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Angiogenic cytokines VEGF, TGF-β1, IL-8 and TNF secretion by human ovarian cancer cells

Wydzielanie angiogennych cytokin VEGF, TGF-β1, IL-8 i TNF przez ludzkie komórki nowotworów jajnika

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Abstract

Objectives: Angiogenesis is a process that is indispensable in cancer progression. A complex network of tumor and microenvironment stimuli regulate angiogenesis. VEGF, TGF-β1, IL-8 and TNF belong to the angiogenic factors that are key points in vessel formation. The aim of the study was to assess h-VEGF, TGF-β1, IL-8 and TNF secretion by human ovarian cell lines. **Material and methods:** OVA 2, OVA 4, OVA 9, OVA 11 and OVA 14 cell lines were established in our laboratory. The cells derived from primary and metastatic tumors of epithelial and non-epithelial origin. SK-OV-3, MDAH 2774, CAOV-1 and OVP-10 were the cell lines obtained from other sources. The concentration of VEGF, TGF-β1 and IL-8 was determined in culture supernatants by using the ELISA tests. **Results:** OVA 11 secreted all the evaluated cytokines. MDAH 2774 was the source of h-VEGF, TGF-β1, IL-8. SK-OV-3 secreted h-VEGF and IL-8. OVA 4 secreted TGF-β1 and TNF. TNF was the only studied cytokine secreted by CAOV-1, OVA 2 and OVA 9 cell lines. OVA 14 did not secret any of the cytokines. **Conclusions:** The investigated cell lines present heterogeneous profile of angiogenic cytokine secretion and seem to be an interesting set of models for the study of angiogenic signaling, or target therapy.

Keywords: VEGF, TGF-β, IL-8, TNF, ovarian cancer, cell line

Streszczenie

Cel: Angiogeneza jest procesem niezbędnym do progresji raka. Złożona sieć bodźców pochodzących od guza i z mikrośrodowiska reguluje angiogenezę. VEGF, TGF-β1, IL-8 i TNF należą do czynników angiogennych, które odgrywają kluczową rolę w tworzeniu naczyń. Celem pracy była ocena wydzielania h-VEGF, TGF-β1, IL-8 i TNF przez ludzkie linie raka jajnika. **Materiał i metoda:** Linie OVA 2, OVA 4, OVA 9, OVA 11 oraz OVA 14 zostały ustalone samodzielnie. Komórki pochodziły z pierwotnych lub przerzutowych guzów jajnika pochodzenia nabłonkowego lub nienabłonkowego. Linie SK-OV-3, MDAH 2774, CAOV-1 oraz OVP-10 pochodziły z innych źródeł. Stężenie VEGF, TGF-β1 i IL-8 określano w supernatantach hodowli komórkowych w teście ELISA. **Wyniki:** Linia OVA 11 wydzielala wszystkie badane cytokiny. Linia MDAH 2774 była źródłem h-VEGF, TGF-β1, IL-8. Linia SK-OV-3 wydzielala h-VEGF oraz IL-8. Linia OVA 4 wydzielala TGF-β1 i TNF. TNF był jedyną cytokiną wydzielaną przez linie CAOV-1, OVA 2 oraz OVA 9. Linia OVA 14 nie wydzielala żadnej spośród badanych cytokin. **Wnioski:** Badane linie komórkowe stanowią heterogenną grupę nowotworów wydzielających cytokiny o właściwościach angiogennych i wydają się interesującym panelem do badań nad procesami angiogenezy czy terapii celowanej.

Słowa kluczowe: VEGF, TGF-β, IL-8, TNF, rak jajnika, linie komórkowe

INTRODUCTION

Angiogenesis is a process involving the growth of new blood vessels from pre-existing vessels and occurs in normal processes related to the female reproductive cycle, and in pathological processes such as tumor growth and metastasis⁽¹⁾. A tumor requires a continuous stimulation of growth of new capillary blood vessels for the tumor itself to grow. Furthermore, the new blood vessels embedded in a tumor provide a gateway for tumor cells to enter the circulation and to metastasize to distant sites⁽²⁾.

Tumor angiogenesis is regulated by numerous stimulators and inhibitors of both tumor and host origin. Vascular endothelial growth factor (VEGF) is one of the most important regulators of angiogenesis. VEGF acts at most stages of angiogenesis: stimulates extracellular matrix degradation and endothelium proliferation and migration, increases vascular permeability resulting in spillage of plasma proteins, such as fibrinogen, into the perivascular environment, which contributes to a provisional matrix and regulates vascular remodeling⁽³⁾.

