Am J Cancer Res 2012;2(5):566-580
www.ajcr.us /ISSN:2156-6976/ajcr0000136

Original Article

Profiling of cytokines in human epithelial ovarian cancer ascites

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Received July 9, 2012; Accepted August 2, 2012; Epub August 20, 2012; Published September 15, 2012

Abstract: Background: The behavior of tumor cells is influenced by the composition of the surrounding tumor environment. The importance of ascites in ovarian cancer (OC) progression is being increasingly recognized. The characterization of soluble factors in ascites is essential to understand how this environment affects OC progression. The development of cytokine arrays now allows simultaneous measurement of multiple cytokines per ascites using a single array. Methods: We applied a multiplex cytokine array technology that simultaneously measures the level of 120 cytokines in ascites from 10 OC patients. The ascites concentration of a subset (n = 5) of cytokines that was elevated based on the multiplex array was validated by commercially available ELISA. The ascites level of these 5 cytokines was further evaluated by ELISA in a cohort of 38 patients. Kaplan-Meier analysis was used to assess the association of cytokine expression with progression-free survival (PFS) in this cohort. Results: We observed a wide variability of expression between different cytokines and levels of specific cytokines also varied in the 10 malignant ascites tested. Fifty-three (44%) cytokines were not detected in any of the 10 ascites. The level of several factors including, among others, angiogenin, angiopoietin-2, GRO, ICAM-1, IL-6, IL-6R, IL-8, IL-10, leptin, MCP-1, MIF NAP-2, osteprotegerin (OPG), RANTES, TIMP-2 and UPAR were elevated in most malignant ascites. Higher levels of OPG, IL-10 and leptin in OC ascites were associated with shorter PFS. IL-10 was shown to promote the anti-apoptotic activity of malignant ascites whereas OPG did not. Conclusion: Our data demonstrated that there is a complex network of cytokine expression in OC ascites. Characterization of cytokine profiles in malignant ascites may provide information from which to prioritize key functional cytokines and understand the mechanism by which they alter tumor cells behavior. A better understanding of the cytokine network is essential to determine the role of ascites in OC progression.

Keywords: Ascites, ovarian cancer, tumor environment, cytokines, mulitplex array, IL-10

Introduction

Epithelial ovarian cancer (EOC) is the leading cause of death among gynecological cancers [1, 2]. EOC is a highly heterogeneous disease. There are four major types of EOC including serous, mucinous, endometrioid and clear cell [3]. High-grade serous ovarian carcinomas (HGSOC) are the most frequent and the majority of these patients presents with advanced (stage III/IV) diseases [1, 4]. Most women presenting with advanced HGSOC have ascites [5], which in itself constitutes a unique form of tumor environment. Accumulation of ascites is the combined result of lymphatic obstruction, increased vascular permeability and secretion of soluble factors by tumor and activated mesothelial cells. HGSOC tumorigenesis is a complex process involving functional alterations in both tumor cells and their surrounding environment. The importance of the interactions between these two components is being increasingly recognized in the tumorigenic and metastatic processes.

Ascites affects tumor cell behavior such as cell growth, invasion, and survival [6-8]. Specifically, ascites from HGSOC exert a protective environment against drug-induced apoptosis by inducing survival signaling pathways such as PI3K/Akt in tumor cells [6, 7]. Newly diagnosed women with protective ascites against drug-induced apoptosis had significantly shorter progression-free survival (PFS) [9]. These data suggest that knowledge of the tumor environment in which OC develops is critical for understand-

Table 1. Description of the OC ascites used for the multiplex cytokine array.

Ascites	Histopathology	Grade	Stage	Prior chemotherapy
OVC346	Serous	3	IIIC	No
OVC361	Serous	2	IIIC	No
OVC451	Mixed cell	3	IC	No
OVC461	Serous	1	IA	No
OVC472	Mixed cell	3	IIC	No
OVC503	Serous	N/A	IV	No
OVC508	Serous	3	IV	No
OVC509	Serous	2	IV	No
OVC530	Mixed cell	2	IIC	No
OVC552	Serous	3	IIC	No

ing cancer progression. Ascites contain a variety of soluble factors, including cytokines, which taken individually affect cancer progression through different mechanisms [10-18]. These cytokines are produced by both tumor cells and peritoneal mesothelial cells, and concentrate in ascites [19, 20]. Ascites also act as an immune suppressive environment for EOC cells. Cytokines such as interleukin (IL)-6 and IL-10 enhance the immunosuppressive status of the tumor environment by inducing, for example, B7-H4 expression on tumor-associated macrophages (TAM) and by promoting apoptosis in these cells [21]. Cytokines were also reported to promote ovarian tumor growth *in vivo*. For example, IL-6 contributes to EOC progression by inhibition of apoptosis, stimulation of angiogenesis, increased migration and invasion, and stimulation of cell proliferation [22-26]. Furthermore, elevated IL-6 levels in EOC ascites correlated with shorter progression-free survival [10]. Taken together, these data suggest that the biological effects of malignant ascites are influenced by the composition and concentration of cytokines present. Recent studies have provided preliminary data on the cytokine composition of ascites [10, 16, 17, 27]. Although these studies have offered useful insights, issues such as inclusion of malignancies other than OC and the limited number of cytokines measured are shortcomings. Given the complexity of ascites with regards to the cytokine network, it is likely that many more cytokines may be present in ascites. Therefore, the analysis of cytokine profiles in EOC ascites may provide an improved understanding of the complex interactions between tumor cells and the tumor environment as well as a valuable tool for the ongoing search for biomarkers of EOC.

In the present study, a multiplex cytokine array, which detects simultaneously 120 different cytokines, was used to profile 10 OC ascites. The aim of the study was to gain a better understanding of the cytokine network present in ascites in order to understand the impact of the tumor environment on OC progression. Our data demonstrate that out of 120 cytokines tested, close to 60% are detectable in OC ascites with a wide variability of expression between different cytokines and between ascites. Moreover, we correlated higher ascites levels of IL-10, osteoprotegerin (OPG) and leptin with shorter PFS and demonstrated that IL-10 neutralization in ascites inhibits the survival-promoting activity of ascites against TRAIL-induced apoptosis.

