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Prevention of Surgery-induced Suppression of Granulocyte Function by Intravenous Application of a Fermented Extract from *Viscum album* L. in Breast Cancer Patients

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Abstract. *Surgical stress and anaesthetics are able to suppress the immune system. This may accelerate the growth and metastasis of residual cancer cells. As Viscum album L. extracts (VA-E) are known to exert both effects, immunomodulating and apoptosis-inducing properties, a Good-Clinical-Practice-guided, prospective bi-centric phase II study was conducted to measure the influence of a perioperative intravenous application of a VA-E on granulocyte function. In 98 patients with breast cancer, it was shown that a single intravenous application of the standardized VA-E "Iscador® M special" in a final concentration of 1 mg/individual prior to surgery prevented the surgery-associated inhibition of the oxidative burst. As no VA-E-related side-effects were observed, this distinct route of application may be a rationale to restrict immunosuppression by surgical stress and anaesthesia.*

It is well known that both surgical stress and anaesthetics are able to suppress the immune system (1-11). However, in cancer patients anaesthesia- and surgical stress-induced immunosuppression may accelerate the growth and metastasis of residual cancer cell and, thus, it is desirable to avoid or diminish these deleterious side-effects on the immune system (8).

Previous work of our group has shown that the viscotoxins, a group of 5 kDa basic polypeptides from *Viscum album* L., which possess especially necrotic but also apoptosis-inducing effects on lymphocytes due to their membrane permeabilising properties (overview in 12-15), enhance *Escherichia coli*-(*E. coli*-) induced phagocytosis and oxidative burst of human granulocytes *in vitro* (16, 17). Moreover, *Viscum album* L. extracts (VA-E) as infusions (or instillations) can increase the number and function of phagocytic cells (18-20).

As surgery particularly affects the granulocyte function, we investigated the influence of an intravenous application of a viscotoxin-containing VA-E during surgery on the functional capacity of human granulocytes. From a pilot study with 10 patients with carcinoma *in situ* of the cervix uteri, there was evidence for a statistically significant improvement of the depression of the oxidative burst and the suppressed proliferative capacity of T lymphocytes during surgery by intravenous VA-E application (Büssing *et al.*, unpublished). We, thus, conducted a Good-Clinical-Practice-guided, prospective bi-centric phase II study enrolling 105 patients with breast cancer and focussed on the oxidative burst of their granulocytes stimulated with *E. coli* or phorbol-12-myristat-13-acetate (PMA). Here, we report that the intravenous application of VA-E (*Iscador*® M special; 1 mg) significantly prevented the depression of granulocyte function in breast cancer patients.

Patients and Methods

Patients. One hundred and five patients with breast cancer were enrolled in this bi-centric controlled, open label, prospective, phase II study: 52 patients in the control centre (CC, Marienhospital Witten, Germany) and 53 patients in the application centre receiving a perioperative infusion of a VA-E (AC, Communal Hospital, Herdecke, Germany) in addition to standard anaesthesia. The inclusion criteria were: women with breast cancer and planned surgical intervention, adequate blood counts (*i.e.* leukocytes ≥3,000/μl, thrombocytes ≥100,000/μl), adequate kidney and liver function, Karnofsky's Index ≥70%, life expectancy >12 weeks, and informed consent for data collection according to the requirements

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Table I. *Characteristics of the intention-to-treat patients.*

	Control group Witten	VA-E group Herdecke
age (years)	56.7±11.5	57.4±11.8
weight (kg)	71.1±15.1	69.5±13.8
smoker (n=x)	11	7
former smoker (n=x)	1	5
oral contraceptives (n=x)	47	49
nullipara (n=x)	6	6
Karnofsky's Index 90-100% (n=x)	52	49
blood counts and serum proteins (n=x) within normal range	52	49
duration of surgery < 1 h (n=x)	14	15
duration of surgery 1-3 h (n=x)	38	38

of the Ethics Committee of the University of Witten/Herdecke, Germany. The exclusion criteria were: women <18 years and >80 years, pregnancy or lactating patients, significant pre-existing medical or psychiatric conditions (including history of heart disease, leukaemia, autoimmune diseases *etc.*), patients in final stages of their disease, persisting toxicity of a prior chemotherapy or radiation at the onset of the study, protein allergy (especially against proteins from mistletoe), use of immunomodulating drugs, and participation in another study during the previous 4 weeks.

