

The immune response is affected for at least three weeks after extensive surgery for ovarian cancer

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ABSTRACT

INTRODUCTION: The treatment of women with ovarian cancer in advanced stages consists of extensive surgery followed by chemotherapy initiated three weeks after surgery. In this study, selected immune parameters were investigated to elucidate when the immune system is normalised following the operation.

METHODS: Ten women undergoing extensive surgery for ovarian cancer were compared with a control group of ten women undergoing abdominal hysterectomy for a benign diagnosis. Blood samples were collected over a period of 21 days post-operatively. The levels of interleukin-6, interleukin-8, interleukin-10 and the activity and total frequency of natural killer cells were measured.

RESULTS: Interleukin-6 and interleukin-10 were significantly elevated immediately after the operation and also after 21 days. The total population of natural killer cells and the total activity were reduced. The total activity of natural killer cells did not normalise within 21 days.

CONCLUSIONS: The level of the cytokines interleukin-6 and interleukin-10 is increased 21 days after the operation, and the function of natural killer cells is not normalised at 21 days after surgery.

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The survival of women with ovarian cancer (OC) in advanced stages (stages IIIa-c and IV, according to The International Federation of Gynaecology and Obstetrics) has improved considerably over the past ten years. The treatment has changed from either traditional surgery or chemotherapy to a combination of both treatment options offering the affected women extensive surgery followed by chemotherapy [1-3]. Extensive surgery includes hysterectomy and bilateral salpingo-oophorectomy, peritonectomy, resection of parts of the colon or the small intestine and splenectomy in cases with macroscopic metastasis visible on the spleen. All para-aortic and pelvic lymph nodes are removed if metastases are suspected.

In Denmark, the treatment protocol dictates that chemotherapy should be initiated three weeks post-operatively (PO) unless the white blood cell count (WBC) is reduced.

Two theories have been proposed in relation to the effect of surgery followed by chemotherapy. The first theory is that resection diminishes the amount of tumour cells which enhances the cytotoxicity of chemotherapy in relation to the residual cancer. The second theory is that the tumour cells induce immunosuppression and thus enhance a vicious circle with tumour growth suppressing the remaining immune system. Extensive surgery reduces the tumour burden and thus slows this vicious circle [4]. The effect observed after extensive surgery is probably a combination of both.

In order to elucidate the changes in the immune system in women undergoing extensive surgery, we analysed their immune system and compared changes with changes encountered in women undergoing abdominal hysterectomy due to benign disease.

The following immune parameters of special relevance to the innate immune system were chosen to describe the immune profile: Interleukin (IL) 6, IL-8, IL-10 and natural killer (NK) cells.

IL-6 is a pleiotropic cytokine that acts in a pro-inflammatory way by attracting neutrophils to the area where damage has occurred. Moreover, it promotes the upregulation of adhesion molecules and L-selectin. IL-6 also stimulates the hepatic acute-phase protein synthesis with release of C-reactive protein and procalcitonin. Furthermore, IL-6 stimulates the production of prostaglandin E2 which, in turn, stimulates the secretion of IL-10 production. In addition, IL-6 has its own direct anti-inflammatory properties. The secretion of IL-6 correlates with the magnitude of the trauma and the duration of surgery, and elevated levels of IL-6 increase the risk of post-operative complications. Elevated IL-6 serum levels in patients with OC are correlated with shorter relapse-free periods and overall poor survival [5].

IL-8 plays an important role in inflammation as it recruits T-cells and accelerates wound healing. In several studies, the presence of IL-8 in women with OC has been correlated with a poor outcome [6]. IL-8 is produced by monocytes, neutrophils and epithelial cells, but also by the tumour cells.

IL-10 is responsible for T helper cell proliferation. IL-10 also hampers the maturation of dendritic cells and hinders the function of co-stimulatory molecules.

ORIGINAL ARTICLE

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TABLE 1

Summary of study details.