Material and methods

Patients

The study population consisted of patients with newly diagnosed epithelial ovarian cancer admitted at the Centre Hospitalier Universitaire de Sherbrooke. This study was approved by the Institutional Review Board of the Centre de Recherche Étienne-LeBel. Informed consent was obtained from women that underwent surgery by the gynecologic oncology service between 2000 and 2010. All samples were reviewed by an experienced pathologist. Ascites were collected at the time of the initial cytoreductive surgery. Baseline characteristics and serum CA125 levels were collected for all patients. All patients had a follow up > 2 years. Disease progression was defined by either serum CA125 ≥ 2 X nadir value on two occasions, documentation of lesion progression or appearance of new lesions on CT-scan or death [28]. Patient's con-

Table 2. Clinical and pathological characteristics of the 38 patients with OC.

Characteristic	Patients (N = 38) No (%)
Age (years)	
Median	60
Range	27-85
Stage (FIGO)	
I	8 (21)
II	4 (10.5)
III	17 (43.6)
IV	9 (23.7)
Grade	
1	4 (10.5)
2	8 (21)
3	19 (50)
Histologic subtype	
Serous	26 (68.4)
Mucinous	2 (5.3)
Endometrioid	1 (2.6)
Mixed cell	9 (23.7)
Debulking status±	
Optimal < 1 cm	26 (68.4)
Suboptimal > 1 cm	7 (18.4)
Prior chemotherapy*	
Yes	6 (15.8)
No	30 (78.9)
Median CA125 at surgery (range)	327 (11-7024)

FIGO: International Federation of Gynecology and Obstetrics. ±The debulking status could not be determined for 5 patients. *Whether patient had received chemotherapy before surgery could not be established for two patients

ditions were staged according to the criteria of the International Federation of Gynecology and Obstetrics (FIGO). PFS was defined by the time from the initial surgery to evidence of disease progression. Because of the limited number of death that occurred in our cohort, PFS, instead of overall survival, was used as the main outcome. The first cohort consisted of 10 ascites from women with OC and was used for cytokine multiplex arrays. Their characteristics are shown in **Table 1**. The second cohort consisted of 38 patients with OC from which ascites concentrations of IL-10, IL-6, OPG, II-8 and leptin were measured by ELISA. The characteristics of these patients are shown in **Table 2**.

Peritoneal fluid specimens

Peritoneal fluids were obtained at the time of initial cytoreductive surgery for all patients. Peritoneal fluids were centrifuged at 1000 rpm for 15 min and supernatants were stored at -80°C until assayed. All acellular fluids were supplied by the Banque de tissus et de données of the Réseau de Recherche en Cancer of the Fonds de la Recherche en Santé du Québec affiliated to the Canadian Tumor Repository Network (CTRNet).

Determination of cytokine concentration by ELISA

IL-6, IL-10 and leptin levels in peritoneal fluid samples were determined by ELISA using the commercially available human Quantikine kits from R&D Systems (Minneapolis, MN). OPG levels were determined using an ELISA from Bender MedSystems (Vienna, Austria). The assays were performed in duplicate according to the manufacturer's protocols. The detection thresholds were 0.79 pg/ml for IL-6, 3.9 pg/ml for IL-10, 7.8 pg/ml for leptin and 4.5 pg/ml for OPG. The intra-assay variability was 5-10% for IL-6, 2.5-6.6% for IL-10, 3-3.2% for leptin and 4.3-7.9% for OPG. The inter-assay variability varied from 3.5% to 7.6% depending on the cytokine.

Ascites analysis using multiplex cytokine array

Cytokine levels in peritoneal fluid samples were determined using human cytokine antibody assay (G series 1000) from RayBiotech Inc. This multiplex assay measures simultaneously 120 different cytokines on a glass chip format (see supplementary Table S1 for the complete list of cytokines). In this method, the levels of cytokines are expressed as relative fluorescent units and can be used to compare cytokine levels in different ascites. This method does not provide the concentration (pg/ml) of cytokines. The signal intensities were quantified using the ScanArray express dual-color confocal laser scanner (Perkin Elmer). Data were collected in Cy3 channel and stored as paired TiFF images. Spots were identified and local background substracted using the TiGR_Spotfinder 3.1.1 software. By comparing the signal intensities, relative levels of cytokines can be established.

In vitro cell survival assay

Cell survival was measured with XTT assay. Briefly, EOC cell line CaOV3 was plated in 96-well plates at a density of 20,000 cells/well in 200 µl of medium containing either no ascites, 10% ascites, or 10% ascites with 10 µg/ml of anti-IL-10 antibody. Two hours later, human recombinant TRAIL or cisplatin was added to the

medium and cells were incubated for 48 h. At the termination of the experiment, the culture media was removed and a mixture of PBS and fresh media (without phenol red) containing phenazine methosulfate and XTT was added for 30 min at room temperature. The O.D. was determined using a microplate reader at 450 nm (TecanSunrise, Research Triangle Park, NC). The percentage of cell survival was defined as the relative absorbance of untreated versus drug-treated cells.

Statistical analysis

Comparison between unpaired groups was made using the Mann-Whitney test or the Kruskal-Wallis test. Data from the multiplex cytokine array and commercial ELISA were plotted against each other, and the correlation coefficients were determined by linear regression analysis using Microsoft Excel. The Kaplan-Meier method was used for the PFS graphs and log-rank test were used for the statistical analysis. PFS was defined as the interval between the surgery and the time of disease progression. Because of the limited number of patients in the cohort and the number of variables, multivariate analysis was not performed. The threshold for statistical significance is $P < 0.05$.