Among the 105 initial intention-to-treat patients, 4 did not match the inclusion criteria for the 'intention-to-treat' analysis set (1 in CC, 3 in AC). From the remaining patients, 3 dropped out after the third contact (1 in CC, 2 in AC). Although these 101 patients (Table I) were treated as intended, according to the study protocol (measurement of side-effects *etc.*) only 51 in the CC and 47 in the AC can be regarded as per-protocol patients.

Drug. One hour before anaesthesia and surgery, the patients of the application centre received a mistletoe lectin-standardized infusion with VA-E "*Iscador*® M special" (1 mg) along with sodium chloride or Ringer solution (given within 30 to 60 min). The 5-mg ampoules of "*Iscador*® M special" were provided by Weleda Heilmittel, Schwäbisch Gmünd, Germany. The mistletoe lectin (ML) content of the drug was 210-290 ng/ml (ML II reference).

Methods. The oxidative burst of granulocytes of the patients was measured by flow cytometry (EPICS XL-MCL, Coulter, Krefeld, Germany) prior to VA-E application, and on days 1 and 3 after surgery using the Phagoburst-kit from Orpegen (Heidelberg, Germany). Whole blood cells (50 µl) were stimulated for 10 min with $1x10^7$ *E. coli* or phorbol-12-myristat-13-acetate (PMA, 1.35 µM), as described (16,17). Burst activity resulted in the the oxidation of the non-fluorescent dihydrorhodamine 123 (Orpegen) to the fluorescent rhodamine 123 (R123, x-axis of Figure 1). The cells were counterstained with the DNA intercalating dye propidium iodide (y-axis of Figure 1). Lymphocytes, monocytes and cell debris were excluded by adequate gating and, thus, only granulocytes were in the relevant gate. Serum cortisol and C-reactive protein were measured by nephelometry.

Statistics. The primary aim of the study was the analysis of the influence of a single VA-E infusion on the oxidative burst of the granulocytes. Therefore, the *E. coli*- and PMA-stimulated oxidative bursts were measured at discrete time-points over a specified interval, *i.e.* prior to surgery (day 0), and on day 1 and day 3 after surgery. The data represent the cell function as evidenced by an increase or decrease of the oxidative burst. We used the Trapezoidal Rule as a numerical approximation of the integral of the burst curve, whereby the area between the first and last measurement is divided into segments and the area of each segment is calculated and summed up to obtain the total 'area under curve' (AUC). This AUC is a statistical means to summarize information from a series of burst measurements on one individual.

The null hypothesis (H_0) that a single intravenous application of a VA-E has no influence on the oxidative burst was tested with Wilcoxon's signed rank test (SAS 8.2 for Windows), both for the intention-to-treat (ITT) and the per-protocol (PP) set with an alpha error (which is the probability of rejecting a true null hypothesis) of 0.05 (two-tailed).

To account for suggested differences between both non-randomised groups, comparisons between groups were performed by a matched-pair analysis (21) on the basis of Propensity Scores (22, 23) with the following variables: age, Karnofsky's Index, smoking habit, oral contraceptives, nullipara, baseline oxidative burst, clinically relevant serum parameter and blood cell counts, and duration of the given surgical intervention. Descriptive analysis of the baseline characteristics revealed no significant differences between the CC and the AC, either in the ITT set (Table I) or in the PP set (not shown).

Wilcoxon's signed rank test was used for all other analyses either within (change from baseline) or between (CC *vs.* AC) treatment groups.

Results

Granulocyte function. To analyse the burst activity of the 98 per-protocol patients as a function of time, the AUC of the patients from CC and AC was measured. The median AUC (PMA stimulus) of CC was –541.00 and –46.75 in the AC. This difference was significant (median difference 389.00, interquartile range 544.00, $p<0.0001$). Also the *E. coli*-stimulated burst was significantly different: median AUC –110.25 in the CC and median –24.75 in the AC (median difference 85.00, interquartile range 104.00, $p<0.001$).

Subsequent detailed analyses revealed that, after surgery, the amount of granulocytes with burst activity decreased slightly but significantly in the 101 treatment patients, however, it was more pronounced in the CC (Table II). The strongest effect was observed after PMA stimulation.

On the single cell level, it became evident that the capacity to induce the oxidative burst in response to PMA was significantly affected in the CC, but not in the AC (Table III). Also the *E. coli*-stimulated burst activity decreased in both groups, but it was significantly more pronounced in the CC.

