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Tumor necrosis factor-α and its receptors in epithelial ovarian cancer

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Abstract: The aim of the present study was to characterize the expression pattern of tumor necrosis factor (TNF)-α and its receptors (TNF-Rs) in the epithelial ovarian cancer (EOC) and compare these results with the outcome of 126 patients. Presence of TNF-α, TNFR-1 and TNFR-2 were studied by Western blotting and immunohistochemistry. The proportion of samples positive for TNF-α and TNF-R2 was higher in epithelial ovarian cancer patients than in benign ovarian diseases ($p<0.001$ and $p=0.016$, respectively). Immunostaining intensity of TNF-R2 were correlated with tumor stage ($p<0.001$) and with reduced mean survival time (MST) ($p=0.002$). The results of the present study suggested that tissue expression of TNF-R2 in epithelial ovarian cancer was correlated with the highest risk of cancer progression. Thus, the clinical value of activated TNF system in epithelial ovarian cancer needs to be further investigated.

Keywords: tumor necrosis factor-α (TNF-α); tumor necrosis factor receptor 1 (TNF-R1); tumor necrosis factor receptor 2 (TNF-R2); epithelial ovarian cancer (EOC)

Introduction

Epithelial ovarian cancer comprises the majority of malignant ovarian tumors in adult women. About 190,000 new cases and 114,000 deaths from ovarian cancer are estimated to occur annually [1].

Surgical determination of tumor stage is the most important prognostic factor. Early stage disease has a very good prognosis. Overall, five year survival rates for all stages were in the 30 50% range. Most women, however, present with late stage disease which is associated with a five-year survival rate of about 20% [2].

The pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α), is disturbed in malignant tumors compared with normal ovarian surface epithelium. Several studies associated inflammation with ovarian tumorigenesis, with TNF-α playing a key role in modulating invasion, angiogenesis and metastasis [3,4].

The biological activity of TNF-α can be modulated by two distinct receptors, named TNF-R1 (p55TNF) and TNF-R2 (p75TNF), both of which show a similar affinity for TNF-α in humans [5,6].

Several reports associated detection of abnormally high levels of TNF-α in the blood of ovarian cancer patients with a wide range of tumor types [7-9]. However, circulating TNF-α is not always detectable in cancer patients and can vary within individual patients over time and course of disease [10,11].

Regulation of TNF-Rs is critical to tumor cell responsiveness to TNF-α, therefore, tumor tissue expression of TNF-α might be more relevant than blood levels in explaining pro-tumorigenic associations. TNF-R1 is the major mediator of most TNF-α activities, including apoptosis. TNF-R2 is activated by the membrane-integrated form and the binding of ligand to TNF-R2 leads to activation of the p38 mitogen-activated protein kinase, c-Jun N-terminal kinase, nuclear factor (NF)-κB, extracellular signal

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regulated kinase and phosphatidylinositol-3-kinase pathway to mediate proliferation of certain cells [12-14].

The influence of TNF-α and TNF-Rs on the behavior of epithelial ovarian cancer is unknown. The aim of the present study was to characterize the expression pattern of TNF-α and its receptors in the epithelial ovarian cancer and compare these results with the patients' outcome.

Materials and methods

Tumor samples. A total of 126 patients with epithelial ovarian cancer (ages 18-79 years; mean age – 58.3 years), all treated at the Department of Gynecology District Hospital in Bialystok (Poland) were included in this study. Tumor samples were compared with 25 benign samples, consisting of 6 normal tissue and 19 cystadenomas of the ovary from women (ages 19-71 years; mean age – 52.8 years) served as controls.

In each case the tumor stage was established according to the criteria of the International Federation of Gynecology and Obstetrics (FIGO) and the histological typing system of the World Health Organization (WHO), respectively. Histologically, 64 patients (50.8%) had a serous adenocarcinoma, 18 (14.3%) – a mucinous adenocarcinoma, 26 (20.6%) – an endometrioid adenocarcinoma and there were 18 (14.3%) cases of other types (therein 10 cases of clear cell carcinomas and 8 of different histologic types).

