12.5:1, 6.25:1, and 3.13:1 (in triplicate). T cell proliferation was evaluated by stimulating peripheral blood mononuclear cells (PBMC) with phytohemagglutinin (PHA) and Concanavalin A (Con A) at 2.5, 5.0, and 10.0 µg/mL, and using a standard MTT assay [35].

Flow Cytometry for Myeloid-Derived Suppressor Cells

PBMC were suspended at a concentration of 1×10^7 /mL in flow buffer (PBS plus 1% FBS). Cells were incubated with fluorochrome labeled antibodies at 4°C. Specific antibodies included CD15 FITC (eBioscience), CD33 PE (BD Biosciences), HLA-DR PERCP-Cy5.5 (eBioscience), CD11b APC (BD Biosciences), and CD14 Pacific Blue (BD Biosciences). Cells were then washed with flow buffer, fixed with 1% formalin, and stored at 4°C until analysis. All analysis was conducted on a BD LSR II flow cytometer (BD Biosciences) [10, 16, 17].

Salivary Cortisol

Cortisol content was determined using the Cortisol Coat-A-Count RIA (Diagnostic Products Corp., Los Angeles, CA). Sensitivity was 0.025 µg/dl. Intra-assay variation was 4.3% and inter-assay variation was 5.2% [36].

Plasma Catecholamines (Norepinephrine, Epinephrine)

Determinations were made by HPLC with ElectroChemical Detection using Standards and Chemistry from ChromSystems (Thermo-Alko, Beverly, MA), and C-18 Columns from Waters Corporation (Milford, MA). Intra-assay variation for norepinephrine and epinephrine was 3% and 6%; inter-assay variation was 6% and 13%; and sensitivity was 15 pg/ml and 6 pg/ml, respectively [36]. Catecholamine samples were available for n=15 patients.

Adrenocorticotropin Hormone

An Immulite 1000 device was used with reagents manufactured specifically for this instrument (Diagnostic Products Corp., Los Angeles, CA). Intra assay coefficient of variation was 5.6% and inter-assay coefficient of variation was 7.8%. Sensitivity was 9 pg/ml [36].

Statistics

The Mann-Whitney U test was used to compare high and low stress groups on MDSC populations, plasma cytokine levels, and hormone levels. The Mann-Whitney Wilcoxon test is a nonparametric analogue of the Student's t-test and is appropriate for these data due to the sample size. We also tested for relationships between MDSC populations and immune cell function (NK cell cytotoxicity, T cell blastogenesis) using Spearman's rank correlations. Follow-up analyses tested for relationships between MDSC populations and patient background information (number of pre-cancer stressful life events and surgery type) using Spearman correlation or Mann-Whitney U tests, as appropriate.

Results

Hormones, Pro-inflammatory Cytokines and Stress

Chronic stress is associated with increases in cytokines which are known to have effects on MDSC generation and expansion. To delineate two different patient stress groups, patients

with higher or lower than the average cancer-specific stress IES score (20–30) were chosen for study (Table I; $n=16$; $P = 0.001$). Stress hormone levels were evaluated in the high and low IES groups to determine the relationship between these two factors. Salivary cortisol levels were significantly elevated in the high stress group (13 $\mu\text{g/dl}$ vs. 9.74 $\mu\text{g/dl}$, $P = 0.013$, Figure 1A), while epinephrine levels were moderately increased in this group as compared to the low IES group (31 pg/mL vs. 22 pg/mL , $P = 0.118$, Figure 1B). Adrenocorticotropin hormone (ACTH) levels were moderately decreased, and norepinephrine levels were comparable ($P > 0.1$ Figure 1C and 1D). A panel of pro inflammatory cytokines was also evaluated. Interleukin-1 receptor alpha (IL 1R α), interferon-inducible protein 10 (IP-10), and granulocyte-colony stimulation factor (G-CSF) were all higher in the high stress group ($P = .010$, $.317$, and $.662$ respectively; Figure 2A–C). Interleukin 6 (IL 6) levels were comparable in both groups of patients (3.06 pg/mL in the high IES group vs. 2.01 pg/mL in the low IES group, $P > 0.1$; Figure 2D). Monocyte chemoattractant protein-1 (MCP-1), granulocyte macrophage colony stimulating factor (GM-CSF), interferon gamma (IFN γ), platelet derived growth factor (PDGF), and interleukin-8 (IL 8) were comparable between the two groups (P values > 0.1 ; data not shown). Plasma levels of vascular endothelial growth factor (VEGF) were elevated in patients with low IES but not to a significant degree ($P > 0.1$; data not shown).

