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CLINICAL REPORT

Psychological Stress and Immunological Modulations in Early-stage Melanoma Patients

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Mental stress may have a negative impact on the immune state of cancer patients, in whom immunologic surveillance is essential for survival. This study investigated the immunological response of 19 patients with early-stage melanoma and a matched control group undergoing the Determination Stress Test before surgery. Cytokine and chemokine levels and lymphocyte subpopulations were measured at baseline and post-stress test time-points. Following the stress test lower levels of interleukin (IL)-6 were observed in the melanoma group compared with healthy volunteers ($p=0.044$). IL-10 increased significantly in the control group 30 min after the stress test ($p=0.002$) in comparison with the melanoma group ($p=0.407$). CCL5/Rantes decreased significantly in the melanoma group, whereas CD16/CD56$^+$ natural killer cells increased in both groups, with a sharp decrease below baseline after stress in the melanoma group ($p=0.001$). This pilot study shows an altered immunological response to stressors in melanoma patients.
Key words: melanoma; cytokines; chemokines; lymphocytes; stress.

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Melanoma is a highly immunogenic tumor, a biological characteristic that has led to the design and implementation of successful therapeutic interventions for metastatic disease. Drugs such as interferon (IFN)-α or ipilimumab have shown favorable results in adjuvant, therapeutic, and palliative settings (1, 2). Several experimental observations describing the influence of melanoma on the activity of the immune system have been reported. Analyses of serum cytokines have demonstrated higher levels of a variety of interleukins (IL), tumor necrosis factor alpha (TNFα), and other cytokines in resected high-risk melanoma patients compared with healthy controls (3). In particular, IL-6 serum levels have been correlated with higher tumor load and worse prognosis in melanoma patients (4–9). IL-6 mediates the production of IL-10 in metastatic melanoma cell suspensions (10), with elevated IL-10 serum levels observed in patients with metastatic melanoma (11). Additional findings include the impairment of natural killer (NK) cells activity and decreased serum levels of lymphocyte subpopulations, such as cluster of differentiation (CD)3$^+$, CD4$^+$, CD8$^+$, CD19$^+$ cells, in melanoma patients with advanced or metastatic disease (12, 13).

The immune system is highly sensitive to stress (14, 15). To date, several reports have demonstrated a variety of lymphocyte and cytokine responses in relation to acute psychological stress in healthy individuals. Some of these responses include the modulatory effect on CD3$^+$ and CD4$^+$ cells (increased and decreased counts) (16–19) and an up-regulation of CD16/CD56$^+$ NK cells in post-stress conditions (17, 19). Cytokines also show different responses to psychological stress. Some reports have shown increased serum levels of IL-1β (20, 21), IL-6 (16, 20–22), IL-10 (21) and TNF (21) in post-stress settings in healthy controls, while other studies have failed to replicate these findings (18, 23–25). Such contradictory results probably reflect the complexity of the experimental models and the significant number of confounders when evaluating the immunomodulatory effect of stress in humans.

Several studies have more specifically evaluated the role of stress in the immune status of cancer patients. In ovarian cancer patients stress was associated with impaired NK cell activity, as described by Lutgendorf et al. (26). Cognitive behavioral stress management also indicated distinct differences in Th1 cytokine regulation compared with a control group in patients undergoing treatment for breast cancer (27).

Little is known about changes in levels of cytokines, chemokines and lymphocyte subpopulations due to stress in patients with melanoma, especially at an early stage of the disease. The reports in the melanoma field have been primarily limited to experimental investigations showing that stress significantly increases the production of vascular endothelial growth factor (VEGF), IL-8 and IL-6, with an associated impaired anti-tumor T-cell response by suppression of CD4$^+$ cells (28, 29).

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The aim of this pilot study in a small cohort of melanoma patients was to detect potential differences in levels of cytokines, chemokines and lymphocyte subpopulations after acute psychological stress. We hypothesized that melanoma patients at an early stage of the disease, e.g. small, early detected tumors, might react differently compared with healthy controls after psychological stress induced by a test system. We analyzed different panels focusing on IL-6 and IL-10, with the hypothesis that we would observe a comparable increase in both cytokines after psychological stress compared with healthy controls, but higher levels of IL-6 at baseline in the melanoma group, according to the observations of higher levels of IL-6 in cancer patients (3, 30). In addition, we focused on $CD4^+$, $CD8^+$ and CD16/$CD56^+$ NK cells, hypothesizing an increase in these cells after stress in both groups.

