

RESEARCH ARTICLE

Thalidomide distinctly affected TNF- α , IL-6 and MMP secretion by an ovarian cancer cell line (SKOV-3) and primary ovarian cancer cells

Benjamin Piura^{1,4}, Liat Medina^{2,4}, Alex Rabinovich^{1,4}, Victor Dyomin^{3,4}, Mahmoud Huleihel^{2,4}

¹ Unit of Gynecologic Oncology, Division of Obstetrics and Gynecology, Soroka University Medical Center, Beer-Sheva, Israel

² The Shraga Segal Department of Microbiology, Immunology and Genetics, Beer-Sheva, Israel

³ Institute of Pathology, Soroka University Medical Center, Beer-Sheva, Israel

⁴ Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Correspondence. Prof. Mahmoud Huleihel, Ph.D., The Shraga Segal Department of Microbiology, Immunology and Genetics, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel.

<piura@bgu.ac.il>

<Huleihel@bgu.ac.il>

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ABSTRACT. *Background.* Thalidomide inhibits TNF- α production in lipopolysaccharide-stimulated monocytes. The aim of this study was to evaluate the effect of thalidomide on TNF- α , IL-6 and MMP secretion in epithelial ovarian carcinoma cells. *Materials and methods.* SKOV-3 cells and primary epithelial ovarian carcinoma cells were cultured in the presence of various concentrations of thalidomide. Cell proliferation was examined by MTT proliferation assay. TNF- α and IL-6 levels were determined in the supernatants of the cell cultures by ELISA, and MMP activity was examined by gelatin zymography. *Results.* Thalidomide did not significantly affect the proliferation and growth of SKOV-3 cells. However, it decreased significantly the capacity of SKOV-3 cells and primary epithelial ovarian carcinoma cells to secrete TNF- α . Thalidomide also significantly decreased the capacity of SKOV-3 cells, but not primary epithelial ovarian carcinoma cells, to secrete MMP-9 and MMP-2. However, thalidomide did not affect IL-6 secretion in SKOV-3 cells or primary epithelial ovarian carcinoma cells. *Conclusion.* Our study suggests that thalidomide distinctly affected TNF- α , IL-6 and MMPs secretion by an ovarian carcinoma cell line (SKOV-3) and primary ovarian cancer cells. This might suggest a different susceptibility of these two types of cells to thalidomide, and/or that the mechanisms of secretion of the factors examined are differently regulated in these cells. Our results may deepen our understanding the mechanism/s of action of thalidomide in ovarian carcinoma cells. The results might have important implications in future therapeutic strategies that will incorporate thalidomide and other cytokine inhibitors in the treatment of epithelial ovarian carcinoma.

Key words: thalidomide, ovarian cancer, TNF- α , IL-6, MMP

Thalidomide (α -N-phthalimidoglutarimide, $C_{13}H_{10}N_2O_4$), which was initially marketed as a sedative-hypnotic drug with anti-emetic activity against morning sickness of early pregnancy, was withdrawn from the market in the early sixties as it was found to cause severe fetal malformations [1-5]. In 1965, thalidomide was observed to be very effective in the treatment of erythema nodosum leprosum (ENL), an acute inflammatory state occurring in lepromatous leprosy characterized by fever, painful cutaneous lesions, arthritis, glomerulonephritis and circulating immune complexes [6]. It has been shown that the serum tumor necrosis factor-alpha (TNF- α) level, which is markedly elevated in ENL patients, was reduced after treatment with thalidomide [7]. Sampaio *et al.* [8] demonstrated that thalidomide selectively inhibits the production of TNF- α , but not the production of the interleukins IL-1 β , IL-6 and GM-CSF, in lipopolysaccharide (LPS)-stimulated human monocytes. The dramatic effect of thalidomide in ENL, decreasing inflammation

and enhancing patient well-being, led to its approval in 1998 by the FDA for the treatment of ENL, and recently for multiple myeloma [1, 5]. Thalidomide's properties have been classified as immunomodulatory (inhibition and stimulation of cytokines, co-stimulation of primary human T cells, modification of cell surface adhesion molecules, stimulation of Th1 immunity, induction of natural killer cells, and inhibition of nuclear factor-kappa B [NF- κ B] activity), and non-immunomodulatory (anti-angiogenic activity, anti-proliferative and pro-apoptotic activity, and cyclooxygenase-2 [COX-2] inhibition) [9]. This broad spectrum of activities and the exertion of these effects through multiple mechanisms indicate that thalidomide may be used in a variety of clinical conditions including cancers such as epithelial ovarian carcinoma (EOC) [3].