Myeloid-Derived Suppressor Cells and Cancer-Specific Stress

Patients treated surgically for Stage II or III breast malignancy were evaluated for the presence of myeloid-derived suppressor cell (MDSC) populations by flow cytometry (Figure 3A). We evaluated multiple subsets of MDSC that have been associated with immune suppressive function in previous studies to determine if these subsets were affected by stress [10, 16, 17]. Interestingly, patients who self reported low levels of stress related to their cancer diagnosis exhibited higher levels of MDSC as compared to patients with lower IES scores. Patients in the low stress group had elevated numbers of CD33+/HLA-DR- MDSC (3.51% vs. 1.17%; $P = 0.005$) and CD33+/CD14- MDSC (2.05% vs. 0.7%; $P = 0.013$; Figure 3B–C). Further phenotypic characterization revealed that patients with lower stress had elevated levels of the CD33+/HLA DR /CD15+/CD11b+ MDSC population (27.28% vs. 9.4%; $P = 0.009$; Figure 3D).

Myeloid-Derived Suppressor Cells, Life Stress, and Surgery

In addition to the IES stress measure, patients were also asked to indicate if they had experienced any of five stressful life events during the previous year [29, 32, 36]. The Life Events test evaluates a longer time period of stress as compared to the IES questionnaire which focuses on the relatively acute time since cancer diagnosis. Interestingly, patients that scored higher on the Life Events test exhibited higher levels of MDSC ($\rho = 0.385$, $P = 0.064$; Figure 4A). These results indicate that the relationship between MDSC and stress could be dependent on both the type and duration of the stressor. Of note, patients who underwent more aggressive surgical treatment (total or modified radical mastectomy) exhibited lower levels of CD33+/HLA DR /CD15+/CD11b+ cells as compared to patients that received less extensive surgery (breast conservation therapy or partial mastectomy; $P = 0.030$) (Figure 4B).

MDSC and Immune Function

Increased levels of MDSC levels were associated with decreases in the number of NK cells (Spearman's $\rho = -.493$, $P = 0.062$). While there was no significant relationship between the percent of MDSC and NK cell cytotoxicity (P values > 0.3) or T cell proliferation (P values > 0.1), there was a trend towards low MDSC levels being associating with increased T cell proliferation (Figure 5A–B).

Stress and Survival

In the high-stress group, four patients experienced breast cancer recurrence (at 23 to 71 months) and four are currently disease-free after a minimum of 88 months of follow-up (range 88 to 104 months). In the low-stress group, one patient recurred at 42 months, and seven are currently disease-free after a minimum of 88 months of observation (range 88 to 93 months).

Discussion

This study was initiated to evaluate the effects of cancer-specific stress on MDSC levels in post-surgical breast cancer patients. Patients at the two extreme ends of the IES scale were selected for evaluation. Those with below average scores were designated as the low IES group while those with elevated scores were designated as the high IES group. Cortisol and IL-1R α were significantly elevated in the high IES group as compared to the low IES group. Low IES patients had significantly elevated levels of CD33+/HLA DR /CD15+/CD11b+ MDSC, which is the converse of the anticipated relationship. Interestingly, when the same patients were evaluated for objective, longer-term stressors by the Life Events measure of stress, the inverse relationship between stress and MDSC was observed: Patients with higher stress levels exhibited higher baseline numbers of MDSC. MDSC levels at baseline did not correlate with measures of NK or T cell function. Levels of MDSC correlated with the extent of surgery, as patients who underwent more extensive surgical procedures had significantly lower levels of MDSC. Importantly, the current study is the first to specifically measure MDSC levels in post operative breast cancer patients who were considered to be macroscopically disease-free.