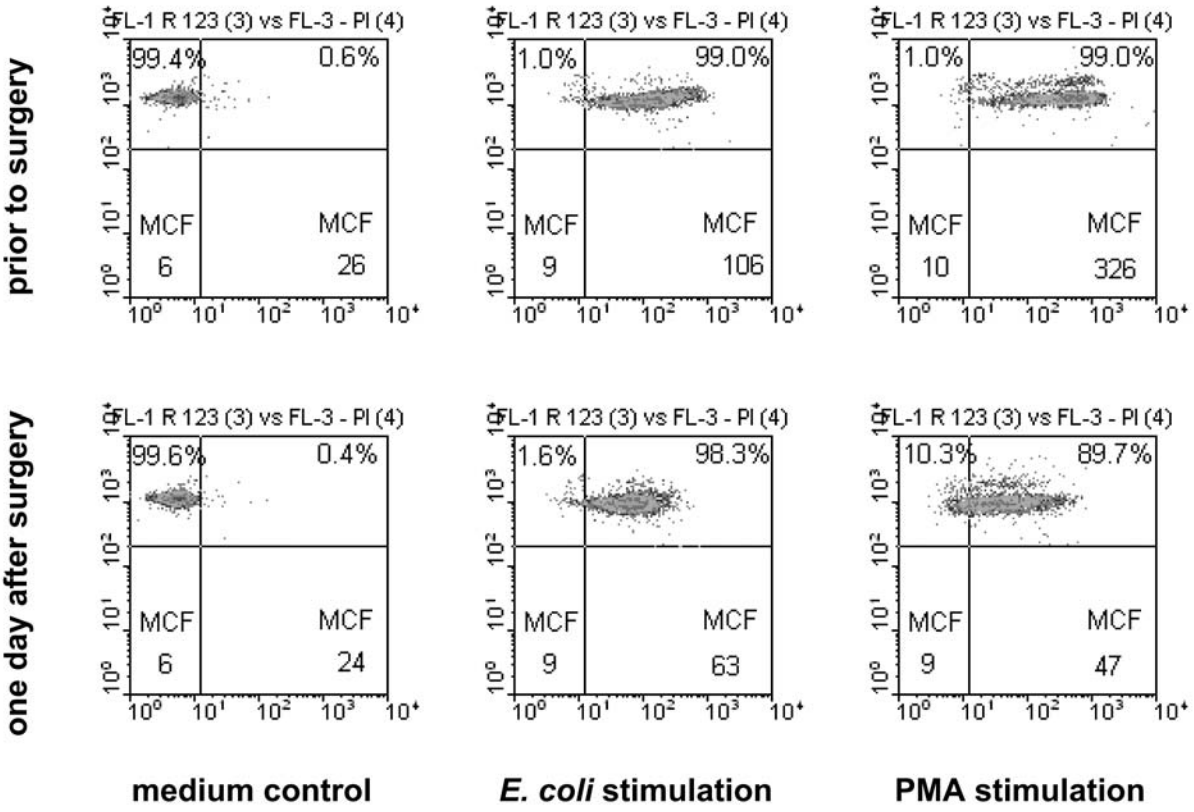

Figure 1. *Representative flow cytometric analysis of the oxidative burst prior to surgery and one day later. Given is the amount of granulocytes with burst activity (% of granulocytes with R123 fluorescence), and the mean channel of R123 fluorescence (MCF, x-axis) which represents the amount of R123 per single cell. Cells with burst activity are found in the upper right quadrant.*

White blood cell counts. Neither group of the 101 patients differed in their baseline leukocyte numbers (x1,000/μl) 6.88±1.63 *vs.* 6.87±1.98 (CC *versus* AC) prior to surgery. After surgery their values increased: 9.09±1.79 *vs.* 8.03±2.02 on day 1, and 7.02±1.88 *vs.* 6.90±1.97 on day 3. However, the difference between both groups at day 1 was not statistically significant.

In contrast, the lymphocyte number slightly decreased in the CC and remained stable in the AC: 1.97±0.64 *vs.* 1.95±0.53 (x1,000/μl; CC *versus* AC) prior to surgery, 1.76±1.79 *vs.* 1.96±0.81 on day 1, and 1.85±0.62 *vs.* 1.87±0.64 on day 3. Again, the difference between both groups was not significant.

Serum proteins. The C-reactive protein (μg/l) in the patients serum increased within the observation period: 1.01±0.96 *vs.* 0.91±0.39 (CC *versus* AC) prior to surgery, 2.38±1.87 *vs.* 2.16±1.75 on day 1, and 2.96±3.26 *vs.* 1.71±1.50 on day 3. This difference between both groups was statistically not significant.