MATERIALS AND METHODS (for a full description see Appendix S1[1])

Study design and subjects

This pilot study examined the immunomodulatory response to stress in a cohort of melanoma patients at an early stage of the disease. The melanoma cases were matched for age and gender with controls.

Patients with primary cutaneous melanoma in clinical stages IA and IB (31) were recruited.

The melanoma group consisted of 19 patients, mean age 51 years (standard deviation (SD) 13.4; age range 30.9–71.3 years), with a recent clinical or histological diagnosis of cutaneous melanoma. Patients with prior history of melanomas were excluded. None of the patients required further adjuvant treatment. The control group consisted of 19 patients, mean age 51.1 years (SD 13.8; 27.8–71.8 years) with benign tumors who were also scheduled for surgery. The gender distribution was 9:10 (female: male) in both groups. Mean tumor thickness in the melanoma group was 0.54 mm (SD 0.23; 0.1–1.03 mm). All melanomas were primary tumors and all patients were at an early stage of the disease without evidence of distant metastasis.

Explanatory information about melanoma and associated risks was given 2–9 weeks prior to the study intervention to avoid further confounding psychological factors at the time of the stress test. All patients participated in the test procedure under equal pre-surgical conditions.

Stress test procedures

The psychological stress test procedures were performed at the University of Graz, Medical School, Department of Dermatology, in the perioperative period under standardized conditions 1–1.5 h prior to scheduled surgery according to the timeline in Fig. 1.

A peripheral venous catheter was placed in the upper extremity by the study personnel. The test procedure was sub-divided into a period of rest (POR) in the lying position with closed eyes for 10 min (POR1), followed by a standardized sensory/mental stress of approximately 15 min (Determination Stress Test: DT), followed by a period of rest in the lying position with eyes closed for 15 min (POR2), and followed by another period of rest in the lying position for 15 min (POR3) (Fig. 1). The test procedure was conducted in a private and soundproof setting.

Study personnel obtained blood samples (BS) to measure cytokines, chemokines, and lymphocytes immediately after first POR (BS1), after the DT (BS2), after POR2 (15 min post-stress) (BS3), and after POR3 (30 min post-stress) (BS4). Time intervals for acquisition of the blood samples were chosen in accordance with previously performed studies on cytokine reactions on acute psychological stress (18, 21, 25).

Determination test

The DT is used to measure reaction ability and reactive stress tolerance (32). For all test forms the internal consistencies for the main variables, reactive stress tolerance and reaction ability, lie between $r=0.98$ and $r=0.99$ (32). The validity of the DT has been demonstrated in several studies (32–34).

Laboratory methods

Cytokine and chemokine assessments. The BD™ Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit was used to quantitatively measure IL-8, IL-1β, IL-6, IL-10, TNF and IL-12P protein levels in a single sample.

BD™ CBA Human Chemokine Kit was used to quantitatively measure IL-8 (CXCL8/IL-8), RANTES (CCL5/RANTES), monokine induced by IFN-γ (CXCL9/MIG) and IFN-γ-induced protein-10 (CXCL10/IP-10) levels in a single sample.

Cell subtype assessments. BD™ Multitest IMK kits were used with CD3/CD8/CD45/CD4 reagent and CD3/$CD16^+$CD56/CD45/CD19 reagent. Flow cytometry analysis measures the emission of optical signals, after passing through a laser beam. In our investigation a "lyse – no wash" method was used (35). The measurement was performed using CellQuest Protocol according to the guidelines of the manufacturer on a BD™ FascCalibur.

Assessment of cortisol levels. Serum for analysis was obtained between 07.40 h and 10.15 h. Cortisol levels were analyzed using Siemens ADVIA Centaur® cortisol assay, a competitive immunoassay using direct chemiluminescent technology.

Statistical methods

Differences between the groups (melanoma group vs. control group) and changes in the parameters in the blood samples of each group (BS1 to the following 3 blood samples, BS2 to the following 2, and BS3 to BS4) were analyzed. These analyses were performed using non-parametric analysis (Mann-Whitney *U* test or Wilcoxon signed-rank test), since most of the parameters were not normally distributed and transformation (log) did not result in a normal distribution. Due to the exploratory nature of this pilot study Alpha-adjusting was not undertaken.

For data analysis PASW 18 (PASW Statistics; SPSS Inc., Chicago, IL, USA) was used.

