

IL-6 and IL-8 enhance factor H binding to the cell membranes

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Abstract. The aim of the present study was to assess the role of interleukin (IL)-6 and IL-8 on the expression of fluid-phase complement inhibitor, factor H (FH), and FH-like protein 1 (FHL-1), in the A2780 ovarian carcinoma cell line. This cell line does not normally produce IL-6, however, is IL-6 responsive due to the presence of receptor for IL-6. The presence of FH and FHL-1 in the cell lysates was confirmed by western blotting. The levels of FH and FHL-1 in the medium were determined by enzyme-linked immunosorbent assay. To evaluate gene expression, reverse transcription-quantitative polymerase chain reaction was performed. The cellular localization of FH and FHL-1 in ovarian cancer cells was assessed by immunofluorescence. The present study revealed that FH, contrary to FHL-1, was secreted by ovarian cancer cells, however, this process was independent of IL stimulation. No significant differences were observed in the concentration of FH in the control cells, when compared with the samples treated with IL-6/IL-8. The results of western blotting revealed that the protein expression levels of FH and FHL-1 were not regulated by IL-6 and IL-8 in a dose-dependent manner. Immunofluorescence analysis confirmed that the A2780 ovarian cancer cell line expressed both membrane bound and intracellular forms of FH and FHL-1. The present data revealed that the A2780 cells expressed and secreted FH protein and are also able to bind FH and FHL-1. This may influence the efficiency of complement mediated immunotherapy.

Introduction

Ovarian cancer is the fifth most common cause of mortality among women, following breast, lung, colon and pancreatic cancer (1). Due to the usually late recognition and low therapeutic efficiency, ovarian cancer is a huge challenge for treatment. The risk of occurrence of ovarian cancer during a lifetime is 2% (2). Prognosis at any stage of ovarian cancer is grave and the results of treatment are not satisfactory (2). According to the accepted procedure, the basic treatment at stage I and II of ovarian cancer is laparotomy. Women with cancer at stages III and IV are treated with chemotherapy, using platinum-based drugs (3). The 5 year survival rate following diagnosis of ovarian cancer is 30% (2). However, dynamic development of immunology, molecular biology and genetics creates novel opportunities for cancer treatment and leads to the development of novel therapies. One of the promising novel developments is anticancer immunotherapy, a therapy based on the use of monoclonal antibodies and utilizing the physiological mechanisms of immune response regulation, including the complement dependent cytotoxicity (4). Although anticancer immunotherapy is becoming more and more popular and several novel applications have been described, no reports exist about effective immunotherapy for ovarian cancer.

The complement system consists of >30 proteins and is a major component of the innate immune response. It also acts as a bridge between the innate and adaptive immune responses and promotes inflammatory processes. Activation of the complement system initiates a protein cascade enzymatic reaction. The result of this reaction is sequential formation of convertase C3, convertase C5 and finally attack of the membrane complex, also termed the C5b-9 complex. Three known pathways of complement system activation exist: Classical, alternative and initiated by the lectin association with the cell surface (4,5). A group of proteins responsible for the stability of the complement system reactions is known as regulators of complement activation. The group of complement regulators contains factors present in the serum or associated with the cell membrane. The most important fluid phase factors are inhibitor of the C1 complex

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formation, C4-binding protein, factor H (FH) and FH-like protein 1 (FHL-1). The key complement regulators associated with the cell membrane are: Complement receptor (CR) type-1 [cluster of differentiation (CD)35], CR2, membrane cofactor protein (CD46), decay-accelerating factor (CD55) and homologous restriction factor (CD59) (6). These proteins, associated with the cell membrane, protect normal cells from complement mediated cell lysis. Cancer cells can protect themselves by the production of immunosuppressive agents (7). Secretion of soluble forms of complement system inhibitors, FH and FHL-1, by ovarian cancer cells protect them from humoral immune responses (8). FH is a single polypeptide chain plasma glycoprotein, which is present in the plasma at a concentration of 110-615 $\mu\text{g/ml}$ (9). Expression of inhibitors of the complement system may be stimulated by cytokines.

A higher concentration of cytokines is observed in the tumor microenvironment. In ovarian cancer, the key role is played by interleukin (IL)6. Its concentration in ovarian cancer can be 1,000-fold higher compared with that in cysts and 10-fold higher than in cancer of the digestive system (10,11). It is assumed that IL-6 present in serum and ascitic fluid is very important in the development of ovarian cancer. An elevated concentration of IL-6 has been documented to correlate with a poor prognosis, enhanced survival of ovarian cancer cells and multidrug resistance (12-14). A previous study addressed the role of IL-6 in promoting the chemoresistance of cancer cells (15). Results from *in vitro* research on liver cancer cell lines, Hep3B and HepG2, have shown that IL-6 increased the expression of complement system inhibitors, CD55 and CD59, associated with the cell membrane (16). Tumor cells may also protect themselves by binding soluble complement inhibitors from serum, including complement factors, FH and FHL-1. However, to the best of our knowledge, the role of IL-6 and IL-8 on the expression levels of FH and FHL-1 in ovarian cancer cells remains to be characterized and investigated.