The aim of this study was to evaluate the effect of thalidomide on TNF- α , IL-6, MMP-2 and MMP-9 secretion in SKOV-3 and primary EOC cell cultures. To the best of our

knowledge, this is the first study investigating the effect of thalidomide on the production of various pro-inflammatory cytokines and MMPs in EOC cells.

MATERIALS AND METHODS

Thalidomide

Thalidomide (Chemie Grünenthal, Stolberg-am-Rhein, Germany) was dissolved in dimethyl sulfoxide (DMSO) 1:1000 to give a stock solution of 500 μ g/mL and stored at -20 °C.

SKOV-3 cell culture

SKOV-3 cells were cultured as described by Rabinovich *et al.* [10]. In brief, the SKOV-3 cells were cultured in minimum essential medium- α (MEM- α) containing 5% fetal calf serum (FCS), L-glutamine (2 mM) and an antibiotic combination comprising streptomycin 0.1 mg/mL and penicillin G 100 U/mL (Biological Industries, Beit-Haemek, Israel). The cells were incubated at 37 °C in a humidified air atmosphere containing 5% CO₂. The number of cells used in the experiments was optimized to achieve confluent growth at the end to the experimental system.

Origin and handling of primary EOC tissues

Fresh, primary EOC tissues were collected under sterile conditions from primary EOC patients undergoing surgery at the operating room of the Department of Obstetrics and Gynecology, Soroka University Medical Center, Beer-Sheva, Israel. The institutional review board (IRB) approved the study and informed consent to participate in the study was obtained beforehand from all ovarian carcinoma patients. The histopathological diagnosis of primary EOC was confirmed in formalin-fixed, paraffin-embedded tissues. Fresh tissue samples were immediately washed with cold phosphate-buffered saline (PBS) in order to eliminate residual blood cells. Approximately 1-2 grams of each fresh, primary EOC tissue was used for the establishment of primary EOC cell cultures.

Primary EOC cell culture

Primary EOC cell cultures were established as described by Huleihel *et al.* [11]. Briefly, ovarian tissue was minced with a scalpel into small pieces and dissociated by stirring with collagenase (0.05% w/v) and hyaluronidase (0.01% w/v) for 2-3 hours at 37 °C with stirring until complete dissociation. The cell suspension was filtered through sterile gauze and centrifuged at 1,200 RPM for 10 min. The cells were suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, L-glutamine (2 mM) and a combination of streptomycin 0.1 mg/mL and penicillin G 100 U/mL (Biological Industries, Beit-Haemek, Israel), and cultured in 25 cm² flasks at 37 °C in a humidified air atmosphere containing 5% CO₂. After 7-10 days, monolayers were formed and the cultures were trypsinized and placed into fresh flasks (first passage). At each passage, cells were cultured in fresh bottles to remove adherent leukocytes, mainly macrophages. Assays were performed after six passages.

Preparation of conditioned media from SKOV-3 cell and primary EOC cell cultures

The conditioned media from SKOV-3 cell and primary EOC cell cultures were prepared as described by Rabinovich *et al.* [12]. Briefly, SKOV-3 cells (4 \times 10⁵ cells/mL/well) or primary EOC cells (5 \times 10⁴ cells/mL/well) were seeded in 24-well plates with MEM- α (SKOV-3 cells) or DMEM (primary EOC cells) in the presence of DMSO 1:1000 alone (control) or thalidomide dissolved in DMSO 1:1000 in concentrations of 4 \times 10⁻⁷-2 \times 10⁻⁵ M. After 3-96 h of incubation, conditioned media samples (supernatants) were collected and stored at -20 °C until examination for TNF- α and IL-6 levels (by ELISA) and MMP-2 and MMP-9 levels (by zymography).

Evaluation the effect of thalidomide on SKOV-3 cell growth and proliferation.