Transforming growth factor-beta (TGF- β) is implicated in diverse physiologic and pathophysiologic functions including immunological, inflammatory, and neoplastic processes. It is a multipotent growth factor affecting tumor proliferation, apoptosis and angiogenesis^(4,5). The angiogenic activity of TGF- β is mainly indirect. TGF- β stimulates monocyte attraction and VEGF secretion, and induces matrix metalloproteinases⁽⁶⁾.

Interleukin-8 (IL-8) is another cytokine involved in tumor growth, angiogenesis, and metastasis. IL-8 stimulates VEGF expression but also directly enhances endothelial cell proliferation, survival, and metalloproteinases expression endothelial cells^(7,8).

Tumor necrosis factor- α (TNF) is a multipotent cytokine involved in apoptosis, cellular proliferation, differentiation, inflammation, tumorigenesis, and angiogenesis⁽⁹⁾. TNF generally stimulates angiogenesis through fibroblast

growth factor (FGF), VEGF and IL-8 activation^(10,11). However, under certain conditions TNF inactivates integrin $\alpha_v\beta_3$, which in turn results in apoptosis induction and angiogenesis inhibition⁽¹²⁾. There is strong evidence that at least some cancer cells can secrete cytokines that promote angiogenesis^(4,7,13,14). The aim of the study was to report a secretion profile of angiogenic cytokines (VEGF, TGF- β 1, IL-8 and TNF) in human ovarian cell lines of different origin.

MATERIAL AND METHODS

Human ovarian cancer cells

OVA 2, OVA 4, OVA 9, OVA 11 and OVA 14 cell lines established in our laboratory and SK-OV-3, MDAH 2774, CAO-1 and OVP-10 cell lines were investigated. The SK-OV-3 and MDAH 2774 lines were originally obtained from the American Type Culture Collection (ATCC number HTB 77 and CRL 10303, respectively). The CAO-1 line was established at the Jagiellonian University in Krakow and the OVP-10 was established at the Institute of Oncology in Warsaw. Five primary cell lines were isolated from tumors during surgical operations and cultured in our laboratory. The OVA 2, OVA 4, OVA 11 and OVA 14 cell lines derived from the primary ovarian cancers and the OVA 9 was a metastasis of melanoma to the ovary. The detailed characteristics of the cell lines established in our laboratory are described in Tab. 1.

All the cell lines were cultured in Dulbecco's MEM with 4.5 g/L glucose, sodium pyruvate and Glutamax-1 (high glucose DMEM) supplemented with antibiotic-antimycotic, 50 μ M 2-mercaptoethanol and 10% fetal calf serum (FCS) (all from Gibco BRL, Life Technologies, Paisley, UK). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

The primary cell lines were frozen for further evaluation immediately after they revealed a stable growth. All lines were tested for *Mycoplasma* contamination.

Signature	Cell line			Tumor		
	Time to 1 st passage (days)	Number of passages	Cell culture doubling time (hours)	Histopathology	Grade	Stage*
OVA 2	60	40	40	Serous adenocarcinoma	G3	IIIC
OVA 4	2	30	24	Endometrioid adenocarcinoma	G2	IC
OVA 9	120	20	40	Melanoma malignum	G3	-
OVA 11	120	6	40	Granulosa cell tumor	G2	IA
OVA 14	2	8	30	Serous adenocarcinoma	G1	IIIA

* FIGO 1986.

100 Tab. 1. Characteristics of cell lines established in our laboratory

Preparation of supernatants

Tumor cells were seeded in 24-well plates for 24 h (4×10^5 cells/mL) in 2 mL of culture medium described above at 37°C, 5% CO₂. Then, the supernatant was detached and frozen for further assessments.

ELISA for human VEGF, TGF- β 1, IL-8 and TNF expression

The concentration of VEGF, TGF- β 1 and IL-8 protein in culture supernatants was determined by using quantitative immunometric sandwich enzyme immunoassay (ELISA) kits (BioSource International, USA). A polyclonal antibody specific for human VEGF, TGF- β or IL-8 was coated onto the wells of the microtiter strips. The samples of supernatant were pipetted into the wells and incubated for 2 hours at room temperature. After washing, a biotinylated monoclonal antibody specific for human VEGF (or TGF- β or IL-8, respectively) was added and incubated for 1 hour at room temperature. After removal of excess second antibody, streptavidin-peroxidase complex was added. After a third incubation (30 minutes at room temperature) and washing to remove all the unbound enzyme, a substrate solution (tetramethylbenzidine) was added and incubated for 30 minutes in the dark. The absorbance of each sample was read in Spectra II ELISA microplate reader at 450 nm. The concentrations of the evaluated cytokines were read from the standard curves plotted from the absorbance of the standards used in each experiment. Only the samples for TGF- β 1 measurement required preincubation with the extraction solution (30 minutes at 4°C) to extract TGF- β 1 from latent complexes. The concentration of human TNF in culture supernatants was measured using a DuoSet ELISA development system (R&D Systems, Minneapolis, Minn.) in the way similar to the one described above according to the protocol provided by the manufacturer.