The aim of present study was to assess of the role of IL-6 and IL-8 on the expression levels of fluid-phase complement inhibitors, FH and FHL-1, in the A2780 established ovarian carcinoma cell line, known to not produce IL-6, however, is IL-6 responsive due to the presence of the IL-6 receptor.

Materials and methods

Interleukin and antibodies. Human IL-6 and human IL-8 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The following antibodies were purchased from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA): Monoclonal mouse anti-FH (sc-166613), polyclonal goat anti-FHL-1 (sc-17953), monoclonal mouse anti- β -actin (sc-47778), horseradish peroxidase-conjugated donkey anti-goat secondary antibody (sc-2020) and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (sc-2005).

Cell culture. The human A2780 ovarian cancer cell line was obtained from the European Collection of Cell Culture (Salisbury, UK). A2780 cells were cultured in RPMI-1640 medium (Sigma-Aldrich), supplemented with

L-glutamine (Sigma-Aldrich), penicillin-streptomycin (10 U/ml-100 $\mu\text{g/ml}$; Sigma-Aldrich) and 10% fetal bovine serum (FBS; Sigma-Aldrich), in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Stimulation of cells. A2780 cells were seeded into Petri dishes (3×10^5 cells/ml in 5 ml). Following washing, the cells were incubated in medium containing increasing concentrations of IL-6 or a combination of IL-6 and IL-8. After 24 h of incubation, the supernatant was collected in new Eppendorf tubes and frozen at -80°C for subsequent enzyme-linked immunosorbent assay (ELISA). The cells were incubated with 5 mM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) for 10 min. The cells were subsequently placed into new tubes and centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was removed and the precipitated cells were stored at -80°C for western blotting.

Western blotting. The cells were lysed in radioimmunoprecipitation lysis buffer comprising 1% Tergitol, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1 mM EDTA, 1 mM ethyleneglycoltetraacetic acid, 1 mM NaVO_4 , 20 mM NaF, 0.5 dithiothreitol, 1 mM phenylmethanesulfonyl fluoride and protease inhibitor cocktail in PBS. The lysates were centrifuged at 12,000 x g for 10 min at 4°C. The protein concentration in the supernatant was measured using a bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A total of 20 mg protein from each sample was electrophoresed on a 10% SDS-polyacrylamide gel electrophoresis gel under reducing conditions, and were subsequently transferred onto polyvinylidene difluoride membranes. Non-specific binding sites on the membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.05% Tween-20 for 1 h at room temperature. The membrane was probed with mouse monoclonal anti-FH (1:1,000), goat polyclonal anti-FHL-1 (1:1,000) and mouse monoclonal anti- β -actin antibodies overnight at 4°C. The membrane was subsequently incubated at room temperature for 1 h with horseradish peroxidase-conjugated donkey anti-goat or goat anti-mouse secondary antibody (1:2,000). Visualization of the protein bands was performed using Pierce enhanced chemiluminescence western blotting substrate (Pierce, Thermo Scientific, Inc.). The protein bands were quantified using Image J software (version 1.48; Media Cybernetics, Inc., Rockville, MD, USA) and normalized against β -actin values.

Densitometric analysis. In order to measure the protein expression level, the intensity of specific bands corresponding to the proteins of interest were determined using the commercially available Image J software. Firstly, the photographic film with bands was scanned. The scanned blot images were imported into the software and were contrast adjusted to ensure the bands were clearly visible on the blot image. Background intensity was subtracted from the blot image. The bands were subsequently selected by drawing a tight boundary around them. The intensities of the selected bands were displayed in an excel format, which can be exported for performing further statistical analyses.

ELISA. To determine the quantity of FH or FHL-1 in the medium samples, a Human Complement FH ELISA kit (EIAab Science Co., Ltd., Wuhan, China) and ELISA kit for complement FH-related protein (CFHR1) (USCN Life Science Inc., Wuhan, China) were used, respectively. Each test was performed, according to manufacture's protocol. The FH assay detection range was 0.15-10.00 ng/ml and the FHL-1 assay detection range was 0.625-40 ng/ml.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA was extracted from cultured cells after 24 h incubation with various concentrations of IL-6 or IL-6/IL-8 using a High Pure RNA Isolation kit (Roche, Basel, Switzerland), according to manufacture's protocol. The extracted RNA was purified and diluted in DNase and RNase-free water. The quality and quantity of isolated RNA was measured using a spectrophotometer NanoDrop® (Thermo Fisher Scientific, Inc.). Reverse-transcriptase PCR was performed using High-Capacity cDNA Reverse Transcriptase (Thermo Fisher Scientific, Inc.). The quantities of used RNA was 2,000 ng in a final volume of 20 μ l. Subsequently, 1 μ l of the resulting cDNA solution (100 ng) was used in qPCR, using primers and probes specific for complement factor H (CFH) and CFHR1. TagMan® Gene Expression assays (Thermo Fisher Scientific, Inc.) including specific primers and probes were purchased from Thermo Fisher Scientific, Inc. (Assay ID, CFH-Hs00962373_m1 and CFHR1-Hs00275663_m1). The relative expression was calculated using the $2^{-\Delta\Delta C_q}$ method (17). β -actin mRNA was used as an endogenous control to normalize CFH and CFHR1 input.