SKOV-3 cells were cultured (10⁵ cells/100 μ L/well) in MEM-a in the absence (DMSO) or presence of thalidomide (4 \times 10⁻⁷-2 \times 10⁻⁵ M) in 96 microwell plates. After 24-72 hours (H) of incubation, SKOV-3 cell growth and proliferation were examined by MTT [3-(4,5-dimethylthiazol-2-yl)-2'5-diphenyl-tetrazolium bromide] staining (Sigma) according to Rabinovich *et al.* [10]. Briefly, MTT (1 mg/mL; final concentration) was added to each well which were then incubated at 37 °C in a CO₂ incubator. Three hours later the reaction was terminated by addition of 100 μ L/well of an extraction solution of 10% sodium dodecyl sulfate (SDS), 10% DMF (N,N-dimethylformamide), 2.5% of 1N HCl, and 2.5% of 80% glacial acetic acid. The optical density was examined after an overnight incubation at the wave-length of 540 nm of cleaved MTT molecules (brown color) and was measured using an ELISA Reader (BIO-RAD, Model 550).

Evaluation of TNF- α and IL-6 in conditioned media from SKOV-3 and primary EOC cell cultures.

TNF- α and IL-6 levels in the conditioned media of SKOV-3 and primary EOC cell cultures were examined by ELISA using specific antibody pairs for human TNF- α and human IL-6.

The first antibodies were mouse monoclonal anti-human TNF- α and anti-human IL-6 antibody, respectively. The ELISA was performed with an overnight incubation with the first antibody (1 mcg/mL) in 96-well ELISA plates, followed by washing with 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 °C. The second antibodies were mouse monoclonal anti-human biotin conjugate TNF- α and anti-human biotin conjugate IL-6 antibody, respectively (Biosource, Camarillo, CA, USA). The second antibody (8 mcg/mL) was added for 1 h of incubation at 37 °C. After washing, streptavidin HRP (horse reddish peroxidase-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA, USA) was added for 15 min at 37 °C. After another washing, TMB (tetramethylbenzidine, Dako, Carpinteria, CA, USA) was added for 10 min and the reaction was stopped by adding 2N H₂SO₄ (sulphuric acid, Gadot, Natania, Israel). Absorbance was read using an ELISA reader at 450 nm. The levels of TNF- α and IL-6 were recorded by reference to a standard curve

obtained with human recombinant TNF- α (Pepro-Tech) and human recombinant IL-6 (Genzyme Diagnostics), respectively. Sensitivity was <8 pg/mL and the range of the standard curve was 5-2,500 pg/mL.

Evaluation of MMP-2 and MMP-9 in conditioned media from SKOV-3 and primary EOC cell culture

MMP-2 and MMP-9 protein levels were examined using gelatin zymography bioassay analysis as described by Rabinovich *et al.* [10]. Briefly, aliquots of conditioned media from SKOV-3 and primary EOC cell cultures (40 μ l diluted in 15 μ l of sample buffer) were electrophoresed on 8% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) containing 1 mg/mL gelatin (Sigma). After electrophoresis, the gels were washed twice in 2.5% Triton X-100 (Sigma) at room temperature and then incubated overnight at 37 °C with gentle shaking in a development buffer (200 mM NaCl [Frotarom], 50 mM Tris-HCl [TRIZMA-hydrochloride], 5 mM CaCl₂ [calcium chloride], and 0.02% Brij-35, pH = 7.5 [Sigma]). The gels were then stained with Coomassie Blue 0.25% (Brilliant Blue R, Sigma) in 10% acidic 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 software.

Statistical analysis

Samples were examined in triplicate for each experiment. Each experiment was repeated at least three times unless otherwise mentioned. Results are expressed as the mean \pm SEM. Student's t-test was performed to evaluate the statistical significance of the results. A p-value <0.05 was considered significant.

RESULTS

Effect of thalidomide on SKOV-3 cell proliferation

Our results showed that addition of different concentrations of thalidomide (4×10^{-7} - 2×10^{-5} M) for 24-72 hours did not significantly affect the growth and proliferation of SKOV-3 cells compared to DMSO (control) (figure 1).