Results

Cytokine profiles of OC ascites

Given the complexity and the heterogeneity of OC ascites, measuring specific cytokines may be of limited informative value. Previous analyses of cytokines in ascites samples have been limited by the number of cytokines simultaneously analyzed and by the inclusion of other gynecologic cancers [9, 16, 17, 27]. To gain a more comprehensive understanding of the cytokine network present in OC ascites, a multiplex cytokine array that can detect 120 different cytokines in a single experiment was used in this study. The multiplex cytokine array was applied to the acellular fraction of a group of 10 ascites obtained from women with early (stage I/II; n = 5) and advanced (stage III/IV; n = 5) OC (**Table 1**). Each of these patients underwent debulking surgery and the ascites were obtained at the time of the initial surgery. Most ascites (70%) were obtained from women with serous OC. None of these women had prior chemotherapy before the surgery. The total mean protein concentration was similar between OC ascites ($P > 0.05$) (data not shown). An example of cytokine multiplex array in ascites is shown in **Figure 1**. For analysis, the internal negative controls were used to determine the cut-off intensity for a positive signal. Intensities up to 1500 were considered negative. A difference of signal intensity between ascites greater than twofold was considered a significant difference. Intensities up to 10,000 were regarded as a weak signal, 10,000 to 20,000 as a moderate signal, and > 20,000 as a strong signal. In total, 53 of 120 (44 %) cytokines had signal intensities ≤ 1500 for each ascites and were considered not expressed (for complete list, see supplementary Table S2). As shown in **Figure 2**, strong signal were seen for ACRP30 (adiponectin), angiogenin, angiopoietin-2, GRO (growth related oncogene), ICAM-1 (intercellular adhesion molecule 1), IL-6 (interleukin 6), PDGF-BB (platelet-derived growth factor BB), and RANTES (regulated upon activation, normal T-cell expressed and secreted) in most ascites. HGF (hepatocyte growth factor), IGFBP-1 (insulin growth factor binding protein-1), IL-10, IL-6R, leptin, MCP-1 (monocyte chemoattractant protein 1), MIF (macrophage migration inhibitory factor), MIP-3α (macrophage inflammatory protein-3α), NAP-2 (nucleosome assembly protein-2), sTNF-R1 (soluble tumor necrosis factor-receptor 1), TIMP-2 (tissues inhibitors of matrix metalloproteinases) and UPAR (urokinase plasminogen activator receptor) displayed a moderate signal but intensities were variable between ascites. The other 51 cytokines displayed either a low or a negative signal for each of the 10 ascites.

Correlation between multiplex cytokine array and cytokine concentration measured by ELISA

To validate measurement of signal intensities obtained with the multiplex cytokine array, signal intensities were correlated with the cytokine concentration in the 10 ascites measured using ELISA that were commercially available. The validation of cytokine expression was performed for selected cytokines that give a strong signal (IL-6), a moderate signal (IL-10, leptin) or a low signal (osteoprotegerin (OPG), IL-8). These cytokines were selected because they had compelling biological reasons for their evaluation. Example of correlation plots for IL-6 and leptin are shown in **Figure 3**. They revealed a strong correlation between data from the multiplex array and data from ELISA with correlation coefficient (r) of 0,842 for IL-6, 0,926 for leptin, 0,826 for