Statistical analysis

Statistical evaluations were performed with Statistica 7.0 software (StatSoft, Poland). Comparisons between groups of continuous outcomes were performed by Student's *t*-test after testing for normal distribution by the Kolmogorov-Smirnov test. $P < 0.05$ was considered significant.

RESULTS

The granulosa cell tumor line (OVA 11) and some epithelial cancer cell lines (SK-OV-3 and MDAH 2774) spontaneously secreted h-VEGF. OVA 11 and SK-OV-3 cell lines presented high h-VEGF secretion, compared with a moderate h-VEGF secretion in MDAH 2774 cell line. h-VEGF concentration in cell line culture supernatants was: OVA 11 – 859.0 ± 0.8 pg/mL, SK-OV-3 – 625.1 ± 66.8 pg/mL,

MDAH 2774 – 128.2 ± 0.5 pg/mL and was significantly higher than in control (12.6 ± 7.7 pg/mL).

TGF- β 1 was spontaneously secreted by OVA 4, OVA 11, MDAH 2774 and OVP-10 cell lines. The highest TGF- β 1 concentration was observed in OVP-10 (632.64 ± 18.7 pg/mL) supernatant and was approximately twice higher than in OVA 11 (330.95 ± 3.6 pg/mL). TGF- β 1 secretion was comparable in OVA 4 and MDAH 2774 cell line. The supernatant TGF- β 1 concentration were: OVA 4 – 240.8 ± 3.1 pg/mL, MDAH 2774 – 246.3 ± 0.05 pg/mL. TGF- β 1 concentration in control was 202.4 ± 4.0 pg/mL.

IL-8 spontaneous secretion was found in OVA 11, SK-OV-3 and MDAH 2774 cell lines. Supernatant concentration of IL-8 was: 1880.2 ± 30.9 pg/mL in OVA 11, 1863.9 ± 72.6 pg/mL in SK-OV-3 and 93.9 ± 4.1 pg/mL in MDAH 2774 cell line culture. IL-8 concentration in control was 11.0 ± 0.96 pg/mL (Fig. 1).

The secretion of TNF was observed in both epithelial (CAOV-1, OVA 2) and non-epithelial (OVA 9 and OVA 11) ovarian cancer cell lines.

Spontaneous TNF secretion was the highest in CAOV-1 cell line (9183.7 ± 49.96 pg/mL). Supernatant TNF concentration in other TNF-secreting-lines was as follows: OVA 11 – 6193.2 ± 284.2 pg/mL, OVA 9 – 3536.1 ± 103.7 pg/mL and OVA 2 – 2095.8 ± 20.8 pg/mL. OVA 4, OVA 14, MDAH 2774, SK-OV-3 and OVP-10 did not secrete TNF (Fig. 2).

DISCUSSION

Tumor growth *in vivo* is determined not only by the proliferative potential of cancer cells but also depends on various tumor-environment interactions⁽¹⁵⁾. Angiogenesis and host immune response are the processes that are considerably regulated by the tumor and play a fundamental role in carcinogenesis⁽¹⁶⁾. One of the key factors involved in angiogenesis is VEGF. It acts in many cell metabolic pathways, which in turn makes VEGF the most prominent target of antiangiogenic agents in cancer therapy⁽¹⁷⁾. VEGF is a mediator for other cytokines with high angiogenic activity, including TGF- β 1 and IL-8^(3,18). IL-8 is also a potent autocrine growth factor⁽¹⁹⁾, which in association with immunosuppressive and antiapoptotic activity of TGF- β 1⁽²⁰⁾ makes the secretion profile of cancer cells producing these cytokines unfavorable for the host. IL-8 confers cisplatin and paclitaxel resistance in ovarian cancer cells⁽²¹⁾.

In our study, spontaneous h-VEGF secretion was observed in both epithelial (SK-OV-3 and MDAH 2774) and non-epithelial (granulosa cell tumor – OVA 11) ovarian cancer cell lines. IL-8 was secreted by the same cell lines. Sixty-six percent of studied cell lines secreted neither h-VEGF nor IL-8. Sonoda *et al.* found VEGF expression in all the 5 examined ovarian cancer cell lines and IL-8 expression in 2 lines⁽²²⁾.