Effect of thalidomide on TNF- α secretion in SKOV-3 and primary EOC cells

SKOV-3 cells (10^5 cells/100 μ L/well) were cultured in MEM- α , and primary EOC cells (10^4 cells/100 μ L/well) were cultured in DMEM in the presence of DMSO 1:1000 alone (control) or various concentrations of thalidomide (4×10^{-7} - 2×10^{-5} M) dissolved in DMSO 1:1000. Supernatants were collected from SKOV-3 cell cultures after 8 and 24 hours of incubation (figure 2A), and from primary EOC cell cultures after 24, 48 and 72 hours of incubation (figure 2B) to be examined for TNF- α levels using an ELISA. In SKOV-3 cells cultured in the presence of DMSO alone (control), TNF- α levels were 30 ± 30 pg/mL and 35 ± 32 pg/mL after 8 and 24 hours of incubation, respectively (figure 2A). In primary EOC cells cultured in the presence of DMSO 1:1000 alone (control), TNF- α levels were 10 ± 9 pg/mL, 15 ± 6 pg/mL and 10 ± 3 pg/mL after

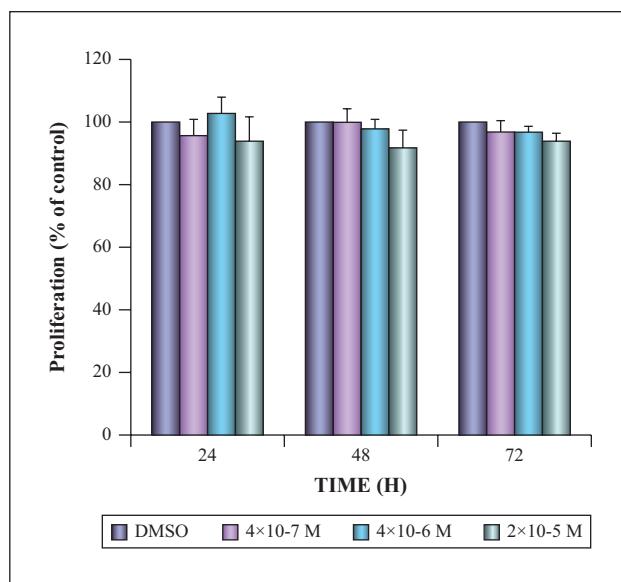
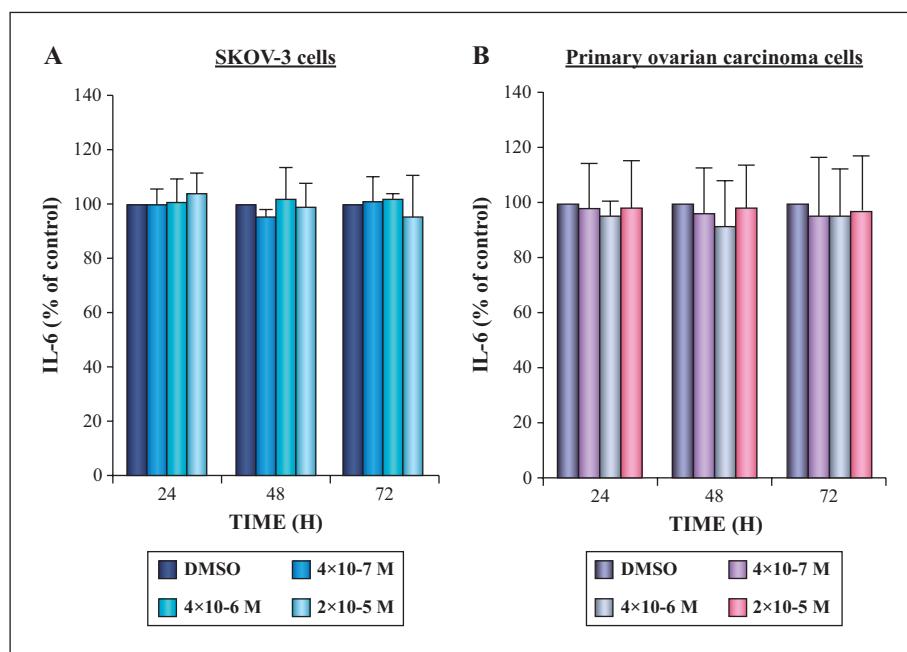
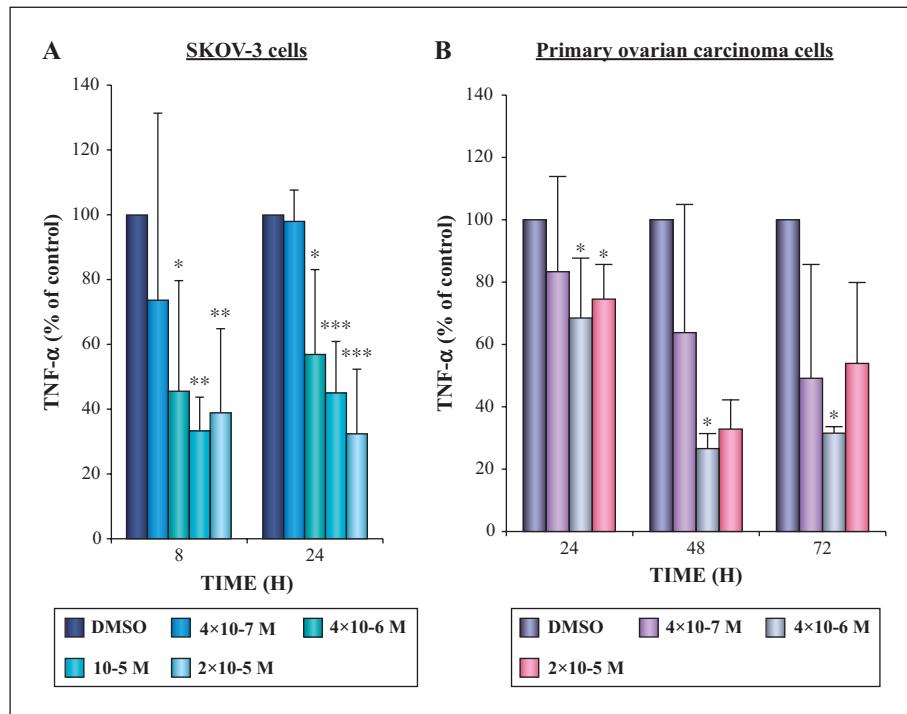


