Regulation of Ovarian Carcinoma SKOV-3 Cell Proliferation and Secretion of MMPs by Autocrine IL-6

ALEX RABINOVICH1,3, LIAT MEDINA2,3, BENJAMIN PIURA1,3, SHRAGA SEGAL2,3* and MAHMOUD HULEIHEL2,3

1Unit of Gynecologic Oncology, Division of Obstetrics and Gynecology, Soroka University Medical Center, Beer-Sheva; 2Department of Microbiology and Immunology, 3Faculty of Health Sciences and BGU Cancer Research Center, Ben-Gurion University, Beer-Sheva, Israel

Abstract. Background: Cancerous ovarian tissues contain and produce high levels of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α). The aim of this study was to assess the mechanisms by which autocrine IL-6 affects the ovarian carcinoma continuous cell line (SKOV-3) tumorigenicity. Materials and Methods: Autocrine IL-6 was neutralized by the addition of anti-IL-6 antibodies to SKOV-3 cell cultures. The proliferation rate was evaluated by MMT staining and the capacity to produce matrix metalloproteinases (MMPs) 2 and 9 was examined by zymography. Results: The SKOV-3 cells secreted IL-6 in a time-dependent manner (24-96 h). The addition of anti-IL-6 antibodies to SKOV-3 cell cultures did not affect their proliferation rate within 96 h of incubation. In addition, SKOV-3 cells secreted MMP-2 and MMP-9 as confirmed by zymography. The MMP-9 levels decreased in a time-dependent manner (3, 8, 24 h) and the addition of anti-IL-6 antibodies to SKOV-3 cell cultures significantly decreased their capacity to secrete MMP-9, particularly after 8 h of incubation. MMP-2 (pro-active and active forms) was also secreted by SKOV-3 cell cultures but could be measured only after 24-96 h of incubation. The levels of MMP-2 increased in a time-dependent manner. The addition of anti-IL-6 antibodies to SKOV-3 cell cultures did not affect their capacity to secrete MMP-2. Conclusion: Our results suggest that IL-6 secreted by SKOV-3 cells could be involved in their tumorigenic potential, particularly potentiating their capacity to secrete MMP-9.

Cytokines are autocrine/paracrine immunoregulatory polypeptide factors. They are produced by virtually all types of cells and may be involved in the stimulation or inhibition of cell growth, regulation of cell differentiation or the induction of cell chemotaxis. Since cytokines may also modulate the expression of other cytokines, it is difficult to assess the role of any particular cytokine in a specific process (1, 2).

Cytokines participate in the process of ovulation, follicle development and ovarian steroidogenesis (3-5); there is, however, increasing evidence that cytokines may play a significant role in the development and progression of ovarian cancer (1). Suggested mechanisms include the stimulation of tumor cell proliferation, promotion of metastasis by increasing cell adhesiveness, enhancing tumor angiogenesis and inducing the immune system to block cell-mediated mechanisms for identifying and destroying the tumor cells (1).

Different cytokines, including pro-inflammatory cytokines, were demonstrated in normal ovarian tissues and cells and were more pronounced in ovarian cancer tissues and cells (6-9).

IL-6, a 184-amino-acid protein with a molecular weight of 20.3 kDa was originally described by Hirano et al. (1986) as a B-lymphocyte differentiation factor (10). It has since been recognized as an important participant in physiological processes in numerous cells (11), but the role of IL-6 in tumor systems is still controversial (12). In the normal ovary, IL-6 may contribute to follicle development by reducing the follicle-stimulating hormone binding capacity of granulosa cells (13). In ovarian carcinoma, IL-6 has been implicated to inhibit spontaneous apoptosis (14, 15) and to promote tumor growth by its effects on cell attachment and migration (16). Nevertheless, IL-6 does not enhance tumor growth directly, but constitutes an autocrine growth factor for ovarian tumor cells (17, 18).

The invasion and spread of metastasizing tumors is known to be mediated by the action of extracellular matrix
(ECM)-degrading proteinases. Proteolytic activity may be required for the destruction of the mesothelial cell layer during invasion of the implanted tumor through the submesothelial basement membrane into the visceral organ stroma and for subsequent tumor-mediated angiogenesis (19, 20). Predominant among the proteinases produced by invading tumor cells are enzymes of the matrix metalloproteinase (MMP) family.

MMPs are a family of zinc-dependent metalloendopeptidases that function in the degradation of collagen, gelatin and other ECM macromolecules. The expression of gelatinolytic MMPs, such as MMP-2 (gelatinase A, 72 kDa type IV collagenase) and MMP-9 (gelatinase B, 92 kDa type IV collagenase), has been linked to enhanced tumor invasion in numerous model systems (19, 21-23).