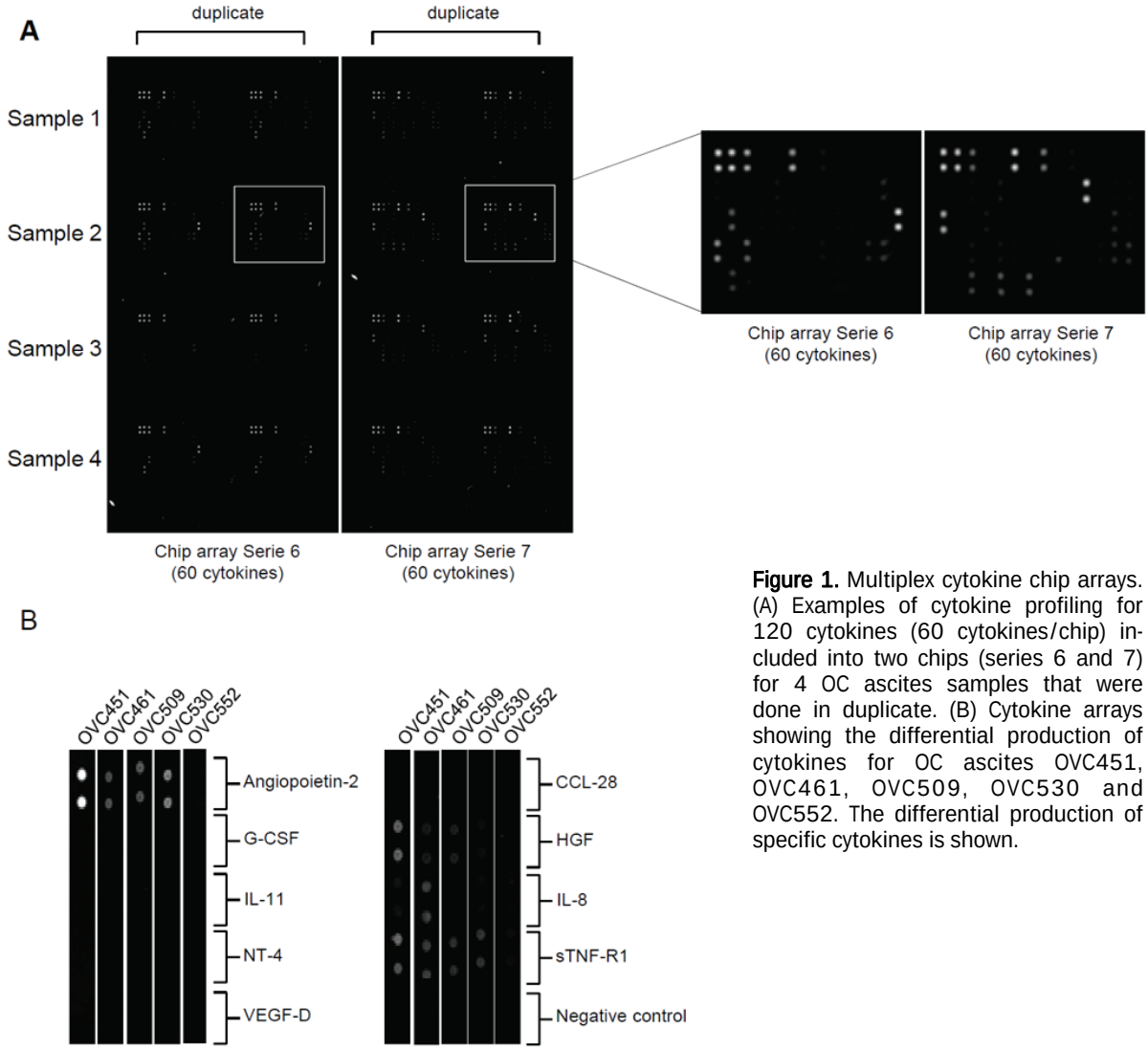

Figure 1. Multiplex cytokine chip arrays. (A) Examples of cytokine profiling for 120 cytokines (60 cytokines/chip) included into two chips (series 6 and 7) for 4 OC ascites samples that were done in duplicate. (B) Cytokine arrays showing the differential production of cytokines for OC ascites OVC451, OVC461, OVC509, OVC530 and OVC552. The differential production of specific cytokines is shown.

OPG and 0.800 for IL-10. IL-8 however showed a moderate correlation between the multiplex array and the ELISA with r = 0,518.

Cytokine concentration in OC ascites

We selected the subset of 5 cytokines that were used to validate the multiplex cytokine arrays and measured their ascites concentrations by ELISA in a cohort of 38 patients with OC. The clinical and pathological characteristics of the 38 patients with OC are shown in **Table 2**. The median age at diagnosis was 60 years (range, 27 to 85 years), and the majority (60.5%) had advanced-stage (FIGO stages III/IV) grade 3 tumors (50%), with serous histology (67.5%). Sixty percent of patients were optimally cytoreduced after initial surgery, and 15.8% received preoperative chemotherapy consisting of a platinum-based regimen combined with paclitaxel. All patients had a follow-up > 24 months (range, 26 to 120 months), with a median PFS of 16 months. The median ascites level in this cohort was 2955 pg/ml for IL-6 (range 0-31303), 485 pg/ml for OPG (range 0-19620), 322 pg/ml for IL-8 (range 31-10066), 24 pg/ml for IL-10 (range 0-488) and 658 pg/ml for leptin (range 0 -10185) (**Figure 4**). Detectable levels of IL-6, leptin and OPG (> 1 pg/ml) were observed in most (> 90%) ascites. No significant correlation was found between serum CA125 levels at the time of initial surgery and cytokine levels (data

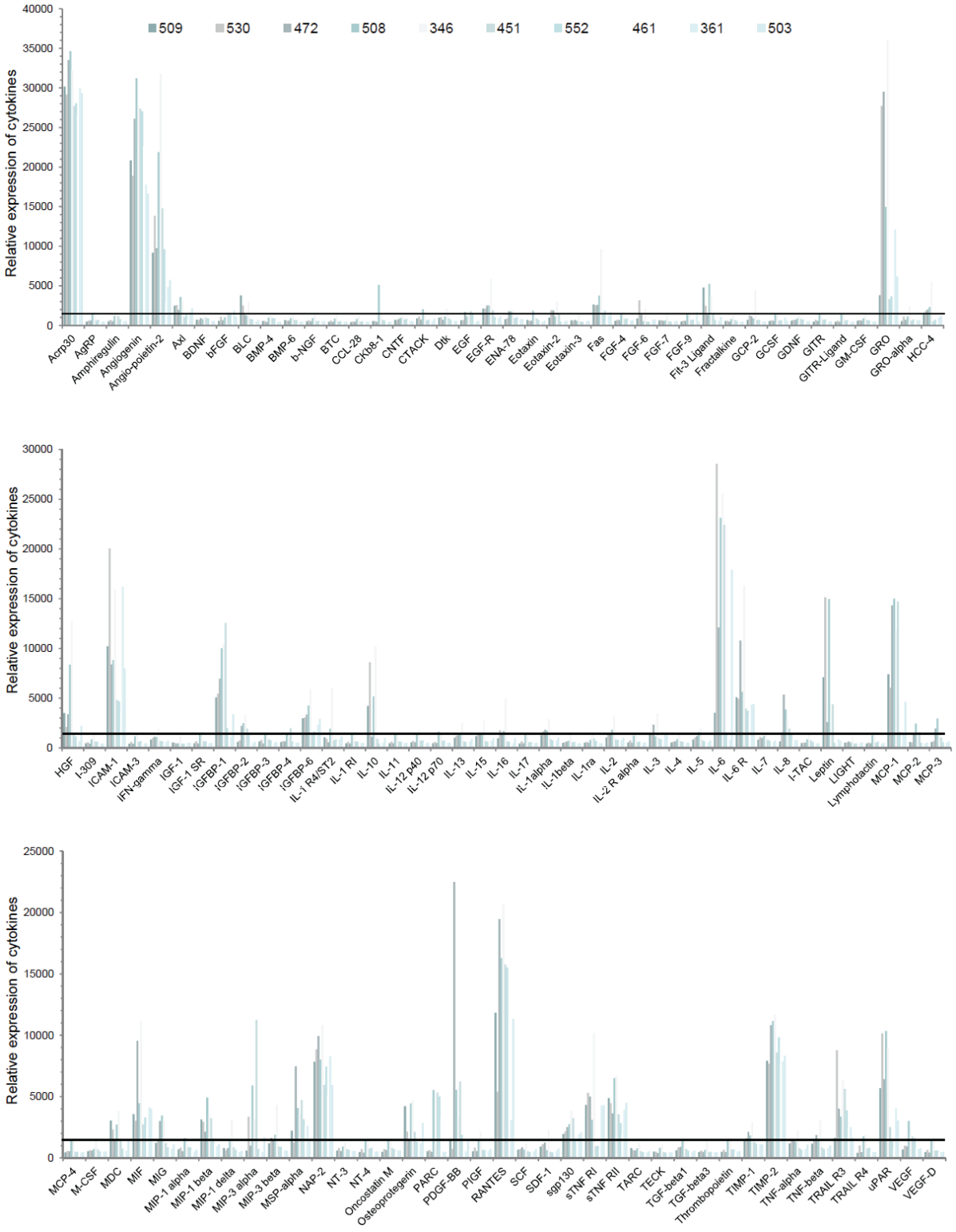

Figure 2. Relative expression of cytokines. Histograms showing the relative expression of the 120 cytokines in the 10 OC ascites. The bar indicates the cut-off intensity for a positive signal.