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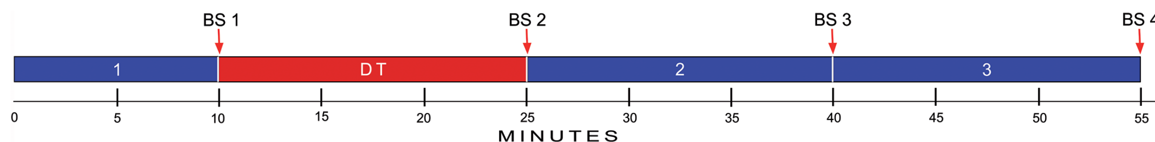

Fig. 1. Timeline of study-related interventions. *Blue bar*: period of rest (POR). 1=POR1 10 min; 2= POR2 15 min; 3=POR3 15 min. *Red bar*: Stress test: Determination Test (DT) 15 min. *Arrow*: blood sample (BS): BS1; BS2; BS3; BS4.

RESULTS

Cortisol

Baseline mean cortisol level was found to be similar between study groups (melanoma group 164 ng/ml, control group 161 ng/ml).

Cytokines

No difference in cytokine levels was observed between the groups at baseline measurement (Table I). Median levels of IL-6 were lower in the melanoma group compared with the control group throughout the duration of the test. The differences between the IL-6 levels reached statistical significance between groups 15 min after the stress test (BS3) ($p=0.044$) (Table SI[1]). In the melanoma group a significant increase in IL-8 level was observed 15 min after the stress test (BS2 to BS3, $p=0.018$). No significant changes in IL-8 expression levels after the stress test were observed in the control group. In the case of IL-10 a significant increase in serum levels was observed 30 min after the stress test in the control group, when compared with the melanoma group (BS3 to BS4, $p=0.002$) (Table SII[1]). The difference in IL-10 serum levels between the 2 groups 30 min after the stress test was not significant ($p=0.111$). No significant changes in the course of IL-1β, IL-12 and TNF levels were observed in the 2 groups (Table I).

Chemokines

No difference in baseline chemokine levels was observed between the study groups (Table I). CXCL8 serum levels decreased in the melanoma group after the stress test, reaching statistical significance 15 min after the DT (BS2 to BS3, $p=0.019$), while an increase in circulating levels was observed in the control group after the DT. The observed changes in these chemokine levels in the control group failed to reach significance (BS2 to BS4, $p=0.050$). In the case of CXCL9 a significant decrease in peripheral levels was observed at BS4 compared with baseline levels in the control group (Table I). In the melanoma group a relevant increase was observed between BS1 and BS2–3 before a decreased expression level was noted at the BS4 time-point (Table I). The CXCL10 serum levels observed in the control group

Table I. Changes in cytokines, chemokines and subpopulations of lymphocytes during the test procedure within the melanoma (M) and control (C) groups