The expression and activation of MMPs appear to play an important role in the process of tumor progression (23, 24). This process involves local tumor expansion through adjacent normal tissues, invasion of metastatic cells into vessels and lymphatic tissue and extravasation at distant sites (25). Moreover, MMPs appear to be involved in the process of angiogenesis, mediating the remodeling and penetration of ECM by new capillaries. An MMP inhibitor should, therefore, have the potential to inhibit tumor growth and spread (25).

Due to their potential destructive effects, MMPs are highly regulated at different levels. The extracellular activity of MMPs is controlled at the level of gene expression, proenzyme activation, and interaction with specific tissue inhibitors of MMPs (TIMPs). At the transcriptional level, various cytokines, such as TNF-\(\alpha\) and IL-1\(\beta\), acting through positive or negative regulatory elements on the MMP genes, control MMP expression (26-29).

The aim of the present study was to evaluate the mechanisms by which autocrine IL-6 is involved in the regulation of the tumorigenic potential of ovarian cancer cells.

Materials and Methods

SKOV-3 cell culture. The SKOV-3 cells were cultured in Minimum Essential Medium-\(\alpha\) (MEM-\(\alpha\)) containing 5% FCS (fetal calf serum), L-Glutamine (2 mM) and an antibiotic combination of streptomycin 0.1 mg/ml and penicillin 100 U/ml (all purchased from Biological Industries, Beit-Haemek, Israel). The cells were incubated at 37\(^\circ\)C in a humidified air atmosphere containing 5% CO\(_2\) from Biological Industries, Beit-Haemek, Israel). The cells were previously washed twice in 2.5% Triton X-100 (Sigma) at room temperature and then incubated overnight at 37\(^\circ\)C with gentle shaking in a development incubator. After washing, the gels were washed with 10% acetic acid and 30% methanol and destained in 10% acetic acid and 30% methanol and destained in 10% acetic acid.

Preparation of conditioned media from SKOV-3 cell cultures. SKOV-3 cells (4x10\(^5\) cells/ml/well) were seeded in 24-well plates with MEM-\(\alpha\) and in the presence of 5 or 20 \(\mu\)g/ml rabbit anti-human IL-6. Control wells remained with MEM-\(\alpha\) alone. After 3-96 h of incubation, conditioned media (supernatants) were collected and stored at –20\(^\circ\)C until examination for IL-6 (by ELISA) and MMP-2, -9 levels (by zymography).

Evolution of IL-6 levels in SKOV-3 cell cultures. IL-6 levels in conditioned media of SKOV-3 cell cultures were examined by ELISA using specific antibody pairs for human IL-6 (first antibody, mouse monoclonal anti-human IL-6; second antibody, mouse monoclonal anti-human biotin conjugate IL-6; Biosource, Canerillo, CA, USA). The levels of IL-6 were recorded by reference to a standard curve obtained with human recombinant IL-6, sensitivity was <8 pg/ml and the range of the standard curve was 5-2500 pg/ml. ELISA was performed by an overnight incubation of the first antibody (1 \(\mu\)g/ml in 96-well ELISA plates, followed by washing PBS (phosphate-buffered saline, Biological Industries) with 0.05% tween-20 (ICN, Aurora, OH, USA)] and the addition of blocking buffer (PBS with 10% FCS) for 2 h at 37\(^\circ\)C. Thereafter, recombinant cytokine (5-2500 \(\mu\)g/ml, Genzyme Diagnostics, Cambridge, MA, USA) and samples were added. After 1 h of incubation at 37\(^\circ\)C, the plates were washed and the second antibody (8 \(\mu\)g/ml) was added for 1 h of incubation at 37\(^\circ\)C. After washing, streptavidin HRP (peroxidase-conjugated streptavidin, Jackson ImmunoResearch, West Grove, PA, USA) was added for 15 min at 37\(^\circ\)C. After another washing, TMB (tetramethylbenzidine, Dako, Carpinteria, CA, USA) was added for 10 min and the reaction was stopped by adding 2N H\(_2\)SO\(_4\) (Sulphuric Acid, Gadot, Natania, Israel). Absorbance was read by an ELISA reader at 450 nm.