With the exception of a higher primary serum cortisol (μg/l) level in the CC, no significant differences were observed during the observation period: 210.3±62.8 *vs.* 141.3±54.3 on day 0, 172.9±68.1 *vs.* 141.2±59.3 on day 1, and 179.3±62.1 *vs.* 155.9±51.1 on day 3.

Discussion

The aim of our study was to analyse the influence of a single perioperative intraveneous application of a mistletoe extract on granulocyte activity. The burst activity of granulocytes from the VA-E-treated patients was significantly less suppressed due to the surgical intervention during the observation period than that of the control patients. This effect cannot be explained by differences in granulocyte number, as surgery increased the number of white blood cells in both groups. Moreover, the C-reactive protein, the main relevant lymphocyte subsets (*i.e.* T cells, B cells, natural killer cells, CD4+ T helper/inducer cells, CD8+ CD28– suppressor cells and CD8+ CD28+ cytotoxic cells,

Table II. *Demonstration of the burst activity of granulocytes (%) stimulated with E. coli or PMA.*

	day 0	day 1	day 3	
Stimulus: *E. coli*				*p*-value
Control group	97.6±2.1	94.3±5.9	96.5±5.3	day 0 *vs.* day 1: <0.0001
VA-E group	96.3±4.7	95.2±6.0	96.3±5.8	day 0 *vs.* day 1: 0.039
	CC *vs.* AC:	CC *vs.* AC:	CC *vs.* AC:	
p-value	n.s.	n.s.	n.s.	
Stimulus: PMA				*p*-value
Control group	98.3±6.6	91.8±10.2	96.8±6.1	day 0 *vs.* day 1: <0.0001
VA-E group	96.6±11.1	98.4±2.9	96.9±12.8	day 0 *vs.* day 1: n.s
	CC *vs.* AC:	CC *vs.* AC:	CC *vs.* AC:	
p-value	n.s.	<0.0001	0.004	

Mean values±standard deviations of 101 treatment patients; Wilcoxon's signed rank test

Table III. *Demonstration of the burst activity of granulocytes stimulated with E. coli or PMA on the single cell level (mean channel of R123 fluorescence).*

	day 0	day 1	day 3	
Stimulus: *E. coli*				*p*-value
Control group	133.9±66.5	61.1±22.5	98.4±41.8	day 0 *vs.* day 1: <0.0001
VA-E group	125.1±61.3	100.1±48.2	117.3±55.6	day 0 *vs.* day 1: <0.0001
	CC *vs.* AC:	CC *vs.* AC:	CC *vs.* AC:	
p-value	n.s.	<0.0001	0.029	
Stimulus: PMA				*p*-value
Control group	367.8±159.4	89.2±87.4	243.8±162.5	day 0 *vs.* day 1: <0.0001
VA-E group	330.7±165.8	296.8±183.0	307.7±181.5	day 0 *vs.* day 1: n.s
	CC *vs.* AC:	CC *vs.* AC:	CC *vs.* AC:	
p-value	n.s.	<0.0001	0.035	

Mean values±standard deviations of 101 treatment patients; Wilcoxon's signed rank test

CD25+ T cells, and the cytokines interleukin 6 and RANTES), as measured in the supernatants of *ex vivo* cultured and stimulated whole blood cells, did not differ between both groups (data not shown), and thus cannot be attributed to the observed immunomodulating effects.

An overproduction of cortisol in response to surgical stress may play an important role in the development of immunosuppression (8). However, the cortisol levels did not differ on the relevant days 1 and 3. Interestingly, it was somewhat higher in the CC group prior to surgery, although the burst activity did not differ significantly between both groups.

Our results indicate that a single intravenous application of 1 mg "*Iscador*® M special" prior to surgery can restrict suppression of the oxidative burst by surgical stress, although our study was a non-randomised study. This suggestion of a prevention of suppression of immunologically relevant cellular function due to surgical stress by the application of a mistletoe extract is further supported by the results of a monocentric randomised study by Schink *et al.* (manuscript in preparation). They observed a significantly improved function of natural killer cells in patients with colorectal tumours who received 5 mg "*Iscador*® M special" intravenously prior to the surgical intervention.

Interestingly, cytotoxic effects were not observed even when high doses of VA-E were applied intravenously (20, 24), although they exert a strong apoptosis-inducing potential *in vitro* especially *via* the mistletoe lectins (25-28).

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