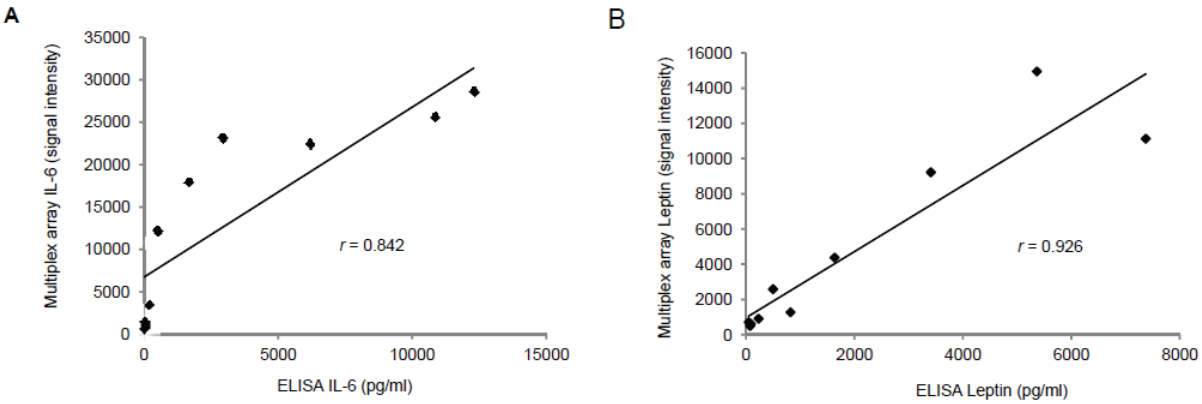

Figure 3. Correlation charts for IL-6 and leptin between the multiplex cytokine array and commercial ELISA.

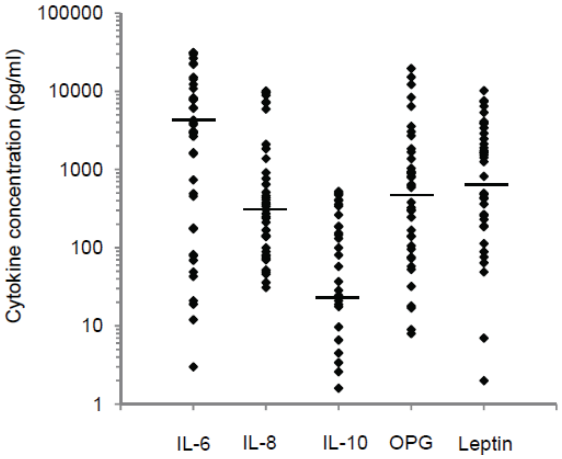

Figure 4. Concentration of IL-6, IL-8, IL-10, OPG and leptin in a cohort of 38 patients with OC. The concentration of each cytokine in ascites samples was determined by using commercial ELISA. Horizontal bars represent median values.

not shown).

Cytokine profiles in stage I/II versus stage III/IV in serous OC

Higher serum levels of IL-6, IL-10 and MCP-1 have been previously reported in patients with advanced OC as compared to stage I disease [29]. We therefore assessed whether IL-6, Il-8, IL-10, OPG and leptin were differentially expressed in stage I/II versus stage III/IV diseases in our cohort. As shown in **Table 3**, median levels of IL-6, IL-10, OPG and leptin were higher in stage III/IV diseases but the differences did not reach statistical significance for any of these 4 cytokines. Because of the limited number of patients with sub-type other that serous OC in our cohort, we could not determine whether IL-6, IL-8, IL-10, OPG and leptin in ascites varied according to the sub-type. Similarly, given that only a few patients have received chemotherapy prior to the debulking surgery (N = 6), we did not assess whether cytokines were differentially expressed with or without prior chemotherapy.

Prognostic value of cytokines in OC ascites

We have previously reported that high IL-6, but not IL-8 levels in OC ascites, correlated with shorter PFS and IL-6 was shown to be an independent marker of poor prognostic [10]. In this study, we thus focused the prognostic value of IL-10, leptin and OPG in our cohort of 38 patients. A cutoff value corresponding to the median of each cytokine was used to separate patients into two groups: those with high ascites levels versus those with low ascites levels. PFS analysis in the overall patient cohort showed a shorter PFS for patients with high OPG ascites levels (> 485 pg/ml) as compared with those presenting with low OPG levels (< 485 pg/ml) (log rank test, P = 0.001) (**Figure 5A**). Median PFS for OPG was 15 months for patients with high OPG levels in OC ascites compared to 36 months for those with low OPG levels (P = 0.001). For patients with high OPG levels, there was a 3.3 fold (95% CI, 1.5-7.2) increased risk of progression. Patients with high levels of IL-10 in ascites (> 24 pg/ml) also showed shorter PFS with a median PFS of 14 months for high IL-10 levels versus 36 months for patients with low IL-10 (P = 0.002) (**Figure 5B**). The hazard ratio (HR) for patients with IL-10 > 24 pg/ml versus

Table 3. Median cytokine concentrations according to disease stage

Cytokines	Stage I - II Median pg/ml (range) N = 11	Stage III - IV Mean pg/ml (range) N = 26	*P*
OPG	96 (17-12220)	793 (0-19620)	0.51
IL-6	455 (0-29669)	3089 (0-31142)	0.91
IL-8	436 (36-9726)	306 (31-10066)	0.74
IL-10	0 (0-488)	81 (0-525)	0.12
Leptin	496 (2-7373)	820 (0-10185)	0.69