Figure 1
Effect of thalidomide on SKOV-3 cell growth and proliferation. SKOV-3 cells were cultured (10^5 cells/100 μ L/well) in MEM- α in the absence (DMSO; 1:1000) or presence of thalidomide (4×10^{-7} - 2×10^{-5} M) in 96 microwell plates. After 24-72 hours (H) of incubation SKOV-3 cell growth and proliferation was examined by MTT staining. Results are presented as percentage of the control (DMSO).

24, 48 and 72 hours of incubation, respectively (figure 2B). The addition of various concentrations of thalidomide (4×10^{-7} - 2×10^{-5} M) dissolved in DMSO 1:1000 to SKOV-3 (figure 2A) and primary EOC cell (figure 2B) cultures caused a decrease in the capacity of the cells to secrete TNF- α . In SKOV-3 cell cultures, the addition of thalidomide (2×10^{-5} M) caused a significant reduction (around 70% decrease) in TNF- α level after 8 hours ($p = 0.0024$) and 24 hours ($p = 0.0001$) of incubation, respectively, as compared to the control (DMSO alone) (figure 2A). In primary EOC cell cultures, the addition of thalidomide (4×10^{-6}) caused a significant reduction in the TNF- α level after 24 hours ($p = 0.046$), and an approximate 70% decrease after 48 hours ($p = 0.028$) and 72 hours ($p = 0.014$) of incubation, respectively, as compared to the control (DMSO alone) (figure 2B).

Effect of thalidomide on IL-6 secretion in SKOV-3 and primary EOC cells

SKOV-3 cells and primary EOC cells were cultured in the absence or presence of thalidomide as described in the previous paragraph. Supernatants were collected after 24-72 hours of incubation to be examined for IL-6 levels using an ELISA. In SKOV-3 cells cultured in the presence of DMSO alone (control), IL-6 levels were 10 ± 2 pg/mL, 50 ± 10 pg/mL and 130 ± 20 pg/mL after 24, 48 and 72 hours of incubation, respectively (figure 3A). In primary EOC cells cultured in the presence of DMSO alone (control), IL-6 levels were 520 ± 80 pg/mL, 610 ± 100 pg/mL and 740 ± 160 pg/mL after 24, 48 and 72 hours of incubation, respectively (figure 3B). The addition of various concentrations of thalidomide (4×10^{-7} - 2×10^{-5} M) dissolved in DMSO 1:1000 to SKOV-3 cell (figure 3A) and primary EOC cell (figure 3B) cultures for 24-72 hours of incubation did not affect IL-6 secretion.