OVA 11 and SK-OV-3 lines secreted 5 to 6 times more h-VEGF and 20 times more IL-8 than MDAH 2774, which

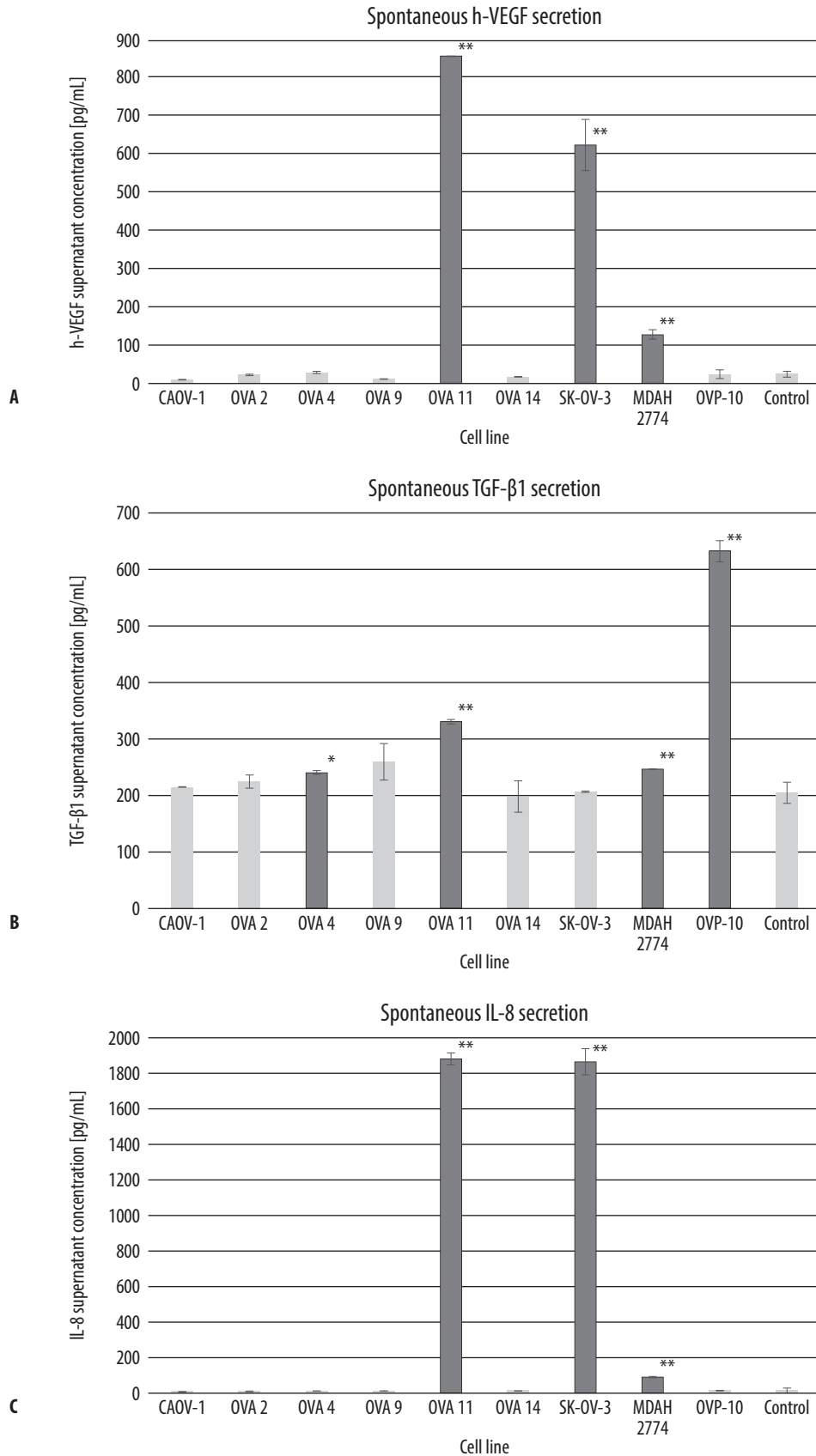


Fig. 1. Spontaneous A. h-VEGF, B. TGF-β1 and C. IL-8 secretion. Tumor cells (2×10^5 cells/mL) were incubated for 24 h. Cytokine concentration was measured in supernatant. Values are mean \pm SD. * $P < 0.001$, ** $P < 0.0001$