Immunofluorescence. The cells were grown on Lab-Tek Chamber Slides (Nunc, Roskilde, Denmark) in RPMI-1640 medium, containing 10% FBS. After 24 h, the cells were incubated in medium containing various concentrations of IL-6/IL-8 (1, 10 or 100 ng/ml) for a further 24 h. Following incubation, the slides were fixed in 3.7% formaldehyde for 15 min and were next permeabilized in 0.1% Triton X-100 for 10 min. Following permeabilization, the slides were blocked in 3% bovine serum albumen solution for 15 min at room temperature, and following washing were incubated overnight at 4°C with mouse monoclonal anti-FH (ab118820) and anti-FHL-1 (ab76912) primary antibodies (both dilutions, 1:200; both purchased from Abcam, Cambridge, UK) at a concentration of 5 μ g/ml. Subsequently, the secondary antibody, donkey anti-mouse immunoglobulin G Alexa Fluor® 488 conjugated (green) (ab150105; Abcam) was used at a 1:1,000 dilution for 1.5 h at room temperature. Fluorescence labeling was analyzed under a fluorescent microscope (BX51, Olympus, Tokyo, Japan).

Statistical analysis. Statistical analysis was performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). Multiple comparisons were performed using one-way analysis of variance followed by Tukey's post hoc test. The data are presented as the mean \pm standard deviation. All statistical tests were two-sided and $P < 0.05$ was considered to indicate a statistically significant difference.

Table I. FH secretion in response to stimulation with various concentrations of IL-6 or IL6/IL-8 mixture.

A, Secretion of FH in culture medium from A2780 cells stimulated with various concentrations of IL-6

IL-6 concentration (ng/ml)	Concentration of FH (ng/ml)
0 (control)	10.20 \pm 0.33
1	9.72 \pm 0.21
10	10.10 \pm 0.33
100	9.76 \pm 0.18

B, Secretion of FH in culture medium from A2780 cells stimulated with a mixture of various concentrations of IL-6 and IL-8

IL-6/IL-8 concentration (ng/ml)	Concentration of FH (ng/ml)
0 (control)	10.23 \pm 0.43
1	10.88 \pm 0.30
10	10.79 \pm 0.26
100	11.07 \pm 0.17

Data are presented as the mean \pm standard deviation (n=3). FH, factor H; IL, interleukin.

Results

FH, compared with FHL-1, is produced and secreted by ovarian cancer cells, independent of different doses of IL stimulation. The quantity of FH and FHL1 in the culture medium from A2780 cells stimulated by various concentrations (1, 10 or 100 ng/ml) of IL-6 alone or IL-6/IL-8 combination was determined after 24 h by an ELISA. The results revealed that ovarian cancer cells produced and secreted FH into the medium (Table I). However, the production of FH by A2780 cancer cells was unaffected by the addition of IL-6. No significant difference in the concentration of FH was observed in the control cells when compared with the samples incubated with IL-6 alone (Fig. 1A) and IL-6/IL-8 (Fig. 1B). The quantity of FHL-1 in the culture medium was either below the detection limit of the ELISA used, or these cancer cells did not secrete FHL-1. A total of two independent tests were performed under the same conditions. In this study, we present the results from only one analysis. Each test had control samples from medium and fetal bovine serum used for research. FH and FHL-1 in the control sample were absent.

Intracellular protein expression levels of FH and FHL-1 protein is not regulated by the IL-6/IL-8 in a dose-dependent manner. The presence of FH and FHL-1 in cell lysates was confirmed by western blotting. A2780 cells were incubated with various concentrations of IL-6 alone or IL-6/IL-8. The results revealed that A2780 cells produced FH

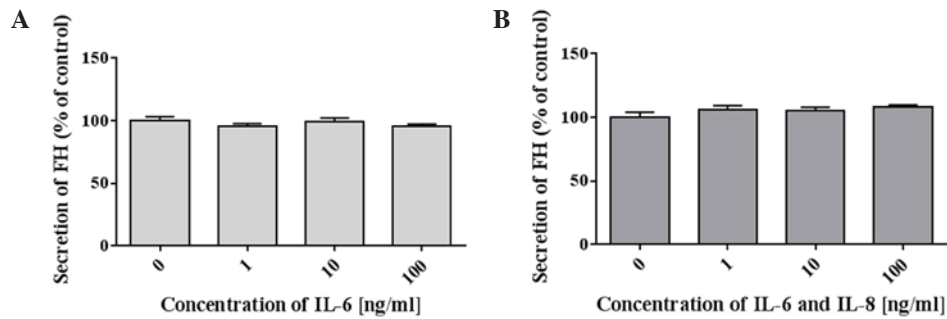


Figure 1. Secretion of FH by A2780 ovarian cancer cells following stimulation with various concentrations of (A) IL-6 and (B) IL-6/IL-8. The results are presented as a percentage of the control untreated cells and the data are presented as the mean \pm standard deviation (n=3). FH, factor H; IL, interleukin.