Evolution of MMP-2,9 levels in SKOV-3 cell cultures. MMP-2, -9 levels were examined using zymography bioassay analysis (31). Aliquots of conditioned media from SKOV-3 cell cultures (40 \(\mu\)l diluted in 15 \(\mu\)l of Sample Buffer) were electrophoresed on 8\% SDS-PAGE (SDS-polyacrylamid gel electrophoresis) containing 1 mg/ml gelatin (Sigma). After electrophoresis, the gels were stained with Coomassie Blue 0.25% (Brilliant Blue R, Sigma) in 10% acetic acid and 30% methanol and destained in 10% acetic acid and 30% methanol (Bio-lab, Jerusalem, Israel) until satisfactory contrast between the gel and the bands was achieved.

Proteolytic activities were detected as clear bands on a blue background, indicating the lysis of the substrate. The intensity of the bands was evaluated by densitometry, using TINA 2.0 software.
Statistics. Samples were examined in triplicate for each experiment. Each experiment was repeated at least three times. To evaluate the statistical significance of the results, the Student’s *t*-test was performed. A *p*-value <0.05 was considered significant.

Results

**IL-6 secretion by ovarian cancer cells (SKOV-3).** The SKOV-3 cells secreted IL-6, which increased in a time-dependent manner (24, 48, 72, 96 h, 20.5±7.5 pg/ml, 58.9±23.4 pg/ml, 181±68 pg/ml and 156.5±36 pg/ml, respectively). The maximum IL-6 secretion was examined after 72 h (Figure 1).

**Effect of IL-6 on SKOV-3 proliferation.** Our results indicate that the addition of various doses of rabbit anti-human IL-6 antibodies (5 µg/ml, 20 µg/ml) to SKOV-3 cell cultures, for 24-72 h, did not affect their proliferation rate (Figure 2).

**Effect of IL-6 on the capacity of SKOV-3 cells to secrete MMPs.** The MMP-2 and MMP-9 levels were examined in conditioned media of SKOV-3 cells treated and untreated with anti-IL-6 antibodies. SKOV-3 cells produced both MMP-9 and MMP-2 as examined by zymography (Figure 3A, Figure 4A) and evaluated by densitometry (Figure 3B, Figure 4B,C). Gelatinase B (MMP-2), initially secreted as a 72-kDa pro-MMP-2, was cleaved to the active 62 kDa form. MMP-2 secretion increased with time (Figure 4A). During the first 24 h of SKOV-3 cell incubation only basal levels of MMP-2 could be detected. After further incubation (24-96 h), increasingly higher levels of MMP-2 could be measured (Figure 4A). Thereafter, MMP-2 (pro-active and active forms) secretion increased in a time-dependent manner (24-96 h). As with MMP-9, the addition of anti-IL-6 antibodies to the SKOV-3 cells decreased the cells’ capacity to produce MMP-2, in a dose-dependent manner (Figure 4A,B,C), but unlike MMP-9, most of the MMP-2 values did not reach statistical significance. Only the secretion of the active form of MMP-2 was significantly reduced (39%) after 72 h of incubation with anti-IL-6 antibodies 20 µg/ml (Figure 4B; *p*=0.03).

Discussion

IL-6 is one of the most widely studied pro-inflammatory cytokines in ovarian cancer (32, 33). Serum IL-6 levels were shown to be higher in ovarian cancer patients than in healthy controls, however, IL-6 does not appear to be as sensitive or useful as a tumor marker as CA-125 (12). The combination of serum IL-6 with serum CA-125 values only slightly increased the overall sensitivity of CA-125. Furthermore, serial determinations of serum IL-6 levels were not found useful in monitoring the clinical course of disease during chemotherapy (34). Nevertheless, IL-6 may...
play a major role in the promotion of tumor growth, cell attachment and migration (16), but not tumor proliferation (16, 17). Our results corroborate those of previous studies (34) and show that SKOV-3 ovarian cells produce IL-6, but IL-6 is not directly involved in SKOV-3 cell proliferation. The addition of anti-IL-6 antibodies to SKOV-3 cells did not affect their proliferation rate.

The MMPs are a family of zinc- and calcium-dependent proteolytic enzymes capable of degrading most ECM components. High levels of MMP-2 and 9 and enhanced proteolytic activity were shown to be associated with

Figure 3. Effect of anti-IL-6 antibodies on the capacity of SKOV-3 cells to secrete MMP-9. SKOV-3 cells (10^5 cells/100 µl/well) were cultured in a serum-free medium (MEM-a), for 3-24 hours, in the absence or presence of rabbit anti-human IL-6 antibodies [0 (control), 5 µg/ml, 20 µg/ml]. Supernatants were collected and examined for MMP-9 activity by zymography (A) and quantified by densitometry (B). Results are presented as percent of the control. * p<0.05, ** p<0.01. MW, molecular weight.