Type of operation	Extensive ovarian cancer surgery	Abdominal hysterectomy
Disease	Ovarian cancer FIGO stage III-IV which was estimated operable The judgement was made on the background of a magnetic resonance and computed tomography	Benign disease, only women scheduled for abdominal hysterectomy on benign basis, e.g. fibroids or benign metrorrhagia
Mode of anaesthesia	General anaesthesia with epidural	General anaesthesia without epidural
Recovery	Intensive care unit	Recovery room
Blood samples, day no.	0, 1, 2, 8, 21	0, 1, 2, 8, 21
Chemotherapy start, day no.	All on day 20-22	–
ASA classification	1-2	1-2
Exclusion criteria	Immunological disease, e.g. rheumatoid arthritis, systemic lupus erythematosus Patients already diagnosed with cancer	Immunological disease, e.g. rheumatoid arthritis, systemic lupus erythematosus Patients already diagnosed with cancer
Age, yrs	> 18	> 18
Analysed immune markers	IL-6, IL-8, IL-10 NK cells	IL-6, IL-8, IL-10 NK cells
Patients, n	10	10
30-day mortality, n	0	0

ASA = American Society of Anesthesiologists; FIGO = International Federation of Gynecology and Obstetrics; IL = interleukin; NK = natural killer.

Previous investigations have shown that women with OC have higher levels of IL-10, and IL-10 has been suggested as a good surrogate marker for tumour grading [7].

NK cells comprise a subtype of lymphocytes that are important mediators of the innate immunity and have the ability to secrete cytokines and interferon gamma (IFN- γ), among others, and chemokines, and to kill infected or transformed cells, i.e. cancer cells [8, 9].

NK cells are believed to play a crucial role in the defence against cancer.

Our hypothesis was that in women with OC stage III-IV who have been exposed to extensive surgery, the immune system does not return to preoperative status within 21 days.

METHODS

The study was carried out in Odense University Hospital, the Region of Southern Denmark. The Regional Scientific Ethical Committees for Southern Denmark approved the protocol (No. S-20080076).

Interleukin 6, interleukin 8 and interleukin 10

Plasma and serum samples were centrifuged, separated and frozen at -80°C .

The samples were analysed using Human IL-6 ELISA BD, Human IL-8 ELISA BD, and Human IL-10 ELISA BD (all Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's instructions.

Natural killer cells

NK cells cannot be separated directly from whole blood. However, as mononuclear cells (MNC) also contain NK cells, we separated MNC from whole blood as described by Boyum [10]. Whole blood was centrifuged with Ficoll-Paque (GE Healthcare Life Sciences, Buckinghamshire, UK), a density medium, in order to isolate MNC. The isolated MNC were then cryopreserved at -135°C .

The activity of NK cells within the MNC cell pool was analysed using a ^{51}Cr -release assay [11]. The following procedure was performed when all blood samples were collected.

Briefly, the theory is that when exposed to harmful cells, NK cells will destroy them. In this assay, K562, a population of chronic myeloid blast cell, was labelled with ^{51}Cr . The K562 cells were labelled passively by diffusion of ^{51}Cr across the cellular membrane. The suspension with the labelled K 562 was then subjected to the rapidly thawed MNC, which contains NK cells. MNC and K562 cells were now incubated in order for the cytotoxicity reaction to take place. If the NK cells were active, they would kill the K562 cells during the incubation time. This attack would destroy the cell wall and provoke the release of the radioactive ^{51}Cr . The magnitude of radioactivity in the supernatant of the assay was then measured.

The activity of the NK cells was calculated as:

$$\text{Activity of NK cells (\%)} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

In order to quantify the NK cells, the remaining frozen MNC were thawed and analysed by flow cytometry using the directly conjugated MoAbs: anti-CD19 to identify B lymphocytes, anti-CD3 for T lymphocytes and anti-CD56 to identify NK cells. A blocking buffer was added. CD19 is a surface marker for B-cells. CD56 is surface marker for NK cells and some malignant cell lines; CD3 is the surface marker for T-cells.

The samples were analysed in a flow cytometer (Becton Dickinson FACS, Calibur, USA) at wavelength 488 nm or 635 nm lasers.

Forward and side scatter was performed.

Graph Pad Prism (La Jolla, CA, USA) was used for statistical analyses. In order to test differences between groups, the Mann-Whitney test was applied. For comparisons within the group, one-way variance analysis was performed. Differences were considered significant when p-values were below 0.05.

A summary of study details is shown in **Table 1**.

Trial registration: not relevant.

RESULTS

A total of 20 women were included in the study. Three patients were excluded in the OC group; one patient turned out to be inoperable, while two patients were incorrectly diagnosed initially (one had benign cysts on the ovaries, and one patient was diagnosed with a signet cell cancer). In the abdominal hysterectomy (H) group, seven patients completed the study, as three patients in the H group did not wish to have the last blood samples taken. The groups were comparable with regard to age, body mass index (BMI), coexisting diseases and total WBC.