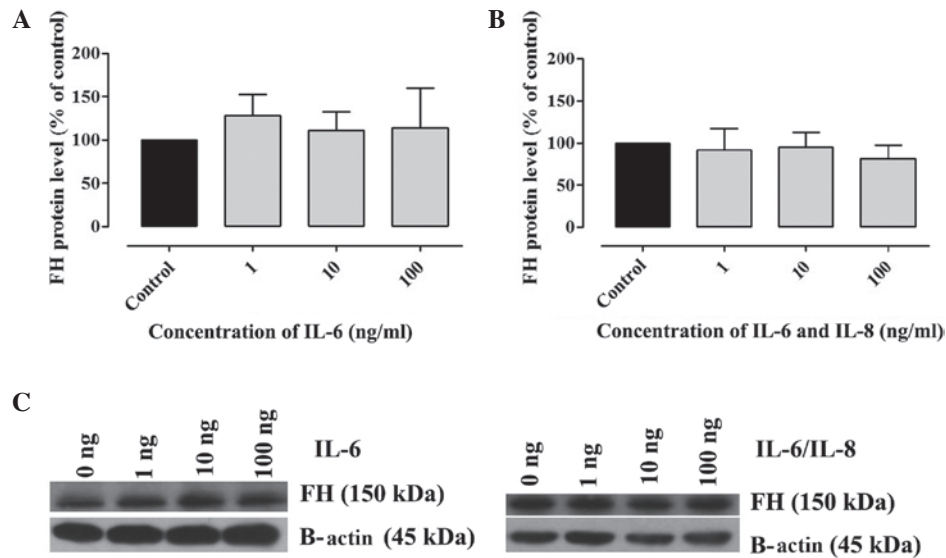


Figure 2. Western blot analysis of the expression levels of FH following incubation with various concentrations of (A) IL-6 and (B) IL-6/IL-8. The graphs show the densitometric analysis, normalized against β -actin. The data are presented as a percentage of the control untreated cells and the data are presented as the mean \pm standard deviation (n=3). (C) Representative western blot of FH and β -actin proteins following stimulation with IL-6 (left) or IL-6/IL-8 (right). FH, factor H; IL, interleukin.

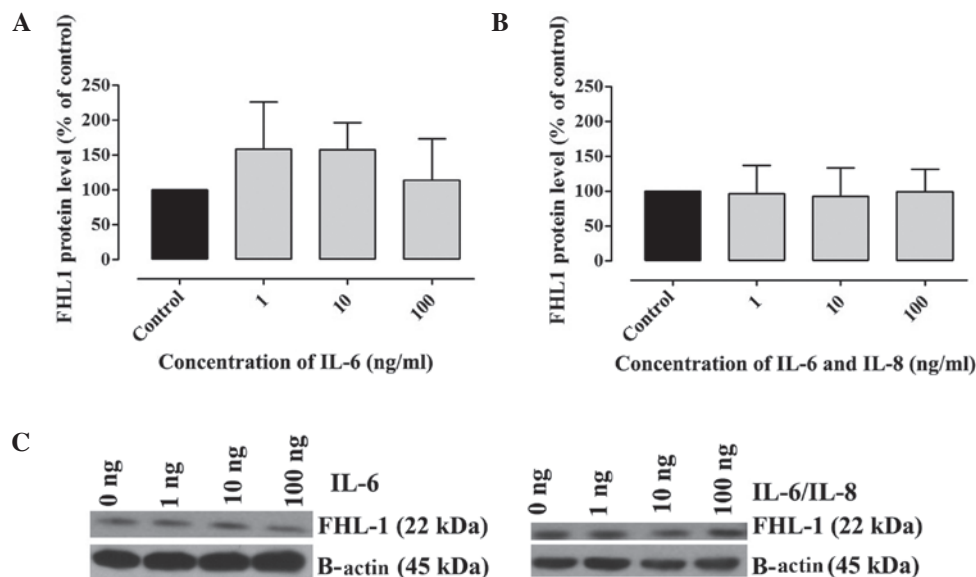


Figure 3. Western blot analysis of the expression of FHL-1 following incubation with various concentrations of (A) IL-6 and (B) IL-6/IL-8. The graphs show the densitometric analysis, normalized against β -actin. The data are presented as a percentage of the control untreated cells and the data are presented as the mean \pm standard deviation (n=3). (C) Representative western blot of FHL-1 and β -actin proteins following stimulation with IL-6 (left) or IL-6/IL-8 (right). FHL-1, factor H-like protein 1; IL, interleukin.