	Group	BS1 Median (IQR)	BS2 Median (IQR)	BS3 Median (IQR)	BS4 Median (IQR)
Interleukin-8	C	15.3 (12.4–17.1)	14.5 (12.0–16.8)	13.9 (10.9–16.7)	14.8 (11.0–19.0)
pg/ml	M	12.3 (10.3–19.9)	12.5 (10.6–16.8)	**13.2 (9.7–17.1)**[b]	13.2 (10.3–17.5)
Interleukin-1β	C	7.4 (5.5–7.7)	5.8 (4.4–6.7)	5.3 (4.4–6.2)	5.7 (4.4–7.2)
pg/ml	M	5.7 (4.6–6.5)	4.8 (0.0–5.6)	5.2 (0.0–5.7)	5.1 (4.2–6.1)
Interleukin-6	C	6.1 (5.5–8.6)	6.2 (4.7–8.5)	7.0 (5.8–9.0)	6.9 (5.7–8.1)
pg/ml	M	5.5 (4.9–6.8)	5.7 (5.1–6.4)	5.9 (5.3–6.5)	6.2 (5.4–7.9)
Interleukin-10	C	5.8 (5.1–6.5)	5.6 (5.2–6.5)	5.7 (4.9–6.4)	6.1 (5.1–7.1)[c]
pg/ml	M	5.4 (5.1–6.2)	5.4 (5.3–6.1)	5.4 (4.8–5.8)	5.6 (4.7–6.0)
Tumor necrosis factor	C	5.6 (0.0–6.3)	5.6 (0.0–7.0)	5.3 (4.7–6.3)	5.2 (4.8–6.6)
pg/ml	M	4.7 (0.0–6.0)	5.0 (4.8–6.4)	5.3 (0.0–5.8)	5.0 (0.0–6.3)
Interleukin-12	C	6.2 (5.1–9.4)	7.4 (5.3–8.5)	6.2 (5.6–7.7)	7.4 (5.5–7.7)
pg/ml	M	5.6 (4.8–7.3)	5.7 (4.8–7.0)	6.1 (5.1–7.4)	5.7 (4.6–6.5)
CXCL8	C	9.2 (5.1–12.3)	9.8 (5.9–11.3)	8.9 (5.9–10.9)	8.1 (0.0–11.6)
pg/ml	M	8.5 (5.3–12.0)	7.7 (0.0–10.9)	**7.8 (0.0–10.9)**[b]	8.3 (0.0–13.1)
CCL5 Rantes	C	845.1 (8.1–1,714.5)	587.8 (6.7–1,089.7)	266.2 (8.1–1,029.9)	82.8 (7.7–1,407.8)
ηg/ml	M	755.8 (7.6–2,198.1)	**157.5 (5.3–1,209.1)**[a]	**676.0 (7.3–1,381.2)**[a]	707.4 (7.6–1,625.8)
CXCL9	C	90.2 (54.1–111.3)	90.0 (45.2–107.0)	91.3 (54.4–106.3)	**85.4 (41.0–108.4)**[a,b,c]
pg/ml	M	73.4 (49.1–151.3)	78.5 (48.3–166.0)	78.1 (50.4–154.9)	**74.4 (50.4–136.9)**[a,b]
CXCL10	C	109 (92–153)	113 (85–166)	112 (92–144)	**116 (77–143)**[a,b,c]
pg/ml	M	103 (88–122)	108 (80–134)	**105 (81–121)**[a]	**103 (78–119)**[a,b]
CD3+ cells	C	1,052 (815–1,474)	**1,234 (933–1,543)**[a]	**1,113 (813–1,601)**[b]	1,154 (688–1,603)
cells/µl	M	1,147 (860–1,360)	**1,260 (930–1,402)**[a]	**1,159 (803–1,329)**[b]	**1,117 (802–1,296)**[b]
CD8+ cells	C	341 (276–572)	**387 (294–640)**[a]	**368 (258–577)**[b]	**373 (236–555)**[b]
cells/µl	M	348 (275–460)	378 (266–454)	**307 (250–457)**[b]	**296 (242–427)**[a,b]
CD4+ cells	C	713 (500–966)	758 (573–997)	**720 (518–959)**[b]	733 (447–1,007)
cells/µl	M	762 (537–910)	**837 (568–958)**[a]	713 (500–974)	751 (525–1,005)
CD16/CD56+ cells	C	169 (99–292)	**358 (153–416)**[a]	**172 (75– 238)**[b]	**147 (75–250)**[b]
cells/µl	M	174 (133–241)	**274 (193–379)**[a]	**132 (111–217)**[a,b]	**162 (104–202)**[a,b]
CD19+ cells	C	184 (83–244)	197 (95–255)	201 (115–274)	204 (98–268)
cells/µl	M	187 (127–226)	178 (131–218)	188 (115–236)	201 (133–208)
CD45+ cells	C	1,521 (1,210–2,198)	**1,836 (1,474–2,178)**[a]	**1,586 (1,260–2,331)**[b]	**1,621 (1,186–2,232)**[b]
cells/µl	M	1,552 (1,248–1,895)	**1,747 (1,408–2,138)**[a]	**1,510 (1,049–1,817)**[b]	**1,439 (1,066–1,683)**[b]
CD4/CD8 ratio	C	2.1 (1.7–2.8)	2.0 (1.6–2.6)	2.1 (1.8–2.9)	2.0 (1.8–2.8)
	M	2.0 (1.7–2.6)	2.0 (1.6–2.5)	**2.1 (1.8–2.7)**[a,b]	**2.2 (1.9–2.9)**[a,b]

Comparison with: [a]BS1, $p<0.05$; [b]BS2, $p<0.05$; [c]BS3, $p<0.05$. IQR: interquartile range. Significant values are shown in bold.

showed a continued increase through the duration of the DT. For the melanoma patients an increase in the serum levels was documented at BS2, before the values decreased to normal levels at the BS4 time-point (Table I) (Table SII[1]).

Whereas no significant changes were observed in the control group, CCL5 Rantes decreased significantly after the stress test (BS1 to BS2, $p=0.010$) in the melanoma group and remained at lower levels 15 min after the DT was completed (BS1 to BS3, $p=0.036$) (Table I).