The blood sampling was carried out from November 2008 to May 2010.

By 1 January 2013, only one patient from the OC group had died. The follow-up period thus spanned from two years and five months to five years and one month.

Interleukin 6, 8, 10

We found a significant difference between the OC group and the H group, the OC group having the highest levels of both IL-6 and 10 at day 1 and day 21 PO (Figure 1A and Figure 1C).

Moreover, IL-8 levels in the OC group significantly increased during the observation period. Although the levels of IL-8 were generally higher in the OC group, no significant difference was found between the groups (Figure 1B).

Natural killer cell frequency and activity

Preoperatively, no difference in either NK cell activity or total number of NK cells was found between groups H and OC. Within the H group, the operation did not impact this finding as no difference in the total number or in the total activity of NK cells occurred in the observation period. In the OC group, however, the activity of NK cells did not change, but as both the total lymphocyte count was diminished and the fraction of NK cells in the lymphocyte pool was reduced at PO day 21, the total NK cell activity was reduced in this group at PO day 21 (Figure 2A-D).

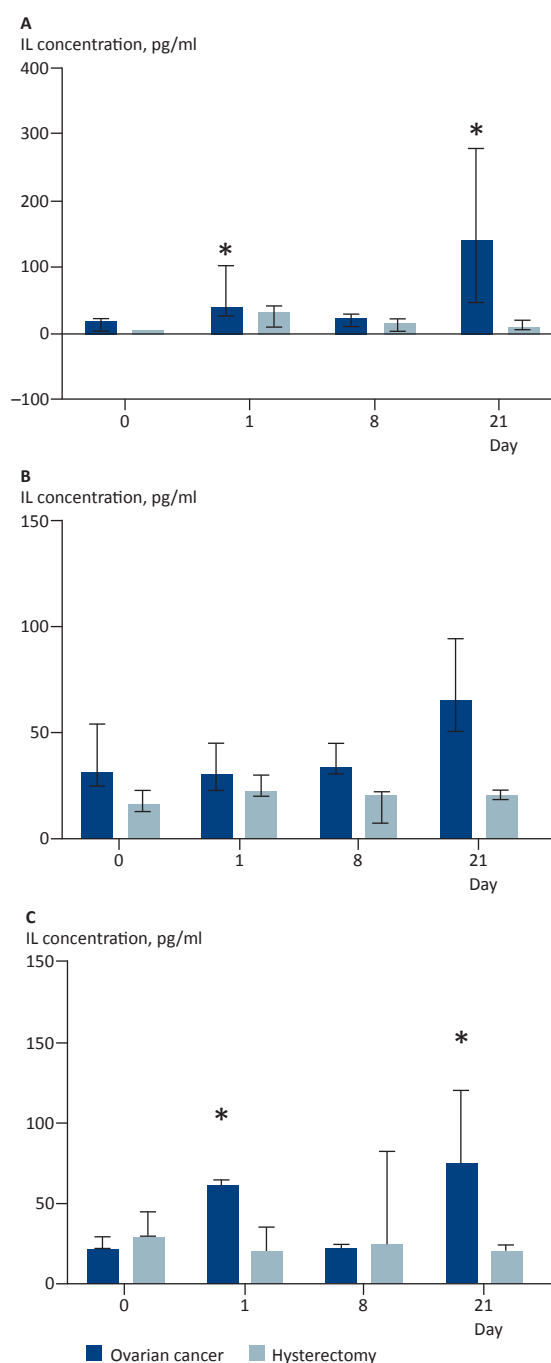
DISCUSSION

Previous studies of IL-6 levels in women with OC have been performed in women with OC stages FIGO I-II [5] which are less advanced stages than the ones included in our study and thus their results are not directly comparable with those of our population (FIGO stages III-IV). In more recent studies, ascites of women with OC was analysed in order to describe the levels of IL-6 and other cytokines [12, 13]. The biology of IL-6 in ascites is different than in blood as the ascites is not exposed to the innate immune system, and therefore tends to have higher levels of cytokines.