Table II. Gene expression levels in response to stimulation with various concentrations of IL-6 or IL-6/IL-8 as quantified by quantitative polymerase chain reaction.

A, Gene expression of complement factor H

Concentration of IL-6 (ng/ml)	Fold change	Concentration of IL-6/8 (ng/ml)	Fold change
1	1.02±0.13	1	1.09±0.11
10	0.96±0.06	10	1.11±0.11
100	1.04±0.03	100	1.04±0.13

B, Gene expression of complement factor H-like protein 1

Concentration of IL-6 (ng/ml)	Fold change	Concentration of IL-6/8 (ng/ml)	Fold change
1	1.01±0.09	1	1.34±0.69
10	0.87±0.10	10	1.25±0.45
100	1.07±0.11	100	1.36±0.18

β -actin was used as a reference gene against which the data were normalized. Gene expression was calculated using the $2^{-\Delta\Delta C_q}$ method. The data are presented as the mean \pm standard deviation (n=3). IL, interleukin.

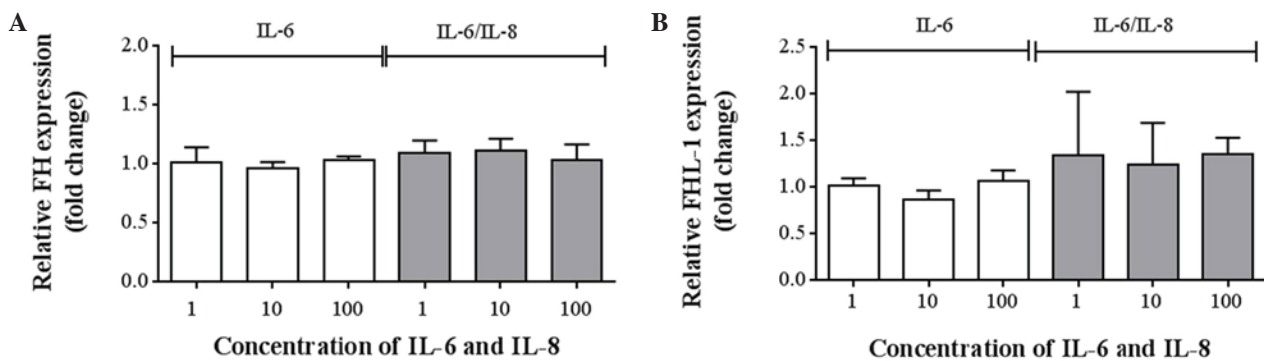


Figure 4. Quantitative polymerase chain reaction analysis of the mRNA expression levels of (A) FH and (B) FHL-1. The graph presents the fold change calculated for the samples incubated with IL-6 and IL-6/IL-8. Sample without interleukin treatment was used as an untreated control and β -actin was used as a reference gene for normalization. The data are presented as the fold change against the control untreated samples and the data are presented as the mean \pm standard deviation (n=3). CFH, complement factor H; CFHR, CFH-related protein 1; IL, interleukin.

and FHL-1 proteins. No significant differences in the concentration of FH (Fig. 2) and FHL-1 (Fig. 3) were observed in the samples incubated with IL-6 alone or IL-6/IL-8 combination, when compared with the control (Fig. 2). However, an upward trend was observed in the concentration of FH and FHL-1 in cell lysates following incubation with IL-6 (Figs. 2A and 3A). A total of three independent tests were performed under the same conditions. In the present study, densitometric analysis results were normalized against β -actin.

mRNA expression levels of FH and FHL-1. To assess the mRNA expression levels of FH and FHL-1, TagMan® Gene Expression assays were performed for CFH and CFHR1. No statistically significant differences were detected between the expression levels of FH (Fig. 4A) and FHL-1 (Fig. 4B), compared with the control (Table II). However, a minimal upward trend was observed in the expression of FHL-1 following incubation with IL-6/IL-8 combined (Fig. 4B; Table II).

A2780 ovarian cancer cell line expresses both membranous and intracellular forms of FH and FHL-1 protein. To assess the cellular localization of the FH and FHL-1 proteins in ovarian cancer cells, immunofluorescence analysis was performed in the A2780 ovarian cancer cell line. Cancer cells were incubated for 24 h with various concentrations of IL-6 alone or IL-6/IL-8 combined. As expected, the protein expression of FH (Figs. 5 and 6) and FHL-1 (Figs. 7 and 8) were detected on the cancer cells membrane.

