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Clinical relevance of regulatory T cells monitoring in the peripheral blood of ovarian cancer patients

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ABSTRACT

Background: Tregs play a suppressive role in the control of antitumour immunity. In this study we evaluated the relevance of prospective monitoring of peripheral blood regulatory T cells (Tregs) as a potential prognostic marker of future outcome of epithelial ovarian cancer in patients with or without a metronomic chemotherapy.
Methods: 46 patients diagnosed with the ovarian cancer were enrolled in the study and divided into groups according to the stage of the disease, outcome of the surgery and treatment received. Proportions of Tregs in the peripheral blood samples were evaluated using flow cytometry.
Results: We show that the early stage of the disease and absence of the tumor residuum after radical surgery are the most important factors predicting a favourable clinical outcome in the ovarian cancer. We did not show any significant effect of consolidation chemotherapy with metronomic doses of etoposide or cyclophosphamide on the peripheral blood Tregs and on the clinical outcome. The slope of the Tregs trend line was a significant predictor of an early relapse, even after controlling for stage and tumor residuum after the surgical debulking by using the Cox proportional hazard model.
Conclusions: This study shows that the faster kinetics of Tregs increase in the peripheral blood, expressed as the slope of the Tregs trend line, is a significant predictor of ovarian cancer early relapse hazard. However, due to its relatively low specificity, the informative value of regular monitoring of Tregs kinetics in the peripheral blood for the subsequent clinical outcome is limited.

1. Introduction

Ovarian cancer is associated with the highest cancer-related mortality among gynecological cancers and is one of the ten most common malignancies in females worldwide (American Cancer Society, 2011) [1]. The 5 year survival rate irrespective of the stage is around 40%. The mortality remains high, despite the efforts for early diagnosis and modern therapeutic protocols. One of the reasons of these unsatisfactory results are the late diagnoses. 75–80% of ovarian carcinomas are presently found in the later stages – namely the third and forth stages (FIGO classification).

Standard treatment employs the use of radical surgery and subsequent combined chemotherapy with platinum agents and taxanes. Today in the late stages (III. a IV.) more than 70% of patients achieve complete clinical remission; however within 2 years more than 50% of women from this group suffer from relapses. In some series of malignant tumours multiple peripheral regulatory CD4+CD25+FoxP3+T lymphocytes (Tregs) were found. Tregs play a suppressive role in the control of antitumour immunity.

In some tumours the percentage of Tregs cells in peripheral blood were found to correlate with the patients' prognosis [2,3]. Monitoring the amount circulating CD4+CD25+FoxP3+ T regulation lymphocytes in the blood might be a promising prognostic marker of disease development.

Presently it is still standard procedure to use 6–8 cycles of chemotherapy (as found for platinic derivation and paclitaxel). In adjuvant chemotherapy adding a third cytostatic agent did not improve treatment, as oppose to the advantages of intraperitoneal chemotherapy over intravenous. The new experimental procedure in consolidatory treatment dictates weekly extended usage of low paclitaxel dosages or peroral low etoposide dosages. Low (metronomic) dosages don't have a direct cytotoxic effect on

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chemoresistant tumour cells but from many experimental animal studies cyclophosphamide was found to have a positive effect on this low doses treatment in stopping growing tumours and their regression – eventually removing disease relapse. The results from the studies are that peroral usage of low cyclophosphamide doses in the patients with advanced tumour caused deep and selective reduction of circulating regulatory CD4+CD25+ T cells [4–6].

The aim of this study was to evaluate the relevance of regular Tregs monitoring for the clinical management of ovarian cancer. Evaluate the clinical relevance of peripheral blood Tregs as a prognostic marker of future disease outcome. We also wanted to compare the effect of metronomic cyclophosphamide and etoposide during consolidation therapy on peripheral blood Tregs numbers, Ca125 and progression-free survival (PFS).

2. Material and methods

2.1. Patients characteristics

46 patients diagnosed with the ovarian cancer were enrolled in the study and divided into groups according to the stage of the disease, outcome of the surgery and treatment received: 7 patients with early stage ovarian cancer (Controls III), 9 patients at stage IIIC with zero residuum after the radical surgery (Controls II), 11 patients at stage IIIC with residuum after the surgery and no consolidation therapy (Controls I), 11 patients with stage IIIC ovarian cancer with residuum treated by etoposide as consolidation therapy (Etoposide group) and 8 patients with stage IIIC ovarian cancer treated by cyclophosphamide during the consolidation therapy (CFM group). Patients underwent radical surgery followed by 6–8 cycles of the first line chemotherapy (platinum compound in combination with taxanes). Twelve blood samples were collected at specific timepoints and used for the monitoring of Ca125 levels and frequency of Tregs in the peripheral blood. Schedule of the blood sampling and the study design is shown on Fig. 1. None of the patients enrolled in the study had received neoadjuvant chemotherapy prior to the surgery. Blood samples were collected with patient consent, and the study was approved by the Institutional Review Board of the University Hospital Motol (Table 1).