Lymphocytes

No difference in lymphocytes levels was observed between the groups at baseline (Table I). After the stress test CD3$^+$ and CD45$^+$ cells increased significantly in both groups and decreased afterwards. In the melanoma group a significant increase in CD4$^+$ cell levels was observed immediately after stress (BS1 to BS2, $p=0.013$) with a non-significant decrease 15 min after the stress test. In the control group no significant changes in CD4$^+$ cell levels were seen between BS1 and BS2, but the decrease 15 min afterwards was significant (BS2 to BS3, $p=0.027$). While CD8$^+$ cells increased in the control group immediately after the stress test (BS1 to BS2, $p=0.010$), these cells remained nearly at baseline level in the melanoma group, followed by decreased levels 15 min after the stress test was completed (BS1 to BS4, $p=0.025$).

Due to the modulation of the different parameters a significant increase in CD4/CD8 ratio was observed in the melanoma group 15 min after the stress test (BS1 to BS3, $p=0.036$) and 30 min after the stress test (BS1 to BS4, $p=0.010$), whereas no significant change was observed in the control group (values and changes are shown in Table I).

In the case of CD16/CD56$^+$ NK cells an increased measurement was observed in the control group (BS1 to BS2, $p<0.001$), which normalized 15 min after the stress test. A similar modulation was observed in the melanoma group; however, the magnitude of change after the stress test was lower, but still significant (BS1 to BS2, $p=0.001$). In both groups levels of CD16/CD56$^+$ NK cells decreased 15 and 30 min after the stress test. Interestingly, in the melanoma group a sharp decrease, below baseline values, was seen 15 min after the stress test (BS1 to BS3, $p=0.003$) lasting until the end of the test (BS1 to BS4, $p=0.011$).

No significant changes in the levels of CD19$^+$ cells were observed in both groups during the study (Table SII[1]).

DISCUSSION

This pilot study focused on the pattern of immune modulation in response to psychological stress in patients with early-stage melanoma. Overall, some distinct differences between the 2 study groups were observed following the stressful stimulus. At baseline no differences in the levels of cytokines, chemokines and lymphocytes was seen between the groups. These results seem to be contradictory to the literature, where higher levels of IL-6 in melanoma patients are described, not only in metastatic patients with shorter relapse-free survival (RFS) and overall survival, but also in patients with clinical stages IIB–III with longer RFS treated with adjuvant IFN (3, 5, 6, 36). The values measured in our study were lower than in the control group, possibly reflecting the early stage of the disease.

After the stress test several findings concerning immunomodulatory activity were observed in our study groups. Whereas levels of IL-6 increased after the DT intervention in the control group, as expected from the literature (20, 22) and remained elevated after the 2 periods of rest, IL-6 increased only slowly in the melanoma group, remaining below the values observed in the control group. The slow response of the pro-inflammatory immune cascade after stress in the melanoma group is an observation that needs to be studied in a larger cohort of patients and in different stages of the disease. Since the melanoma group and the control group were matched with respect to age and gender, we do not anticipate these variables to play a role in the observed results (37, 38).

An additional relevant observation includes the difference in IL-10 modulation between the melanoma group and controls. In contrast to our findings, higher serum levels of IL-10 and IL-12 have been described previously in patients with stage III and IV disease (6, 11, 31). While the melanoma cohort in our study failed to reach significant changes in the modulation of IL-6 and IL-10, the control group did demonstrate a significant increase in IL-10 levels at BS4. These findings may reflect a reduced anti-inflammatory immune response in melanoma patients, but it will require a larger cohort to assess the significance of this finding.

Studies involving melanoma cases with advanced disease have demonstrated an up-regulation in the expression of IL-8 (3). Our failure to demonstrate this finding is probably related to the early stage of disease in our study cohort.

The response observed in the lymphocyte subsets was similar in both groups when CD3$^+$ and CD45$^+$ cells were evaluated. Cell levels increased significantly immediately after the stress test and then decreased significantly, as observed by other authors (18, 19). The most relevant difference in the response of lymphocyte subpopulations after stress between melanoma and control groups was observed for CD16/CD56$^+$ NK cells. In both groups a sharp increase was present immediately following the DT, as expected (17–19) and then cell levels returned to nearly normal levels by the BS4 time-point. Interestingly, in the melanoma group the levels dropped below baseline at the BS3 time-point. A parallel finding included the observed decrease in CCL5 Rantes levels, a chemotactic cytokine

involved in the promotion of proliferation and activation of NK cells. At the BS2-3 time-point cell levels dropped below baseline values in the melanoma group.

In conclusion, this pilot study suggests that, exposed to stress, melanoma patients (even in the early stages of the disease) have a different immunological reaction pattern to that of controls.

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The authors declare no conflicts of interest.

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