Tissue specimens were obtained at the primary surgery and before the start of the adjuvant radiation or chemotherapy. Each sample was divided into two portions: one portion was processed immediately for immunohistochemistry, and the second one was frozen in liquid nitrogen and maintained at -70°C for Western blot analysis. The primary antibodies used were goat antihuman TNF-α, TNF-R1 and TNF-R2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immunohistochemistry. Immunohistochemistry for TNF-α and TNF-Rs was performed on formalin-fixed paraffin-embedded tissue blocks in all the cases. Four-micrometer sections of specimens were deparaffinized in xylene and hydrated in a graded series of alcohols. Antigen retrieval was performed by immersing material in 0.1 M citrate buffer (pH 6), and placing it into a microwave oven for 20 minutes. Endogenous peroxidase activity was inactivated by incubation in 3% H_2O_2 for 10 minutes at room temperature. After rinsing in PBS buffer (pH 7.4) Tris, the sections were incubated with primary antibodies against TNF-R1(1:250) and TNF-R2 (1:50) at 4°C overnight, and TNF-α (1:50), at 37°C for 2 hours. All sections were sequentially treated with biotinylated anti-rabbit or mouse immunoglobulin (DAKO) for 20 minutes at 37°C and peroxidase-labelled streptavidin for 20 minutes at 37°C. A negative control was replaced by phosphate-buffered saline. All sections were counterstained with 3, 3'-diaminobenzidine (DAB, Sigma) followed by hematoxylin nuclear counterstaining. Cases were considered positive for TNF-α or TNF-Rs if cytoplasmic immunoreactivity was present in >10% of tumor cells.

Western blot. For Western blot analysis, tissue samples were suspended in 0.05 M Tris-HCl buffer (pH 7.6) in the ratio 1:3 (wt/vol). The homogenates were prepared with a knife homogenizer (25000 rpm for 45 seconds at 4°C) and sonicated (20 kHz, 4 × 15 seconds at 4°C). After centrifugation (10000 × g for 15 minutes at 4°C), supernatants were stored at -70°C until measurements were performed.

The protein concentration of supernatants was calculated using the Bradford method. Supernatants were then equilibrated with a loading buffer (10% sodium dodecylsulfate [SDS] in Tris-HCl [pH 8], containing 50% glycerol, 0.1 mM 2-β-mercaptoethanol and 0.1% bromophenol blue) at 50 μg/mL. The mixture was then denatured for 5 minutes at 100°C and samples containing 20 μg of protein were subjected to electrophoresis. The following molecular mass standards were used: 75, 55 and 17 kDa (Bio Rad Laboratories, USA). The gels were allowed to equilibrate in 25 mM Tris, 0.2 M L-glycine, 20% (vol/vol) methanol for 5 minutes and the proteins were transferred to 0.2 μm pore diameter nitrocellulose membranes at 100 mA for 1 hour. The membranes were then incubated with one of the following primary antibodies (Santa Cruz Biotechnology, USA) at dilutions: TNF-α (1:500), TNF-R1 (1:1000) and TNF-R2 (1:250) in 5% dried defatted milk in TBS-T (20 mM Tris-HCl buffer, pH 7.4; 150 mM NaCl; 0.05% (vol/vol) Tween 20) for 1 hour. Species-specific secondary antibodies were then added at 1:7500 dilutions. Incubation was continued for 30 minutes with slow shaking. Then, nitrocellulose membranes were washed with TBS-T (5 times for 5 minutes) and treated with Sigma-Fast BCIP/NBT reagent (Sigma Aldrich, Germany). The labeled membranes were photographed, scanned, and optical density was analyzed using imagining QuantityOne software (Bio Rad Laboratories, USA).

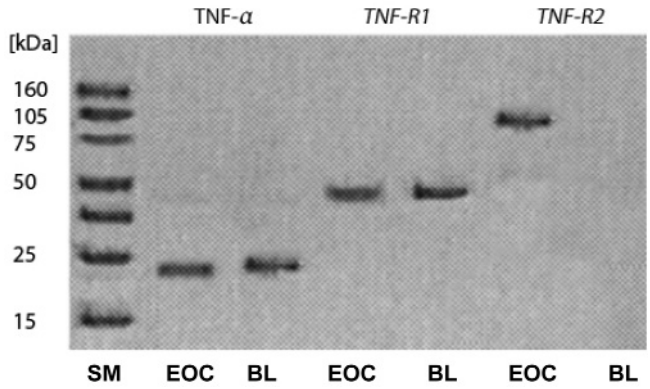

Fig. 1. Western blott analysis of tumor necrosis factor α (TNF-α), TNF receptor 1 (TNF-R1) and TNF receptor 2 (TNF-R2); benign lesions (BL); epithelial ovarian cancer (EOC).

Ethical issues. Patients were informed and gave their consent for the study. The protocol was previously approved by the Bioethical Committee of the Medical University of Bialystok (R-I-003/229/2003).