The conclusion in all these studies is that there is a

FIGURE 1

Interleukin (IL)-6 (A), IL-8 (B) and IL-10 (C) concentrations in blood on post-operative days 0, 1, 8 and 21. Median values and 95% confidence intervals.



*) Significant difference between the two days ($p < 0.05$).

correlation between elevated levels of IL-6 and the patients' outcome. In our study, we found significant differences in the levels of IL-6 between the OC group and the control group – with especially high levels of IL-6 in

the OC group on the 21st post-operative day. The high levels at day 1 may, in part, be explained by the recent surgery.

We interpret the elevated IL-6 levels as a possible combination of regrowth of the microscopic residual ovarian tumour cells in numbers that are sufficient to arouse the innate immune system, production of IL-6 from residual ovarian tumour cells [14] that now have reached a number that can produce IL-6 in measurable amounts, and finally a cessation of the temporary post-operative immune paralysis that always occurs after surgery.

We have not been able to confirm the association found by other researchers linking high IL-8 levels with a poor outcome in women with OC [6, 15]. However, our study differs from other studies as we only examined women in stage III-IV, and the OC patients in our study underwent surgery.

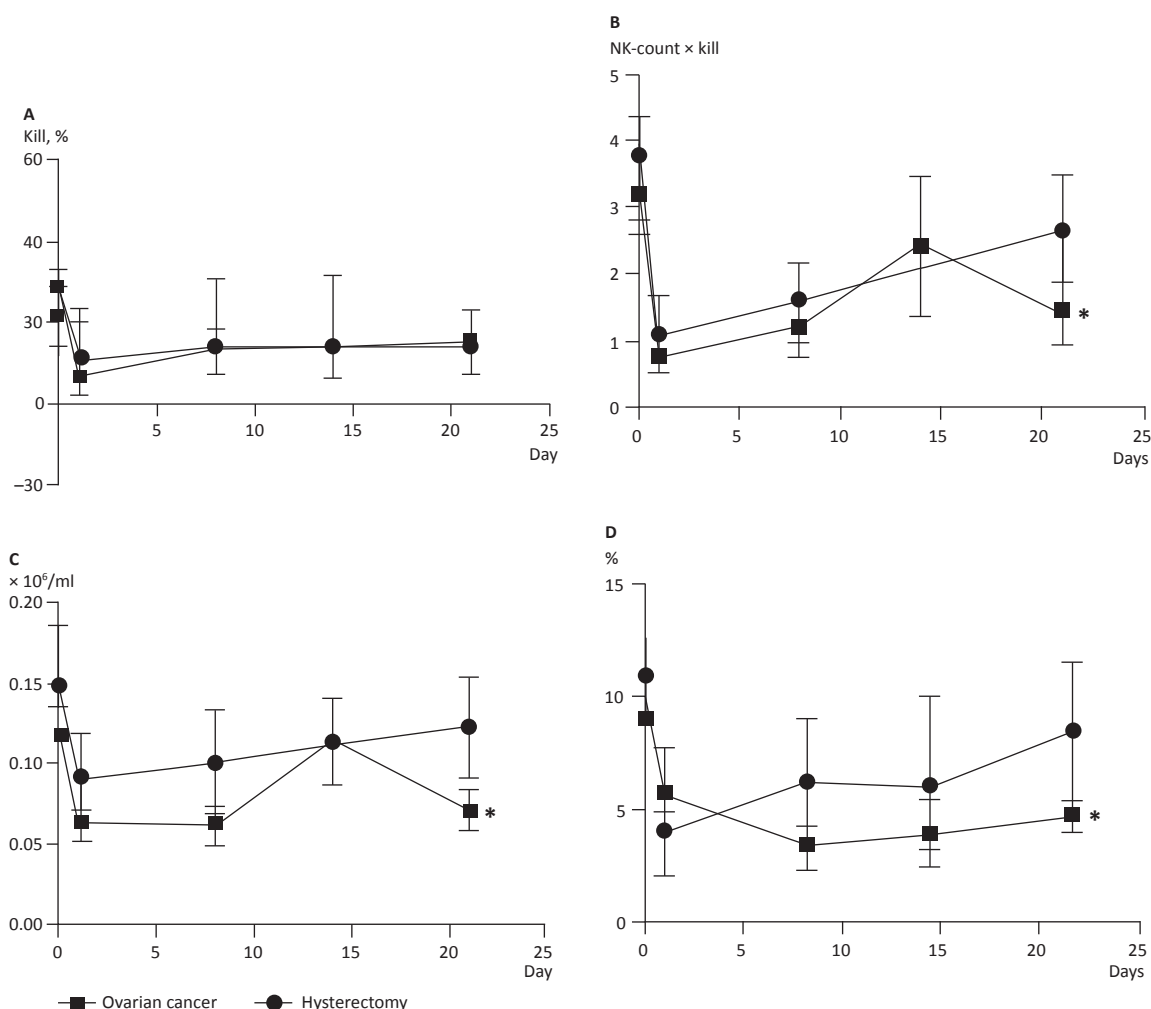
In relation to IL-10, it is interesting to see how the levels are normalised at day 8 PO, and then escalate rapidly at 21 PO in the OC group. The normalisation of IL-10 levels at day 8 PO corresponds with the results found by Mustea et al [16].