Discussion

Cancer cells can escape immune surveillance by developing inhibitory mechanisms that provide resistance to immunological recognition and subsequent complement attack (18). Ovarian carcinoma is the most common primary tumor, which leads to the production of free abdominal fluid or ascites (19). It has been widely reported that IL-6

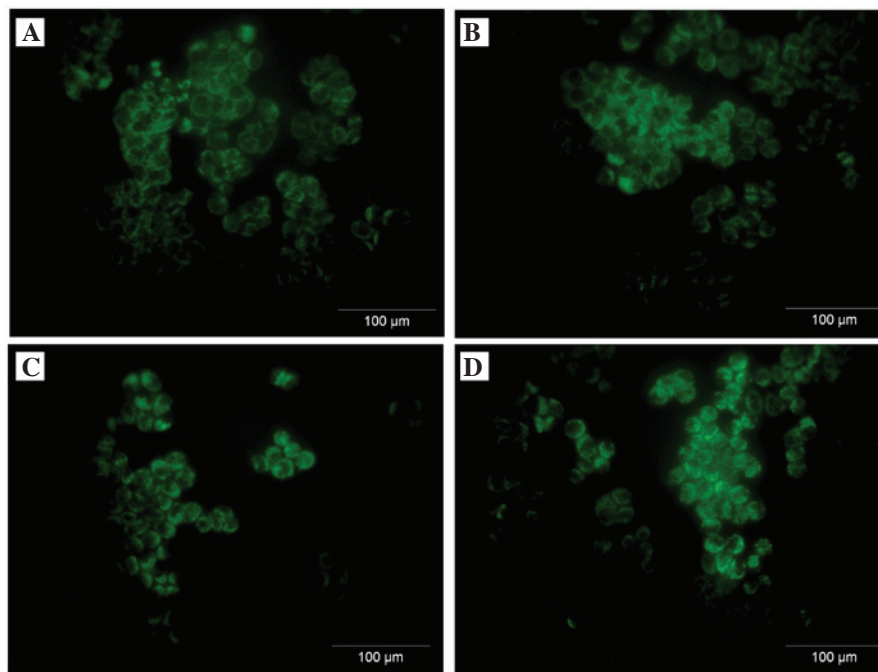


Figure 5. Immunofluorescence staining of factor H protein in the A2780 ovarian cancer cell line after 24 h incubation with various concentrations of IL-6. Representative images of cells treated with (A) 0, (B) 1, (C) 10 and (D) 100 ng/ml IL-6 (magnification, x400). IL, interleukin.

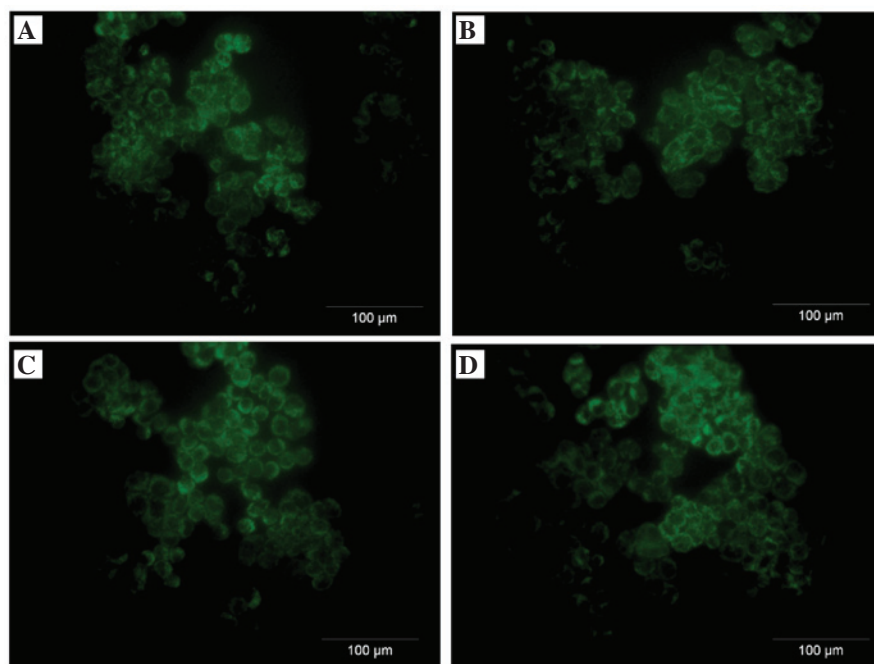


Figure 6. Immunofluorescence staining of factor H protein in the A2780 ovarian cancer cell line after 24 h incubation with various concentrations of IL-6/IL-8. Representative images of cells treated with (A) 0, (B) 1, (C) 10 and (D) 100 ng/ml IL-6 and IL-8 (magnification, x400). IL, interleukin.

is overexpressed in the serum and ascites in patients with ovarian malignancy. Furthermore, the elevated level of IL-6 in these fluids correlates with poor prognosis and survival (14). Wang *et al* (20) demonstrated that IL-6 and IL-8 may promote the cell proliferation of CAOV-3 and OVCAR-3 cells in a time- and dose-dependent manner. Additionally, this cell proliferation induced by IL-6 and IL-8 was suppressed by the use of specific antibodies. However, in the previous study, IL-6 and IL-8 had a synergistic

effect on the proliferation of CAOV-3 cells, however, not on OVCAR-3 cells. This mechanism was not associated with the complement system (20). Unfortunately, the exact role that IL-6 and IL-8 serve in ovarian malignancies remains to be established. The present study attempted to determine the influence of IL-6 and IL-8 on the expression levels of FH and FHL-1.