Effect of thalidomide on MMP-9 and MMP-2 secretion in SKOV-3 and primary EOC cells

SKOV-3 cells and primary EOC cells were cultured in the absence or presence of thalidomide as described in

the previous paragraph. Supernatants were collected from SKOV-3 cell cultures (figures 4A,B,D,E) and from primary EOC cell cultures (figures 4C,F) after various periods of incubation and examined for MMP-9 and MMP-2 activity using gelatin zymography (figure 4B and figure 4E,

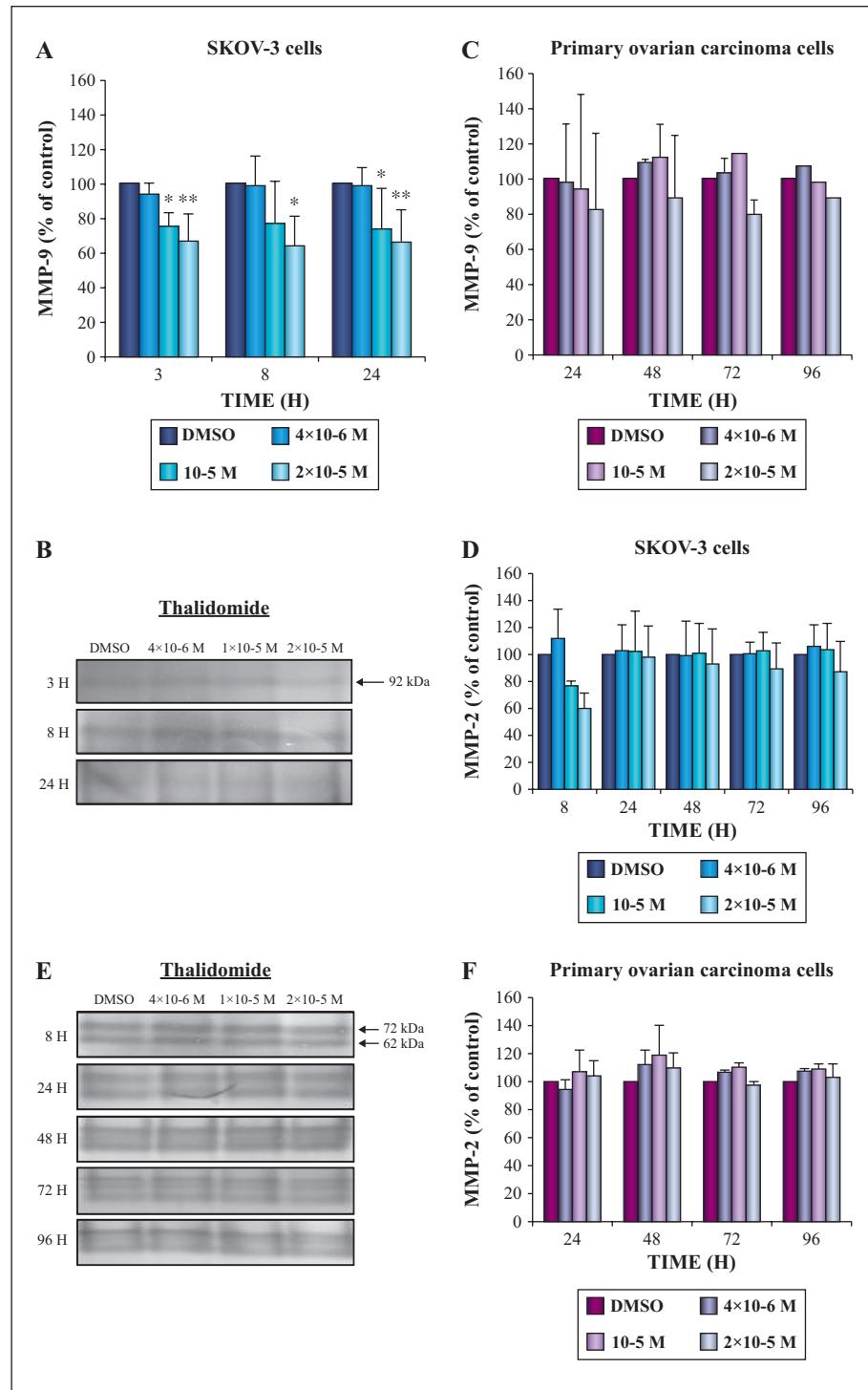


Figure 4

Effect of thalidomide on MMP-9 and MMP-2 secretion in SKOV-3 and primary EOC cells. SKOV-3 cells (A, B, D and E) and primary EOC cells (C and F) were cultured as described in *figure 2*. Supernatants were collected from SKOV-3 cell cultures (A,B) after 3, 8 and 24 hours of incubation ($n = 3-6$) and from primary EOC cell cultures (C) after 24, 48, 72 and 96 hours of incubation ($n = 1-2$) to be examined for MMP-9 activity. For examination of MMP-2 activity, supernatants were collected from SKOV-3 cell cultures (D,E) after 8, 24, 48, 72 and 96 hours of incubation ($n = 3-6$) and from primary EOC cell cultures (F) after 24, 48, 72 and 96 hours of incubation ($n = 2$). MMP-9 (92 kDa) and MMP-2 activity (72 kDa is the pro-active form and 62 kDa is the active form) was examined using gelatin zymography (as presented in *figure B* and *figure E* respectively for SKOV-3 cells) and quantified using densitometry with TINA software. Results are presented as percentage of the control (DMSO alone) for each time group. $n =$ number of repetitions of experiment (each experiment was performed in triplicate). * $p < 0.05$; ** $p < 0.01$.

respectively, for SKOV-3 cultures) and quantified by densitometry using TINA software. In SKOV-3 cell cultures, the addition of various concentrations of thalidomide (4×10^{-6} - 2×10^{-5} M) caused a significant decrease in

the capacity of the cells to secrete MMP-9 (*figure 4A*) and MMP-2 (*figure 4D*). Thalidomide in concentrations of 1×10^{-5} M and 2×10^{-5} M caused a significant reduction (approximately 30% decrease) in MMP-9 level