Figure 4. Effect of anti-IL-6 antibodies on the capacity of SKOV-3 cells to secrete MMP-2. SKOV-3 cells (10^5 cells/100 µl/well) were cultured in a serum-free medium (MEM-a), for 3-96 h, in the absence or presence of rabbit anti-human IL-6 antibodies [0 (control), 5 µg/ml, 20 µg/ml]. Supernatants of pro-active form - 72 kDa and active form - 62 kDa were collected and examined for MMP-2 activity by zymography (A) and quantified by densitometry (B - active form; C - pro-active form). Results are presented as percent of the control. ** p<0.05. MW, molecular weight.
advanced tumor stage and to predict a worse prognosis (35, 36). A complex array of cytokines regulates the production and secretion of MMPs from ovarian carcinoma cells, ovarian carcinoma cell lines and stromal cells surrounding ovarian tumors (25, 27-29). Furthermore, different cell types respond differently to stimulation by the same cytokines, rendering the interpretation and comparison of results even more challenging. Our primary goal was to evaluate the mechanisms by which IL-6 is involved in the regulation of the MMP-2 and -9 production and secretion, which may represent the tumorigenic potential of ovarian cancer cells.

Although MMP-2 and -9 are both considered gelatinases with almost identical substrates (21), their secretion kinetics, as well as their response to anti-IL-6 antibodies differ. While it has been shown that the secretion of MMP-9 is more prone to being affected by pro-inflammatory cytokines like TNF-α and IL-1β, the secretion of MMP-2 is more constitutive and less influenced by cytokines (26-29, 32).

SKOV-3 cells secrete MMP-9 in a time-dependent manner, but after 24 h of incubation, MMP-9 levels were no longer detected. The addition of anti-IL-6 antibodies decreased the cells’ capacity to secrete active MMP-9 in a dose-dependent manner. The most significant decrease in MMP-9 production (45.3%) was achieved after 8 h of incubation with 20 μg/ml of anti-IL-6 antibodies. SKOV-3 cells secrete MMP-2 in a time-dependent manner, but unlike the case of MMP-9, during the first 24 h of incubation the levels of total, active and pro-active forms of MMP-2 could barely be detected. Only continued incubation up to 96 h revealed increasingly higher levels of all forms of MMP-2. The addition of anti-IL-6 antibodies to SKOV-3 cells revealed a tendency towards decreased dose-dependent MMP-2 production. In MMP-9, most of the values did not reach statistical significance with the exception of the secretion of the active form of MMP-2 after 72 h of incubation with anti-IL-6 antibodies 20 μg/ml (39%, p=0.03).

Our results clearly show different time-course secretions of MMP-2 and MMP-9 by the SKOV-3 cells and that these two factors are differently regulated by autocrine IL-6. These results may suggest the involvement of endogenous IL-6, produced by ovarian carcinoma cells in the up-regulation of MMP secretion, thus, increasing their invasiveness and metastatic capacity.

The effect of IL-6 on the production of MMP-2 and -9 has been investigated in other tumor cells. Barille et al. (37) showed that IL-6 had no effect on MMP-2 and MMP-9 production by multiple myeloma cells. Kossakowska et al. (38) found that IL-6 stimulated the production of both MMP-2 and MMP-9 in three malignant lymphoma cell lines. In contrast to our results, IL-6 was also reported to have a growth-promoting function in lymphoma. Sundelin et al. (39) investigated the effect of IL-6 on the expression of MMP-2 and -9 in two oral squamous cell carcinoma cell lines. In both cell lines IL-6 alone had no effect on the secretion of MMP-2. The influence on MMP-9 expression was contradictory; in one cell line IL-6 alone inhibited MMP-9 secretion, while in the other IL-6 had a stimulatory effect.

In conclusion, our results suggest that IL-6 could be involved in the tumorigenic potential of ovarian carcinoma cells by increasing the secretion of MMPs, mainly of MMP-9. Thus, anti-IL-6 antibodies could be considered, under certain circumstances, to play a role in future therapeutic strategies for ovarian carcinoma.

Acknowledgements

This study was partially supported by the Israel Cancer Association and the Goldman Foundation, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel.

References


36 Rauvala M, Puistola U and Turpeenniemi-Hujanen T: Gelatinases and their tissue inhibitors in ovarian tumors; TIMP-1 is a predictive as well as a prognostic factor. Gynecol Oncol 99: 656-663, 2005.