Certain tumor cells have also been identified to secrete the soluble complement regulators, FH and FHL-1 (19). FH

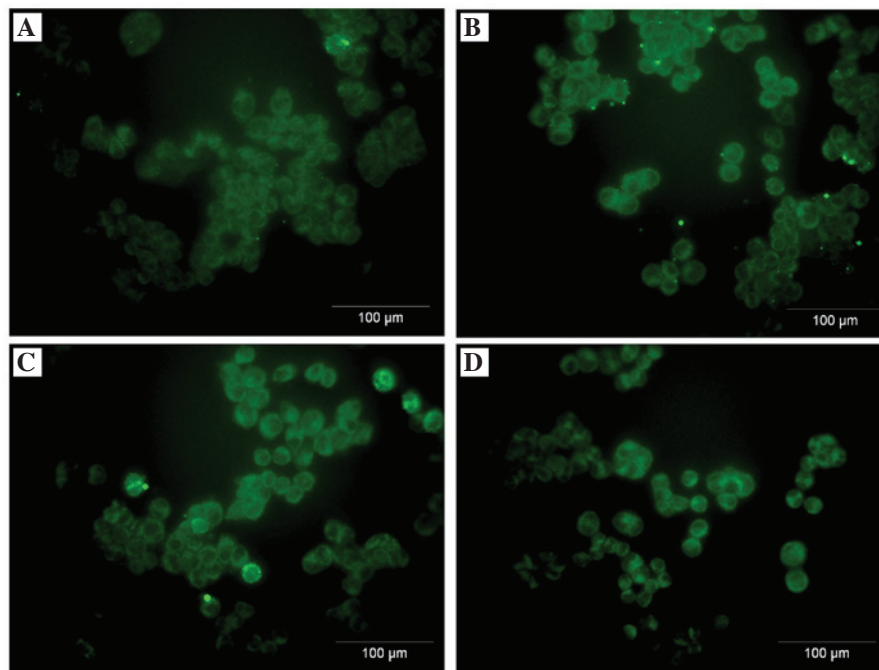


Figure 7. Immunofluorescence staining of factor H-like protein 1 in the A2780 ovarian cancer cell line after 24 h incubation with various concentrations of IL-6. Representative images of cells treated with (A) 0, (B) 1, (C) 10 and (D) 100 ng/ml IL-6 (magnification, x400). IL, interleukin.