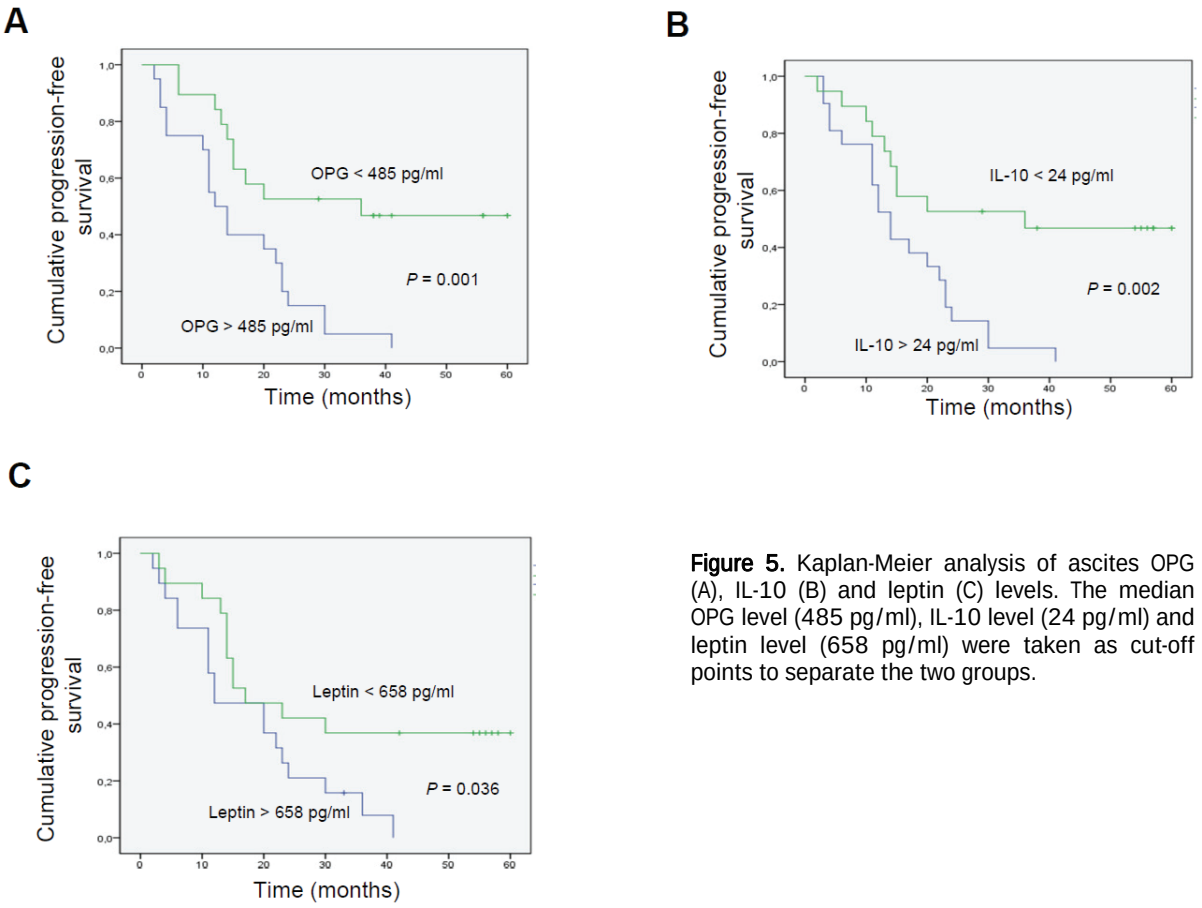

Figure 5. Kaplan-Meier analysis of ascites OPG (A), IL-10 (B) and leptin (C) levels. The median OPG level (485 pg/ml), IL-10 level (24 pg/ml) and leptin level (658 pg/ml) were taken as cut-off points to separate the two groups.

those with low IL-10 for progression was 3.1 (95% CI, 1.4-6.7). High levels of leptin (> 658 pg/ml) were associated with shorter PFS (log rank test, $P = 0.036$) with an HR of 2.1 (95% CI, 1.02-4.51) (**Figure 5C**).

Role of IL-10 in resistance to TRAIL

OC ascites have been previously shown to confer resistance to TRAIL-induced apoptosis in tumor cells [6, 7]. In addition, ascites that conferred TRAIL resistance *in vitro* were associated with shorter PFS [9]. To investigate whether cytokines could contribute to ascites-mediated TRAIL resistance, we initially selected IL-10, for which higher levels were strongly associated with shorter PFS (**Figure 5B**). OVC469 and OVC517 ascites contain 402 pg/ml and 526 pg/ml of IL-10 respectively whereas OVC461 ascites do not contain measurable IL-10 levels. OC cell line CaOV3 was treated with increasing concentrations of cytotoxic drugs in the presence of ascites that were depleted of IL-10 using an IL-10 blocking antibody (10 µg/ml). As

shown in **Figure 6**, incubation of CaOV3 cells with ascites inhibited cisplatin-induced cell death at concentrations up to 1000 ng/ml (**Figure 6A**). Ascites had a significantly more pronounced inhibiting effect on TRAIL-induced cell death (**Figure 6B**). The three ascites tested significantly inhibited TRAIL-induced cell death at all TRAIL's concentrations that were used. Blocking IL-10 in ascites did not affect the prosurvival activity of ascites against cisplatin. In contrast however, the prosurvival activity of OVC469 and OVC517 ascites was partially inhibited by the IL-10 blocking antibody. The prosurival activity OVC461 ascites, which does not contain measurable IL-10, was not affected by IL-10 blocking antibody. Furthermore, pretreatment of CaOV3 cells with recombinant human IL-10 (up to 1000 pg/ml), in the absence of ascites, did not protect tumor cells from TRAIL- or cisplatin-induced cell death (supplementary Figure S1), demonstrating that IL-10 by itself do not block the TRAIL signaling cascade. Taken together, these results suggest that IL-10 is a soluble factor that partially contributes to ascites-mediated TRAIL inhibition through interaction with other soluble factors.