Statistical analysis. Statistical analysis was performed using Statistica software version 8.0 (StatSoft, Inc., StatSoft Polska Sp. z o.o., Poland) using Fisher's exact test. $p<0.05$ was considered statistically significant.

Results

Western blotting

For each antibody used, a single band was found at the corresponding molecular mass: TNF-α (17kDa), TNF-R1 (55kDa) and TNF-R2 (75 kDa) (Fig. 1). These bands appeared in both groups, except for TNF-R2 in benign lesions, which often was undetectable using this technique.

Table 1. Immunoexpression of tumor necrosis factor-α, tumor necrosis factor receptors 1 and 2 in epithelial ovarian cancer and benign lesions.

	No. of cases	TNF-α positive cases n (%)	TNF-R1 positive cases n (%)	TNF-R2 positive cases n (%)
EOC	126	126 (100)	126 (100)	64 (51)
Benign lesions	25	21 (84)	25 (100)	6 (24)
p-value		<0.001	n.s.	0.016

EOC – epithelial ovarian cancer; TNF-α – tumor necrosis factor-α; TNF-R1 – tumor necrosis factor receptor 1; TNF-R2 – tumor necrosis factor receptor 2; n.s. – not significant

Table 2. Immunoexpression of tumor necrosis factor-α, tumor necrosis factor receptors 1 and 2 epithelial ovarian cancer in a different FIGO stage.

	Stage	No. of cases	TNF-α positive cases n (%)	TNF-R1 positive cases n (%)	TNF-R2 positive cases n (%)
EOC	I-II	53	53 (100)	53 (100)	16 (30)
	III-IV	73	73 (100)	73 (100)	48 (66)
p-value			n.s.	n.s.	<0.001

EOC – epithelial ovarian cancer; TNF-α – tumor necrosis factor-α; TNF-R1 – tumor necrosis factor receptor 1; TNF-R2 – tumor necrosis factor receptor 2; I-IV – stage of epithelial ovarian cancer according to International Federation of Obstetrics and Gynecology (FIGO) criteria; n.s. – not significant

Table 3. Immunoexpression of tumor necrosis factor-α, tumor necrosis factor receptors 1 and 2 in patients with epithelial ovarian cancer in correlations with mean survival time.

MST (years)	No. of cases	TNF-α positive cases n (%)	TNF-R1 positive cases n (%)	TNF-R2 positive cases n (%)
<5	69	69 (100)	69 (100)	44 (64)
>5	57	57 (100)	57 (100)	20 (35)
p-value		n.s.	n.s.	0.002

MST – mean survival time; TNF-α – tumor necrosis factor-α; TNF-R1 – tumor necrosis factor receptor 1; TNF-R2 – tumor necrosis factor receptor 2; n.s. – not significant

Immunohistochemistry

No immunoreaction was observed in negative controls incubated with preimmune serum or using the antibodies preabsorbed with an excess of purified antigens. Staining of thymus sections (positive controls) was always positive for all of the antibodies used. For each antibody assayed, the percentage of positive cases and the immunostaining intensities are shown in Table 1.

In the samples positive for TNF-α, labeling was always observed in the cytoplasm of epithelial cells. Statistical differences were found in the percentage of the positive cases between the different groups (Table 1) ($p<0.001$).

All samples were positive to TNF-R1 (Table 1). Labeling was observed in the peripheral cytoplasm of epithelial cells. In 64 samples positive to TNF-R2 (51%) (Table 1) ($p=0.016$), labeling was observed in the epithelial cell cytoplasm (Fig. 2A). The percentage of cases showing positive immunoreaction to TNF-R2 increased with the tumor FIGO stage: I-II (30%) compared to III-IV (66%) and was statistically significant (Table 2) ($p<0.001$).

The relationship between TNF-α, TNF-R1, TNF-R2 immunoexpression and patients' outcome was investigated. Patients with positive immunoreactions to TNF-R2 had a significantly shorter mean survival time ($p=0.002$) (Table 3).

Discussion

It has been reported that TNF-α can exert either pro-apoptotic or survival and proliferation effects, so it is necessary to clarify the role that TNF-α is playing in the different tissues and pathological conditions [15]. It seems that in a normal ovarian tissue, TNF-α regulates cell proliferation through its pro-apoptotic effects, but in the ovarian cancer, both inhibitions of the TNF-R1 apoptotic pathway and the increase in TNF-R2 survival and proliferation effects might be related to the enhanced cell proliferation [7]. The disagreement that this cytokine is both a "necrosis factor" and a "promoting factor" can be explained by the differences in levels of TNF-α in distinct settings. When TNF-α is administered therapeutically in extremely high doses, it acts as an antiangiogenic and necrotic factor. However, when TNF-α is produced by tumors and tumor-associated macrophages or stromal cells at physiological levels, it promotes tumor growth and additional macrophage recruitment, stimulating the elaboration of angiogenic and growth factors from infiltrating cells [5,6,13].