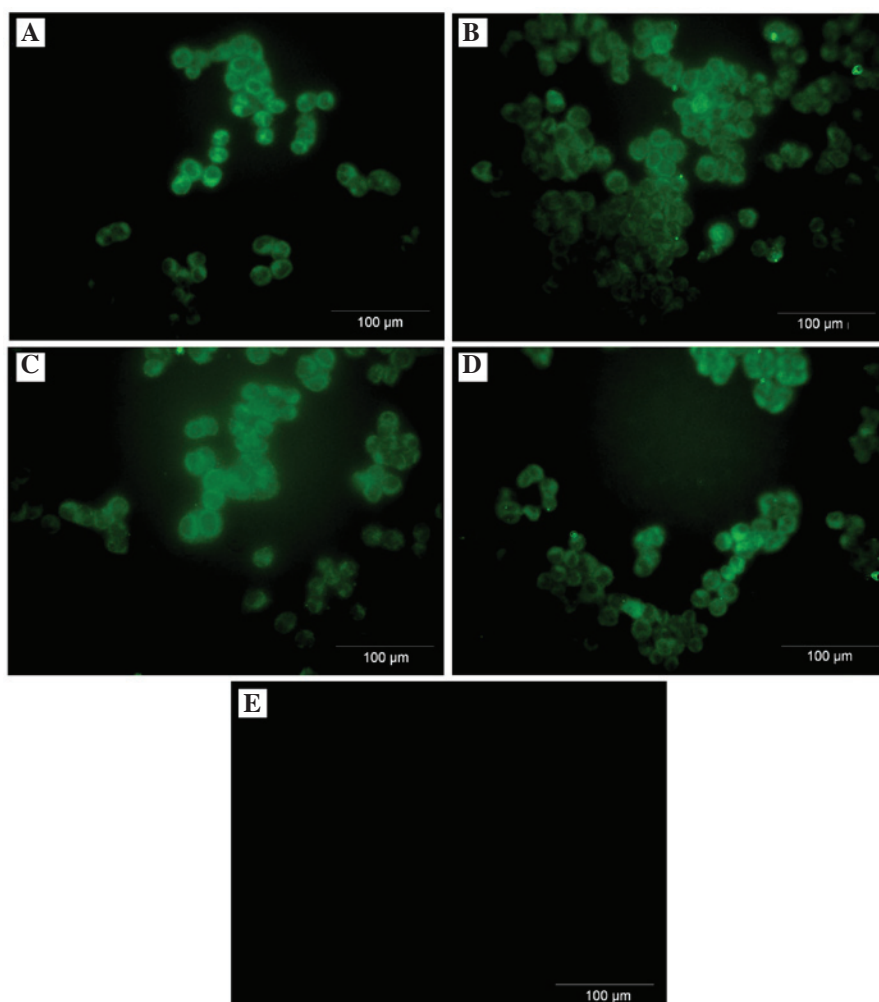


Figure 8. Immunofluorescence staining of factor H-like protein 1 in the A2780 ovarian cancer cell line after 24 h incubation with various concentrations of IL-6/IL-8. Representative images of cells treated with (A) 0, (B) 1, (C) 10, (D) 100 ng/ml IL-6 and IL-8, and (E) control with goat immunoglobulin G. (magnification, x400). IL, interleukin.

is one of the central complement regulators, which belongs to a protein family that includes FHL-1 and five CFHR proteins (21). Junnikkala *et al* (8) demonstrated that ovarian tumor cells produce FH and FHL-1, and additionally that these factors were present in the apical part of the tumor cell layers in tissue sections. The authors revealed that FH and FHL-1 were abundantly present in the ascetic fluids of patients with ovarian cancer, and that a relative proportion of FHL-1 was clearly increased in the malignant ascites specimens (8). Ajona *et al* (18) demonstrated that the majority of non-small cell lung cancer cell lines constitutively produce both CFH and FHL-1 (18).

To the best of the our knowledge, the role of IL-6 and IL-8 on FH and FHL-1 expression in ovarian cancer cells has not been characterized and investigated. In the present results, it was observed that A2780 ovarian cancer cells can secrete inhibitors of the complement system, FH and FHL-1. No differences were observed between the cells incubated with various concentrations of IL-6 and IL-8, and without these cytokines by western blotting and ELISA. The results of western blotting revealed that the protein level of both FH and FHL-1 was not regulated by IL-6 and IL-8. However, in the ELISA, FH, however, not FHL-1, was produced and secreted by ovarian cancer cells, but this process was independent of different doses of IL stimulation. No significant difference in the concentration of FH was detected in the control cells when compared with the samples incubated with IL-6/IL-8. No differences in the mRNA expression levels of FH and FHL-1 were confirmed by qPCR (Table II). Only a minimal upward trend in the expression of FHL-1 was observed following incubation with IL-6 and IL-8 combined (Fig. 4B). Kapka-Skrzypczak *et al* (22) previously demonstrated the results of qPCR analysis of FH and FHL-1 expression in four groups of tissue: Ovarian cancer, normal ovary, endometrial cancer and normal endometrium (22). The authors detected no differences between the expression of FH and FHL-1 in all experimental groups, particularly between normal and cancer tissues (22).

In vitro conditions differ from *in vivo* conditions. In the *in vivo* tumor microenvironment, the affect of IL-6 and IL-8 is rather constant. IL-6 is secreted by mesothelial cells, fibroblasts, macrophages, ovarian tumor cells, and IL-8 is secreted by endothelial cells, mesothelial cells, monocytes and ovarian tumor cells (23). Tumor microenvironment is involved in all processes of ovarian cancer progression (23). Based on the present results, it was concluded that A2780 cells express FH and secrete this protein into the environment, however, it is independent of IL-6 and IL-8. Additionally, these cancer cells are able to bind FH and FHL-1 to their cell membrane. The present study demonstrated the binding of the soluble complement regulators, FH and FHL-1, to the surface of ovarian cancer cells. Binding of FH to cell surfaces is a composite and complicated occurrence (24). FH protein is composed of 20 short consensus repeat (SCR) domains. Two functional regions are located at the N- and C-terminal of the FH. SCRs 1-4 N-terminal domains mediate the complementary regulatory activities of FH, and the C-terminal domains SCRs 19-20 are responsible for target recognition. The SCRs 19-20 allow the attachment of FH to cancer cells, and also inhibit the complement

activation directly at the cell surface (25). Binding of FH to cell surfaces is relevant for the protection of cancer cell membranes and surfaces from unwanted complement activation (24). Based on the results form the present study, it was determined that IL-6 and IL-8 enhance the binding of FH to the membranes of cancer cells. It is likely that this process may be important with regards to the enhancement of the efficacy of complement-mediated immunotherapy.

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