after 3 hours ($p = 0.004$), 8 hours ($p = 0.026$) and 24 hours ($p = 0.007$) of incubation, respectively, as compared to the control (DMSO only) (figure 4A). Thalidomide in concentrations of 1×10^{-5} M or 2×10^{-5} M caused a significant reduction (approximately 30%-decrease) in MMP-2 level after 8 hours of incubation (1×10^{-5} M, $p = 0.009$; 2×10^{-5} M, $p = 0.007$) as compared to the control (DMSO alone), but this effect vanished after 24-96 hours of incubation (figure 4D). In primary EOC cell cultures, the addition of thalidomide, at all concentrations, did not significantly affect the capacity of the cells to secrete MMP-9 (figure 4C) and MMP-2 (figure 4F).

DISCUSSION

TNF- α , a pro-inflammatory cytokine produced by monocytes, macrophages, lymphocytes and natural killer (NK) cells, has been implicated in the pathophysiology of infections and autoimmune diseases, and has been shown to be overproduced in several malignancies [5, 8-11, 13-17]. While high pharmacological doses of TNF- α induce vasculotoxic tumor-regressing effects, pathophysiological levels of autocrine/paracrine endogenous TNF- α induce angiogenesis, tumor genesis and growth [13, 18]. Previous studies have demonstrated that thalidomide inhibits TNF- α production in LPS-stimulated human monocytes and induces TNF- α mRNA degradation [8, 19]. The anti-inflammatory, anti-angiogenic and anti-neoplastic functions of thalidomide, that are exerted through its ability to block TNF- α production, can be explained by inhibition of the TNF- α -induced translocation of the transcription factor NF- κ B from the cytoplasm to the nucleus [3, 20-22]. The inability of NF- κ B to bind DNA results in the suppression of NF- κ B-dependent genes, such as IL-8, and VEGF, that are factors necessary for angiogenesis and tumorigenesis [18, 20, 21]. In a previous study [16], we demonstrated that the neoplastic epithelial cells of EOC, and not the tumor infiltrating immune cells, are the main source of TNF- α and IL-6. In this study, we have shown, for the first time, that the addition of thalidomide to SKOV-3 cells and primary EOC cells depleted of tumor-infiltrating leukocytes, caused a significant decrease in the capacity of the neoplastic epithelial cells to secrete TNF- α .