2.2. Identification of regulatory T cells

Regulatory T cells (Tregs) were identified by surface staining with anti-CD3 (Exbio), anti-CD4 (eBioscience), anti-CD8 (Exbio), anti-CD25, anti-CD127 and anti-CCR4 (BioLegend) antibodies followed by fixation and permeabilization with a Foxp3 Staining Buffer Set (eBioscience) and intracellular staining with anti-FoxP3 (eBioscience) antibody. Samples were analyzed on a BD FACSAria (BD Biosciences) using FlowJo software (TreeStar, Ashland, OR). To calculate the slope of the Treg trend line for each patient, the Treg values obtained during each time point were graphed on an *x*, *y* dot plot and a regression line was drawn to fit the values using Statistica® 10.0 software (StatSoft, Tulsa, OK). The slope of the regression line represents the slope of the Treg trend line.

2.3. Statistical analysis

Statistical analyses were performed using Statistica® 10.0 software. The parametric assumptions of the data were verified using the Kolmogorov–Smirnov test for normality. The homogeneity of variances was tested by the Levene test. Correlations between investigated parameters were evaluated using the Pearson *r* coefficient. The remaining data were analyzed using an ANOVA followed by a Scheffé test. Additionally, the Cox proportional hazard model was done to assess the prognostic value of the stage of the disease, presence of tumor residuum after surgical debulking, levels of Ca125 and slope of the Tregs trend line for disease-free survival. The results were considered statistically significant when $p < 0.05$.

3. Results

Progression free survival (PFS) of the five groups of patients showed an excellent prognosis of patients with stage I/II of the disease and very good outcome in stage IIIC patients with no residual tumor after first surgery. There was no difference in the PFS of patients with residual tumor after surgery, irrespective of the consolidation therapy received (Fig. 2). In all three groups of stage IIIC patients with tumor residuum after surgery (no consolidation therapy, cyclophosphamide or etoposide treatment), 70% of patients relapsed within 36 months. Similarly there were no significant differences in the distribution of patients with early or late relapses between these three groups (Fig. 3).

Patients with the late relapse of the ovarian cancer (>12 months after the end of the first line chemotherapy) had significantly higher serum Ca125 levels at B8 timepoint ($p < 0.01$). Patients with the early relapse had significantly higher Ca125 starting from B6 timepoint ($p < 0.01$) (Fig. 4).

Patients with the early relapse had significantly faster kinetics of the rise of Tregs in the peripheral blood, which translated into the higher slope of Tregs trend line (Fig. 5A). The slope of the Tregs trend line was a significant predictor of an early relapse ($p = 0.02$), even after controlling for stage and tumor residuum after the surgical debulking by using the Cox proportional hazard model. The slope of Tregs trend line does not have any predictive value for late relapses. A rising slope of the Tregs trend line was observed in 100% of patients with an early relapse, 15.38% of patients with the late relapse and 31.82% of patients in remission.

Administration of consolidation therapy does not lead to the decrease of Tregs in the peripheral blood.

4. Discussion

Standard treatment of ovarian cancer consists of radical/cytoreductive surgery and subsequent combined chemotherapy with platinum-based agents in combination with taxanes. Despite the high primary response, the majority of stage III and IV patients experience relapse which is ultimately fatal. The three major prognostic factors of ovarian cancer outcome are stage at diagnosis,

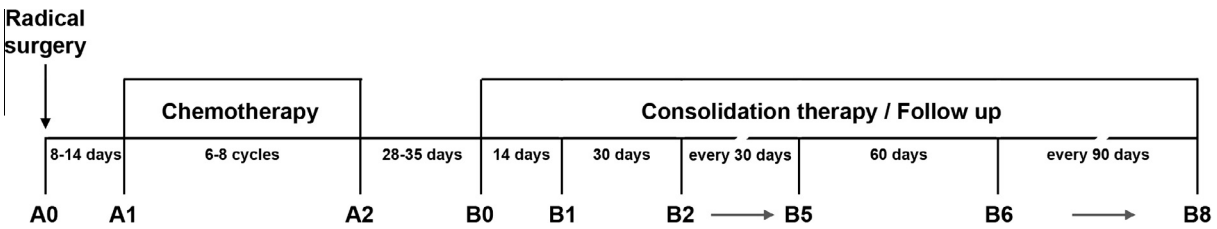

Fig. 1. Schedule of the therapy and blood sampling during the study.