Discussion

Exploratory cytokine analysis of ascites from OC patients has been limited to date. Only a few studies focused on characterizing cytokines levels in OC ascites and the difference in the cytokine profile expression pattern that distinguishes between non-malignant and malignant ascites and between the various OC subtypes. Furthermore, the number of cytokines analyzed in these studies has been limited to 20 cytokines or less. One of the distinctive features of our study was the simultaneous measurement of 120 cytokines in OC ascites. The characterization of the tumor environment in which OC develops and metastasizes is essential for understanding how this environment alters the behavior of tumor cells. In the present study, the cytokine production patterns of OC ascites obtained from newly diagnosed patients were determined using a multiplex cytokine array that measures 120 different cytokines. Interestingly, our analysis has identified multiple cytokines present at high levels in most malignant ascites that, to our knowledge, have not been previously reported, including adiponectin, angiogenin, angiopoietin-2, GRO, ICAM-1, HGF, IGFBP -1, leptin, MIP-3α, NAP-2. It is also interesting to note that cytokines such as HCC-4, IL-6, IL-8, IL-10, leptin, MIP-1β, MIP-3α, PARC, PDGF, and TNF-R1 are differentially expressed in the serum of healthy women versus patients with OC [29, 30]. The differential production of some cytokines in ascites points toward an important role of these cytokines in the progression of OC. It is therefore not surprising to find growth factors or growth factor-related proteins such as EGF-R, Fit -3 ligand, and HGF, proinflammatory cytokines (IL-6, IL-6R and IL-10), and pro-angiogenic factors (angiogenin, angiopoietin, IL-8 and PDGF) among the cytokines present in OC ascites. For example, angiopoietin is an angiogenic factor that support angiogenesis during tumor growth [31]. Angiogenesis in OC also plays a major role in the growth of disseminated tumors [32]. Patients with OC have higher serum levels of angiopoietin than patients with benign or borderline tumors [33]. The high production of angiopoietin in OC ascites is therefore consistent with its known function in angiogenesis.

Cytokine profiling of ascites could serve as a valuable tool to identify putative key soluble factors that determine the biological activity of ascites. Applying the multiplex cytokine array to a small number of malignant ascites enabled us to identify candidate cytokines which expression was subsequently validated in a larger cohort by ELISA. Using this approach, we show that IL-10 is one of the factors that promote the prosurvival activity of ascites. We demonstrate that IL-10 depletion from IL-10 containing ascites by an IL-10 blocking antibody partially inhibits the survival-promoting activity of these ascites against TRAIL, whereas the IL-10 neutralizing antibody does not affect the survival-promoting activity of an ascites that does not contain measurable levels of IL-10. Interestingly, the inhibitory effect of the IL-10 neutralizing antibody is selective for TRAIL as it does not alter the prosurvival activity of ascites against cisplatin. To our knowledge, IL-10 has not been previously associated with TRAIL resistance and further work is therefore needed to understand the mechanism by which IL-10 selectively decreases TRAIL-induced cell death but not cisplatin-induced cell death. Importantly, our data underscore the interplay that exists between different cytokines in OC ascites to regulate TRAIL-induced apopotosis. As OPG was identified as another potential candidate, we incubated different ascites with an OPG neutralizing antibody but we could not show any effect on the survival-promoting activity of ascites against TRAIL or cisplatin (data not shown). Nonethe-