Previous studies have demonstrated that stress can lead to elevations in pro-inflammatory cytokines and stress hormones in cancer patients. Some of these same factors, such as IL-6 and VEGF are associated with the development and/or promotion of immune suppressor cell populations [7, 10, 11, 16, 21, 37–39]. Since stress induces alterations in multiple factors associated with MDSC accumulation and generation, stress may very well lead to alterations in MDSC levels and/or function. The induction of MDSC by stressful events could therefore lead to reduced immune function. Data from our collaborators and others indicates that stress is associated with decreased immune function [4, 6, 7, 29]. Data from our groups' randomized clinical trial of stress reduction demonstrated that the psychological intervention to reduce stress correlated with improvements in immune function at 4 months post-surgery [35]. We proposed that stress and the associated immune suppressive effects could lead to reduced tumor surveillance and increased risk of recurrence. Indeed, in our previous studies, a multivariate comparison of survival using the Cox proportional hazards analysis showed that patients in the Intervention arm had a reduced risk of breast cancer recurrence and death as compared to patients in the Assessment only arm. From this work, it was hypothesized that patients with high levels of cancer specific stress after surgery might exhibit higher levels or increased activity of MDSC and that this cell compartment could be responsible for the reduced immune function that was observed in the Assessment only arm. As the IES is a subjective measure of stress specific to cancer, it was expected that high IES would correlate with high levels of MDSC. Instead, the opposite relationship was observed. Patients with low IES scores actually had higher levels of MDSC at baseline as compared to patients with high IES scores. While there are fewer MDSC, the direct suppressive effects of these cells has not been fully evaluated. It is possible that with stress, there are fewer MDSC in peripheral blood, but these MDSC could be more active. In addition, the manner in which stress is measured could represent a source of variability.

At this point, we examined other factors that could be affecting patients at this stage of treatment. The patients in this study were post-surgery and had minimal tumor burden. In

addition, while the IES measures subjective stress associated with a cancer diagnosis, this does not take into account other stressful events that could have occurred in their lives. In this cohort of patients, the IES questionnaire represents an acute measure of stress since it specifically looks at the time period since cancer diagnosis (< 2 months). When these same patients were classified by an objective measure of long-term stress, the Life Events Scale, it was found that patients with high stress had elevated levels of MDSC, as had been predicted. It has long been recognized that the duration and intensity of a stressor affects its impact on immune function [40]. A recent meta-analysis compared immune function in healthy volunteers who had either experienced a short period of stress (i.e. short stressful events similar to the stress of a cancer diagnosis) or a longer period of stress (e.g. caring for a spouse with dementia). This study found that only chronic stress was associated with dysfunction of the innate and adaptive immune cell compartments. Chronically stressed participants exhibited significantly decreased NK cell lysis, decreased proliferation of T cells in response to PHA and ConA and reduced levels of interleukin-2 secretion by PBMCs [4]. These data suggest that chronic life stress could promote MDSC generation, while acute stress might have the opposite effect on MDSC levels. Further prospective studies with larger patient populations will be needed to prove a direct connection between immune function, MDSC, and chronic stress.

When the patients in this study were evaluated according to surgical procedure received (breast conservation therapy or mastectomy), it was found that patients who underwent the more extensive surgical procedure had significantly fewer MDSC. Of note, surgery type was not associated with self-reported stress levels in this sample. These results indicate that the extent of surgery could have an impact on the generation or survival of MDSC. A more extensive surgery could be associated with increased trauma and/or pain for the patient. These factors could lead to additional psychological changes that might alter MDSC numbers. The use of analgesic medications in the post-operative period could also affect MDSC accumulation. Given the temporal proximity of the surgical procedure to the stress measurement, it is necessary to consider possible influences of this intervention on factors that can initiate and support MDSC expansion [41–43].