Ovarian cancer develops along a continuum of malignant transformation and promotion. Tissue expression and fluid levels of TNF-α have been associated significantly with ovarian cancers, but not with the benign tumors [10,11]. Inflammatory conditions related to the ovarian cancer include exposure to exogenous irritants and ovulation, accompanied by cell proliferation, oxidative stress, vascular permeability and overproduction of biological factors, such as prostaglandins, leukotrienes, TNF-α and other cytokines [5,10,16,17].

In the last decade, several studies have assessed the clinical relevance of different biological variables evaluated in sera/plasma/tissue samples from patients with epithelial ovarian cancer in order to detect bio-

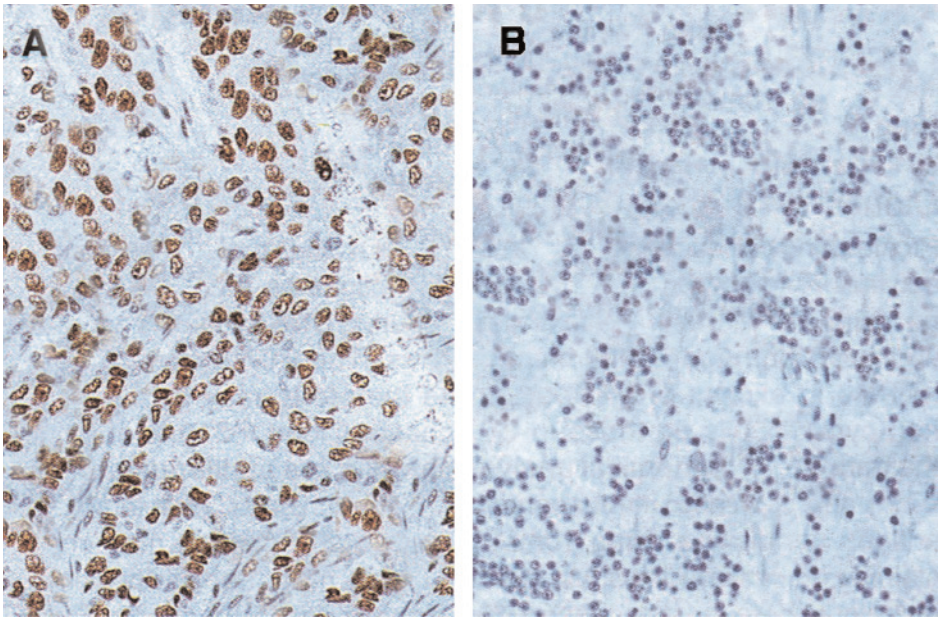

Fig. 2. TNF-R2 immunoexpression in epithelial ovarian cancer (**A**) and benign ovarian lesion (**B**) (original magnification ×40).

markers capable of predicting either the response to chemotherapy or survival [15]. Numerous reports have associated detection of abnormally high levels of TNF in the blood of epithelial ovarian cancer patients [7-9]. Within groups of patients with the same tumor type, higher levels of TNF-α correlated with advanced tumor stage and shorter survival time. However, circulating TNF-α is not always detectable in cancer patients and can vary among individual patients over time and course of disease [10,11].

Several authors have described the presence of TNF-α in different benign hyperplasias [18-20]. In the present study, we detected TNF-α and TNF-R1 in more than 84% of cases with benign ovarian lesions, whereas TNF-R2 was detected in a low number of cases (24%). These results are in accordance with García Tunón *et al*., who reported higher immunoexpression of TNF-α in breast pathologies than in benign breast lesions [21].

In epithelial ovarian carcinomas, TNF-α is more active than in benign pathologies as well as the higher percentage of patients that were positive to both TNF-α and TNF-R1 (100%) and 51% of patients were also positive to TNF-R2. These data suggest that TNF-α plays a significant role in the ovarian cancer progression.

The results of the present study suggested that tissue expression of TNF-R2 in epithelial ovarian cancer is correlated with the highest risk of cancer progression. Thus, the clinical value of activated TNF system in epithelial ovarian cancer needs to be further investigated.

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