IL-6 has been shown to support growth and survival of EOC cells [11, 23-27]. Since it has been reported that TNF- α stimulates the production of IL-6 in SKOV-3 and primary EOC cell cultures, it would be expected that the neutralization of TNF- α by thalidomide or the α hTNF- α antibody would decrease the capacity of the cells to secrete IL-6 [28]. It has indeed been observed that neutralization of TNF- α by the monoclonal antibody against TNF- α , infliximab, caused a decrease in IL-6 levels in primary EOC cell cultures [29]. In addition, several studies have demonstrated that thalidomide, along with TNF- α inhibition, also causes inhibition of IL-6 production by peripheral blood mononuclear cells [24, 30-33]. In this study however, the addition of thalidomide to SKOV-3 and primary EOC cell cultures did not affect IL-6 production. These findings are in agreement with studies that demonstrated that thalidomide selectively inhibited TNF- α production, but not IL-6 production, in LPS-stimulated human monocytes and human macrophages exposed to titanium particles [8, 19, 34].

These results may suggest a similar mechanism in ovarian carcinoma cells and in monocytes/macrophages for the secretion of IL-6, which does not use TNF pathways, and that other cytokines may compensate for the decrease in TNF- α levels.

Invasion and spread of tumors is mediated by extracellular matrix (ECM)-degrading proteinases [10, 35]. Predominant among these proteinases are the matrix metalloproteinase (MMP) family. MMPs are zinc-dependent metalloendopeptidases that play a role in the degradation of collagen, gelatin and other ECM macromolecules. Expression and activation of 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 progression and invasion [10, 35, 36]. Moreover, MMPs appear to be involved in the process of angiogenesis by mediating the remodeling and penetration of ECM by new capillaries [36]. Therefore, MMP inhibitor should have the potential to inhibit tumor growth and spread [37]. In this study, the addition of thalidomide to SKOV-3 cell cultures caused a significant decrease in the capacity of the cells to secrete MMP-9 and MMP-2. However, the addition of thalidomide to primary EOC cell cultures did not significantly affect the capacity of the cells to secrete MMP-9 and MMP-2. However, the decrease in MMP-9 levels was statistically significant only after three hours of incubation, and the decrease in MMP-2 levels was statistically significant only after eight hours of incubation. These findings are in agreement with other studies that demonstrated that thalidomide inhibits MMPs [36, 38].

Our findings that thalidomide inhibits TNF- α and MMPs support the hypothesis that some effects of thalidomide could be mediated through TNF- α inhibition. This may have important implications in the development of future treatment strategies for EOC. So far, there are only a few clinical studies on the efficacy of thalidomide in patients with EOC. Thalidomide was given orally (100-400 mg/day) to heavily pretreated patients with advanced or recurrent EOC either as a single agent [39-42] or in combination with systemic chemotherapy [43]. Thalidomide was well tolerated and had modest activity in heavily pretreated patients with advanced or recurrent EOC. Nevertheless, a recent study showed that thalidomide was no more effective than tamoxifen in delaying disease recurrence among women with biochemical-recurrent ovarian, peritoneal or fallopian tube carcinoma [44]. Obviously, further clinical studies are needed to establish the role of thalidomide in the treatment of EOC.

In conclusion, this is the first study evaluating the effect of thalidomide on TNF- α , MMPs and IL-6 secretion in ovarian carcinoma cells. We have shown that (1) thalidomide caused a significant decrease in the capacity of SKOV-3 cells and primary EOC cells to secrete TNF- α , (2) thalidomide caused a significant decrease in the capacity of SKOV-3 cells, but not primary EOC cells, to secrete MMP-9 and MMP-2, and (3) thalidomide did not affect IL-6 secretion in SKOV-3 cells and primary EOC cells. These findings may suggest different susceptibilities of primary ovarian carcinoma cells and ovarian carcinoma cell lines (SKOV-3 cell line). In addition, the mechanism/s that regulates the secretion of TNF- α , IL-6 and MMPs might be different in the cells examined. Our results may have

important implications for the possible incorporation of thalidomide and other anti-cytokines and MMPs agents in future treatment strategies for patients with EOC.

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