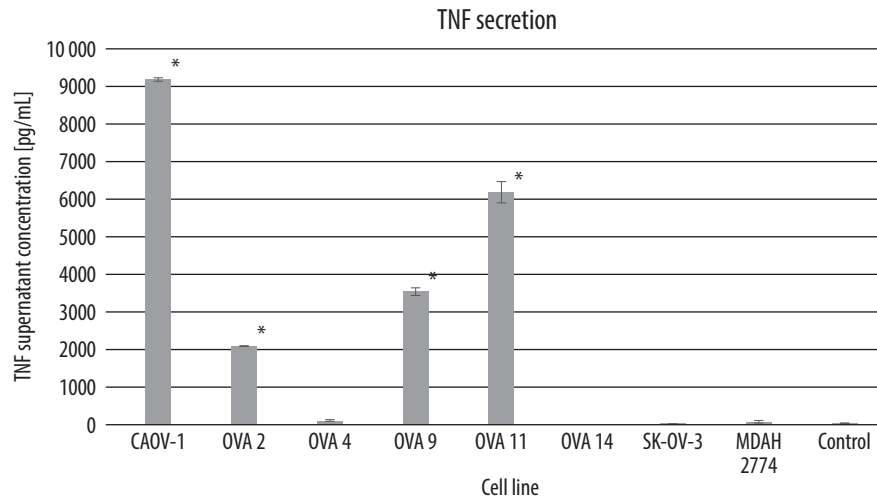


Fig. 2. Spontaneous TNF secretion. Tumor cells (2×10^5 cells/mL) were incubated for 24 h. Cytokine concentration was measured in supernatant. Values are mean \pm SD. * $P < 0.0001$

can make them highly VEGF-IL-8 expressed cell lines compared with MDAH 2774. Wang *et al.* demonstrated that IL-8 secreted by ovarian cancer cells promotes malignant behavior of these cells, alters cell cycle distribution, and enhances cell invasiveness⁽²³⁾. Desai *et al.* found the cooperation of autocrine IL-8 and VEGF in epithelial-mesenchymal transition and invasiveness⁽²⁴⁾. In light of those studies, OVA 11, SK-OV-3 and MDAH 2774 seem to be good candidates for a model for research of distinct VEGF-IL-8 secreting ovarian cancer cell lines.

TGF- β 1 was secreted in our study by 4 out of 9 cell lines. Two lines were established in our laboratory (endometrioid cancer – OVA 4 and granulosa cell tumor – OVA 11). Only in OVP-10 cell line culture TGF- β 1 supernatant concentration was about 3 times higher than in medium control. OVA 4, OVA 11 and OVP-10 secreted statistically significant, but relatively small amounts of TGF- β 1. Our findings are consistent with the study of Inan *et al.* who observed overexpression of TGF- β 1 and TGF- β 2 in immunohistochemically stained epithelial ovarian tumor tissue blocks⁽²⁵⁾. Gordinier *et al.* confirmed overexpression of TGF in both primary and metastatic tumor specimens and suggested that tumor cells expressing both human leukocyte antigen (HLA) and TGF- β may be suitable targets for adaptive immunotherapy⁽²⁶⁾. Wei *et al.* silenced *TGF- β 1* gene in ovarian cancer cells, which generated a stronger Th1/Tc1 immune response of peripheral blood mononuclear cells in *in vitro* studies⁽²⁷⁾. Autocrine secretion of TGF- β 2 remains a point of interest, as this cytokine induces a reactive stroma, which is necessary to stimulate angiogenesis⁽²⁸⁾.

TNF remains another important agent involved in angiogenesis. Autocrine action of TNF generates and sustains a network of other mediators including VEGF that promote angiogenesis, peritoneal cancer growth and spread^(29,30). TNF was spontaneously secreted in our study by four

cell lines. Two of them were epithelial ovarian cancers, and the two others were metastatic melanoma and granulosa cell tumor, respectively.

It seems important to investigate autocrine TNF action in association with other angiogenic agents as some authors suggest TNF-induced apoptosis by affecting the interactions between cells and the basal membrane, which can have an antiangiogenic result.

It is noteworthy that OVA 11 was the only line that secreted all studied cytokines. On the other hand, each line, except for OVA 14, was the source of at least one agent that plays a key role in angiogenesis.

CONCLUSIONS

The cell lines investigated in this study, both the ones established in our laboratory and the reference ones, constitute a set of human ovarian cell lines that present different secretion profiles of main angiogenic factors and can be used as different models for studying angiogenesis.

Conflict of interest

The authors claim neither financial nor personal relations with other persons or organizations, which could adversely affect the content of the publication, or claim their right thereto.

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