

Appendix S1

MATERIALS AND METHODS

Study design

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. The study end-points included IL-6, IL-10, CD4⁺, CD8⁺ and NK cells levels. The study also explored the IL-8, IL-1 β , TNF, IL-12, CXCL8, CCL5 Rantes, CXCL9, CXCL10, CD3⁺, CD19⁺, CD45⁺ cells levels and CD4/CD8 ratio.

Study subjects

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 (SD 13.4; 30.9–71.3 years), with recent clinical or histological diagnosis of cutaneous melanoma. Patients with prior history of melanomas were excluded and 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 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 disease without evidence of distant metastasis. In the control group the following benign tumors were removed: melanocytic nevus (6), epidermal or tricholemmal cyst (5), scar (2), fibroma or neurofibroma (2), lipoma (2), sebaceous gland hyperplasia (1) and condyloma acuminata (1). All diagnoses were made clinically 2–9 weeks prior to study enrolment and confirmed by histopathology.

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.

The study subjects were required to understand the study procedure and written informed consent was obtained. The local ethics committee approved the study (EK Nr. 21-129 ex 09/10) and the investigation followed the rules of the Declaration of Helsinki.

Histological classifications

All excised lesions were processed following routine histological protocols. For all primary melanomas, histological specifications on tumor characteristics were reported, including tumour thickness in mm.

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 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 lying position with eyes closed for 15 min (POR2), and followed by another period of rest in 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 upon cytokine reactions on acute psychological stress (18, 21, 25). The blood samples were brought immediately to the laboratory located in close proximity, centrifuged without delay and frozen at -20°C . Multiplex analysis was chosen for simultaneous measurement of different serum parameters to minimize the processing time. Time between sample acquisition and sample processing varied between 1 week and 3 months. Samples were batched for analyses.

The expression of relevant cytokines (IL-1 β , IL-6, IL-8, IL-10, IL-12, TNF), chemokines (CXCL8, CXCL9, CXCL10, CCL5 Rantes), and lymphocytes (CD3⁺ cells, CD4⁺ cells, CD8⁺ cells, CD16/CD56⁺ NK cells, CD19⁺ cells, CD45⁺ cells) and CD4/CD8 ratio were analyzed.

Cortisol level was measured before start of test procedure, only at baseline (BS1), to detect a chronic stress state that might interact with an acute stressor.

Determination test

The DT is used to measure reaction ability and reactive stress tolerance (32). The participant has to use her/his cognitive skills in order to "distinguish different colors and sounds, memorize the relevant characteristics of stimulus configurations, and to select the relevant responses according to the assignment rules depicted in the instructions and/or learned in the course of the test". The participant's task is to press the appropriate buttons on the response panel by hand or foot according to the presented color stimuli and acoustic signals. The difficulty is determined by demanding continuous, fast and varying responses to rapidly changing stimuli. There are 5 visual stimuli: white, yellow, red, green and blue. The presentation of the stimuli occur in adaptive mode (fixed presentation time per stimulus), in an action mode (unlimited presentation time per stimulus) or in a reaction mode (automatically varied presentation time). The presentation of the stimuli in our test occurred in reaction mode. 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

Becton Dickinson (BD)TM Cytometric Bead Array is a laboratory method for detection of liquid analytes by beads of known size and fluorescence with the help of flow cytometry and can be used for quantitative measurements of cytokines and chemokines in one sample.

The wide dynamic range of the fluorescence detection by flow cytometry and the effective "capture" of analytes by means of suspended particles make it possible to measure concentrations of an unknown analyte in less time and using less sample dilutions in comparison with conventional ELISA methods. The required sample volume is approximately 1/6 of the amount needed for conventional ELISA assays. This makes possible the simultaneous detection of 6 analytes from a single sample.

Standard curves were performed according to the manufacturer's guidelines. The "capture beads" were prepared according to the guidelines and data analysis was performed with the aid of the FCAP Array software.

The so-called "capture beads" were conjugated with a specific antibody. This was a phycoerythrin (PE)-conjugated antibody, which yielded a fluorescent signal in relation to the amount of

bound analyte. If the "capture beads" and detection reagents are incubated with a sample of unknown analytes, sandwich complexes with analyte and detection reagent are built. These complexes can be measured using flow cytometry.

Cytokine assessments. The BD™ Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit was used to quantitatively measure IL-8, IL-1 β , IL-6, IL-10, tumor necrosis factor (TNF) and IL-12P protein levels in a single sample. Values are given in picogram/ml (pg/ml). The kit was provided by BD™ with phycoerythrin (PE)-conjugated anti-human antibodies, property of BD™.

Chemokine assessments. BDT™ CBA Human Chemokine Kit was used to quantitatively measure IL-8 (CXCL8/IL-8), RANTES (CCL5/RANTES), monokine induced by interferon- γ (CXCL9/MIG) and interferon- γ -induced protein-10 (CXCL10/IP-10) levels in a single sample. Values are given in pg/ml. The kit was provided by BD™ with phycoerythrin (PE)-conjugated anti-human antibodies, property of BD™.

Cell subtype assessments. BDTM Multitest IMK kits were used with CD3/CD8/CD45/CD4 reagent and CD3/CD16⁺CD56/CD45/CD19 reagent, provided both in 1 ml of buffered saline with 0.1% sodium azide. The first reagent contained FTIC-labeled CD3, clone SK7; CD8 phycoerythrin, clone SK1; CD45 peridinin chlorophyll protein, clone 2D1 and CD4 allophycocyanin, clone SK3 and the second reagent contained FTIC-labeled CD3, clone SK7; phycoerythrin-labeled CD16, clone B73.1 and CD56, clone NCAM 16.2; peridinin chlorophyll protein-labeled CD45, clone 2D1 and allophycocyanin-labeled CD19, clone SJ25C1. In addition BD™ Multitest IMK kit lysing solution, 10 \times concentrate, a proprietary buffered solution containing <15% formaldehyde and <50% diethylene glycol was used. Values are given in cells/ μ l.

Flow cytometry analysis measures the emission of optical signals, after passing through a laser beam. Cells are marked in front with monoclonal antibodies.

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™ FACS Calibur.

Assessment of cortisol levels. Serum for analysis was obtained between 07.40 h and 10.15 h. Cortisol levels were analyzed using ADVIA Centaur cortisol assay (Siemens Healthcare Diagnostics, Bayswater Victoria, Australia), a competitive immunoassay using direct chemiluminescent technology: cortisol in the patient sample competes with acridinium ester-labeled cortisol in the Lite Reagent for binding to polyclonal rabbit anti-cortisol antibody in the solid phase. The polyclonal rabbit anti-cortisol antibody is bound to monoclonal mouse anti-rabbit antibody, which is covalently coupled to paramagnetic particles in the solid phase. The test was performed using Siemens ADVIA Centaur® (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA).

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.