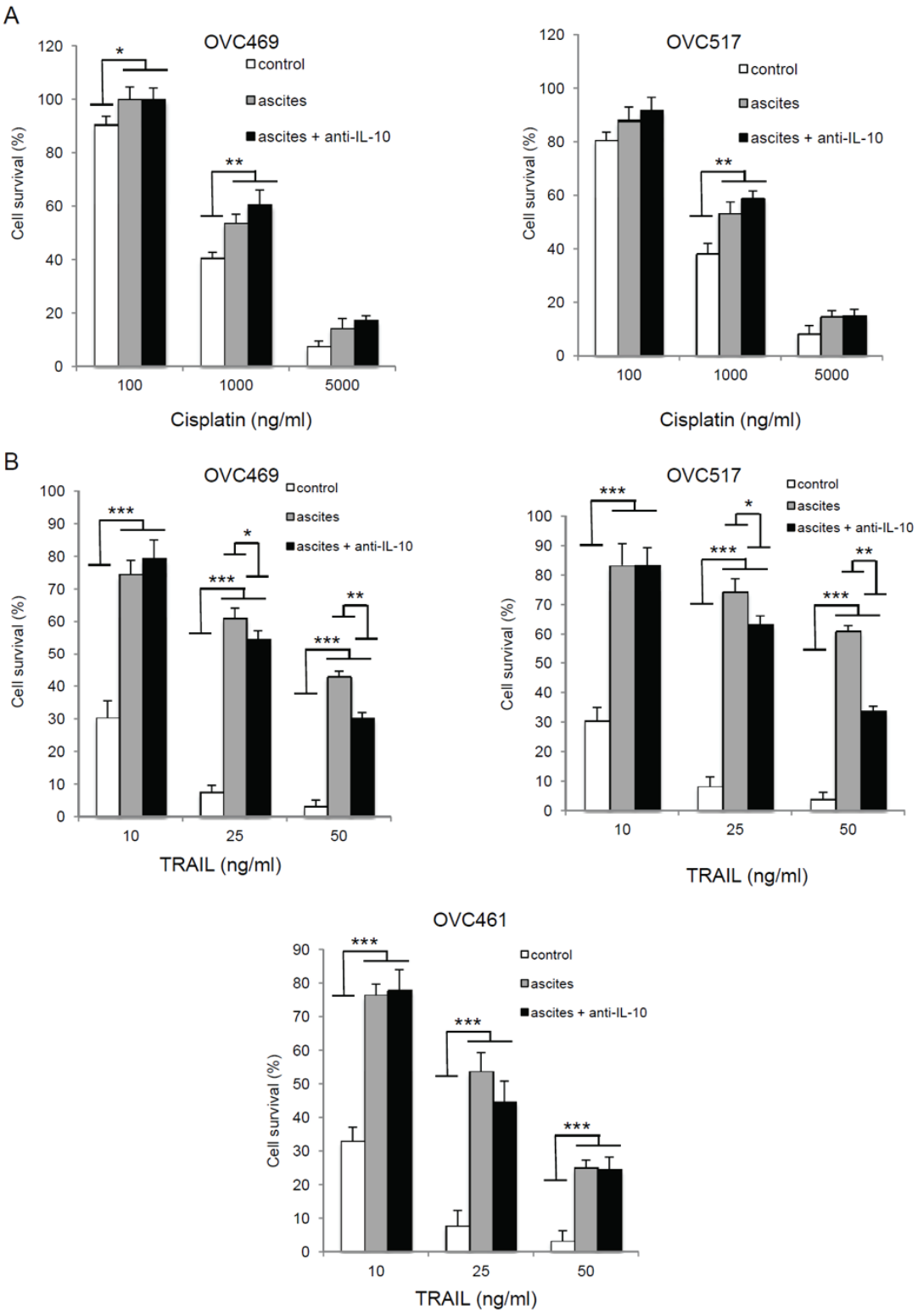

Figure 6. Effect of IL-10 neutralizing antibody on ascites-mediated inhibition of TRAIL. CaOV3 cells were either ascites or ascites + IL-10 neutralizing antibody (10 ug/ml) for 2 hrs and increasing concentrations of cisplatin (A) or TRAIL (B) were added. After 48 hrs, cell viability was measured by XTT assay. Data are representative of three experiments and are expressed as the mean ± SEM. * $P \leq 0.01$, ** $P \leq 0.001$, *** $P \leq 0.0001$.

less, these data underscore the potential of cytokine multiplex array to provide a framework for biomarker discovery in ascites.

In this study, we showed that high ascites levels of IL-10, OPG and leptin correlated with shorter PFS. Whether these cytokines represent independent prognostic factors remain to be established because multivariate analysis was not performed in this study mainly because of the small number of patients. Nonetheless, the presence of these cytokines in OC ascites and their association with patient's outcome suggest that the tumor environment plays an active role in the progression of OC. IL-10 was detectable in about 75% of OC ascites. IL-10 is known to inhibit T helper cell proliferation, hamper dendritic cell maturation and inhibit co-stimulatory molecules [34-36]. In this context, IL-10 is regarded as a potential immune suppressive component during tumor development. The high concentration of IL-10 in ascites and its association with shorter PFS might reflect its ability to allow tumor cells to escape from immune surveillance. IL-10 could also promote the prosurvival of ascites as shown it this study. OPG is a secreted member of the tumor necrosis factor (TNF) receptor superfamily that binds to the ligand for receptor activator of nuclear factor κB (RANKL) and inhibits bone resorption. OPG can also bind and inhibit the activity of TRAIL, raising the possibility that the physiologic mechanism of tumor surveillance exerted by soluble TRAIL may be abrogated in the ascites where OPG expression is high [37]. This is supported by the observation that overexpression of OPG is associated with significantly worse overall survival and relapse-free survival in colon cancer patients [38]. Moreover, overexpression of the OPG protein is an independent risk factor for colon cancer recurrence [38]. Leptin is a 16KD adipokine produced predominantly by adipocytes and leptin mediated signaling pathways play an important role in cancer cell proliferation, invasion and metastasis [39, 40]. The role of leptin in OC development and progression has not been extensively investigated but available studies have shown that leptin stimulates OC cell growth at least i*n vitro* [41, 42]. Further studies are needed to determine the precise role of these cytokines in malignant ascites.

In summary, this study provides the first in depth analysis of cytokine production in OC ascites from newly diagnosed patients. Comparative analysis of the cytokine profiles in OC ascites identified a subset of cytokines produced at high levels in OC ascites, which may contribute to the pathogenesis of OC. A number of these cytokines appeared to correlate with the clinical outcome and high levels in ascites were associated with shorter PFS. Among these cytokines, IL-10 was shown to promote the prosurvival activity of ascites. These data support the need to continue proteomic analysis of malignant ascites to understand the role of soluble factors. Cytokine array is a promising tool with which to characterize and prioritize key cytokines in malignant ascites.

Acknowledgments

This work was supported by grants from the Cancer Research Society and the Canadian Institutes of Health Research (MOP – 115072) (A.P.). We wish to thank the Banque de tissus et de données du Réseau de Recherche en Cancer du Fond de Recherche en Santé du Québec (FRSQ), affiliated to the Canadian Tumor Repository Network (CTRNet) for providing the ascites samples. We also thank Nathalie Carrier for her assistance with the statistical analyses and Éric Boivert, Ph.D., for the cytokine array data analysis.

Authors contributions

DL participated in the design of the study and performed the assays for measuring IL-6, IL-10, OPG and leptin levels in ascites. IM was responsible for obtaining the ascites and the clinical data. She also performed the cytokine chip arrays experiments. CR participated in the design of the study and helped to draft the manuscript. AP conceived the study, participated in its design and drafted the manuscript. All authors read and approved the final manuscript.

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Table S2. List of cytokines that are not detected in OC ascites by the mutliplex cytokine array

Cytokines	Cytokines	Cytokines
AgRP	I-309	M-CSF
Amphiregulin	ICAM-3	MIP-1α
BDNF	INF-γ	MIP-1δ
bFGF	IGF-1	NT-3
BTC	IGF-1 SR	NT-4
CCL-28	IGFBP-3	Oncostatin M
CNTF	IL-1 R1	PIGF
DTK	IL-11	SCF
Eotaxin-3	IL-12 p40	SDF-1
FGF-7	IL-17	TARC
FGF-9	IL-1β	TECK
Fractalkine	IL-1 Rα	TGF-β1
G-CSF	IL-2 Rα	TGF-β3
GDNF	IL-4	Thrombopoietin
GITR	IL-7	VEGF-D
GITR- ligand		
GM-CSF		

Figure S1