Examination of a larger sample of patients in a prospective manner will further our understanding of the connection between stress, stress hormones and pro-inflammatory cytokines, and immune suppressor cells in breast cancer. Future studies will also evaluate the direct effects of MDSC on NK and T cell function and how stress alters these interactions.

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Figure 1. IES scores are associated with alterations in stress hormone levels

Sixteen patients with higher or lower than average IES scores were evaluated for (A) salivary cortisol, (B) epinephrine, (C) ACTH, and (D) norepinephrine. Hormone levels were compared to individual IES scores for each patient.

Figure 2. Patients with higher IES scores exhibit elevations in pro-inflammatory cytokines
 Plasma samples obtained from sixteen patients were evaluated for multiple cytokines by ELISA: **(A)** Interleukin-1R alpha (IL-1Ra), **(B)** interferon response protein 10 (IP-10), **(C)** granulocyte-colony stimulating factor (G-CSF), and **(D)** interleukin-6 (IL 6) levels are shown. Cytokine levels were compared to individual IES scores for each patient.

Figure 3. Acute stress is associated with decreased levels of myeloid-derived suppressor cells Peripheral blood mononuclear cells (PBMCs) were obtained from breast cancer patients post-surgery and stained for multiple subsets of MDSC. **(A)** Representative scatter plots from a breast cancer patient as compared to PBMC obtained from a normal control. PBMCs were labeled with CD33, HLA-DR, CD15, and CD11b, red box indicates gated population, arrows indicate sequence of gating. PBMCs were harvested from sixteen patients with higher than average or lower than average IES scores and labeled with markers to evaluate early myeloid cells. **(B)** CD33 and HLA-DR, **(C)** CD33 and CD14. Graphs represent the percent of total live cells that express each marker. **(D)** MDSC subset analysis, percent positive represents the number of CD15+/CD11b+ cells of the CD33+ HLA-DR- early myeloid population.

a.

b.

Figure 4. Chronic stress and surgery are associated with elevations in myeloid-derived suppressor cells

(A) PBMCs were obtained from breast cancer patients postsurgery and stained for CD33+/HLA-DR-/CD15+/CD11b+ MDSC. These results were compared to the Life Events measure of stress for each patient. (B) Patients were grouped according to surgical procedure: breast conservation therapy or mastectomy. These surgical groups were then compared with levels of MDSC.

Figure 5. MDSC and immune cell function post-surgery

PBMCs were obtained post-surgery from patients and were evaluated by flow cytometry for the expression of CD33, HLA-DR, CD15, and CD11b. NK cells were isolated from each patient and evaluated for the ability to lyse K562 chromium-labeled target cells, and T cell proliferation was measured in response to 72 hr Concanavalin A stimulation. CD33⁺/HLA-DR⁻/CD15⁺/CD11b⁺ MDSC levels were compared to (A) NK cell cytotoxicity and (B) T cell proliferation within the same patient.

Table 1

Patient Characteristics

	Low Stress (n=8)	High Stress (n=8)
Variables	Mean (SD) or %	Mean (SD) or %
Sociodemographic		
Age (years)	49.7 (13.0)	54.5 (13.4)
Education (years)	15.6 (4.1)	13.5 (2.7)
Partner status (% partnered)	87 %	75 %
Family income (thousand \$/year)	88.4 (44.5)	46.4 (40.4)
Race (% Caucasian)	88 %	100 %
Disease		
Stage (II vs. III, % II)	75 %	63 %
Nodes (% positive)	75 %	75 %
ER/PR (% positive)	63 %	63 %
Surgery (% modified radical mastectomy)	50 %	75 %
Days since surgery	41.2 (13.0)	33.4 (11.9)