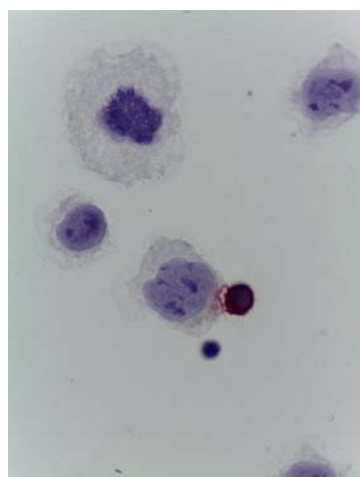
We were unable to investigate any correlation between the post-operative tumour load and the levels of IL-10 as all our patients were considered to have been radically operated (all macroscopic tumour tissue removed).

We speculate that on post-operative day 21, the amount of microscopically residual tumour tissue has reached a level where the tumour cells may again begin to produce IL-10. This is backed by findings reported by Rabinovich et al that OC cells with a high degree of certainty produce IL-10 [17]. Another explanation may be that the measured IL-10 is an indicator that the innate immune system is aroused again, corresponding to the ris-

FIGURE 2

Natural killer (NK) cell frequency and activity on post-operative days 0, 1, 8 and 21: activity (A), total activity (B), total concentration of NK cells (C), % NK cells of lymphocytes (D). Median values and 95% confidence intervals. *) Significant difference ($p < 0.05$).





A natural killer cell (stained with anti-human CD56) and three K562 cells with haematoxylin as contrast staining.

ing levels of IL-6, as IL-6 induces the production of IL-10.

In our study, the activity and the total number of NK cells did not increase from day 8 to day 21. Uchida et al [18] reported that the activity of NK cells was normalised three days after abdominal surgery for non-malignant diseases, while the activity of NK cells was reduced for up to two weeks following breast cancer surgery. With a three-week-long suppression of the NK cell activity, our results are thus not unexpected.

Limitations

The limitation of our study is the small sample size. The processing of the immunological samples, however, was complex, expensive and time-consuming which rendered it impossible to study the immune status in a larger population. In addition, we are aware of the fact that not all immunological aspects have been taken in to account; specifically, we have not measured IFN- γ . IFN- γ can give an indication of the activity and amount of NK cells. This was a deliberate choice as IFN- γ has a short half-life, which makes it very difficult to obtain the perfect time to collect blood samples and, if the timing is not correct, it becomes difficult to compare circulating levels of this cytokine.

CONCLUSIONS

The existing policy concerning initiation chemotherapy following extensive surgery for OC in stage III-IV is to commence chemotherapy on the 21st post-operative day. The reason for this delay has been a belief that a patient's immune system has returned to its preoperative status after a 21-day interval. Our study shows that this is not the case; and thus there is no basis for delaying chemotherapy. The women subjected to extensive surgery for OC demonstrated elevated levels of both IL-6 and IL-10 at 21 post-operative days. This may suggest that the inflammatory response is still in progress or

that regrowth of OC cells has taken place. As the total activity of NK cells is reduced at 21 days PO, this potentially leaves these patients without sufficient ability to combat cancer cells. As a recent study by Lydixsen et al [19] showed that initiation of chemotherapy after day 32 following OC surgery implicated a poorer five-year survival, an intuitive approach would be to commence chemotherapy earlier.

Our results, which do not support delaying chemotherapy, may open the possibility of initiating chemotherapy even before day 21 as the innate immune system is back to its preoperative standard at that time anyhow.

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