Apoptosis and apoptosis-related proteins (Fas, Fas ligand, bcl-2, p53) in macrophages of human ovarian epithelial tumors

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Summary

Apoptosis and the apoptosis-related proteins (ARP) (Fas, Fas ligand (FasL), bcl-2 and p53) were analyzed in macrophages of different human ovarian epithelial tumors. Few macrophages were found in ovaries of women without oncologic disorders. In ovarian benign cysts, macrophagic density reached 4.9±1.2 per 50,000 μm², most were present in lymphoid-macrophagic infiltrates of the sub-epithelial stroma (3.7±0.5% of the area of a slide), and 23.4% were Fas and FasL positive. In borderline tumors, the expanse of lymphoid infiltrates increased to 15.6% of the area of a slide, and the number of macrophages increased 2.4-fold compared to benign cysts. Of the macrophages, 83-88% expressed Fas and FasL, few had bcl-2 and CD25 receptors, and isolated ones were apoptotic. In carcinomas with high lymphoid-macrophagic infiltration, the infiltrate occupied 17.5% of the slide and macrophages amounted to 12.1±1.5/50,000 μm². Many macrophages were in regions of grouping apoptosis of tumor epithelial cells and significantly fewer expressed Fas, FasL and bcl-2. Macrophages destroyed by apoptosis accounted for 4.6%. In carcinomas with low lymphoid-macrophageal infiltration, the area of the last was 5.1% of the slide. There were 8.6±0.8 macrophages/50,000 μm², mainly at the margins of zones of necrosis and of tumor cells' grouping apoptosis. Extensive macrophagic infiltration into tumor parenchyma is one way by which the host immune system destroys tumors. Fas and FasL appear in macrophages of benign cysts, but in borderline tumors and in carcinomas with low infiltration their concentration increases sharply, to 79.8% and 96.6%, respectively. In 4.5% of these cells, apoptosis of macrophages was seen. The findings suggest that macrophages participate in the transfer of ARP to tumor epithelial cells, thereby inducing their apoptosis, but undergoing the simultaneous apoptosis.

Key words: Apoptosis; Bcl-2; Fas; Fas ligand; Macrophages; Ovarian tumors.

Introduction

Tumor-associated macrophages (TAM) are a population of mononuclear-phagocytic cells that express a complex array of proteins related to tumor biology [1]. They were demonstrated to participate in the regulation of tumor proliferation and in the formation of microvessels and are an essential component of solid tumor growth in breast cancer [2]. Recently it was shown that in breast tumors TAM constitute a source of estrogen and regulating not only cell expression but also cytokines expression [3]. In ovarian cancer, TAM promote the synthesis of the matrix metalloprotease MMP-9 [4].

The Fas/Fas ligand system has been proposed as one of the mechanisms by which tumors escape the immune surveillance [5, 6]. Although it is commonly accepted that macrophages together with lymphoid cells play a crucial role in the antitumor efforts of the host [7, 8], extremely little is known about the role of these cells in tumors of different grades [9] and on their relationship to the Fas and Fas ligand (FasL) system. Therefore, our study attempts to investigate this relationship in human ovarian epithelial cancers.

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Material and Methods

Forty-six human ovarian epithelial tumors, belonging to three categories were analyzed: benign cysts (n=10), tumors with low malignant potential or borderline tumors (n=14), and carcinomas (n=22). Each group included serous and mucous tumors, and the third group also included cases of endometroid cancer. The carcinoma group was divided into two subgroups according to the degree of lymphoid-macrophageal infiltration. For comparison, six ovaries of women with non-cancerous diseases were also analyzed.

Histopathological studies were performed on 3 μm sections of formalin-fixed and paraffin-embedded tissue using standard procedures, with uniform conditions of fixation and staining by hematoxylin and eosin. The apoptotic index (AI) was determined using an ApopTag marker (Oncor Inc., CA) and was calculated as the percentage of TUNEL-positive cells, counted in 10-12 fields of 50,000 μm². Apoptosis-related proteins (ARP) such as Fas, FasL, bcl-2 and p53 were determined immunohistochemically using commercial kits (Santa Cruz Biotechnology, CA, USA, and Novocastra Labs., Newcastle, England). In a similar fashion, macrophages were identified through their CD68 marker (Novocastra Labs., Newcastle, England) and IL-2-positive cells were evaluated through the CD25 antigen (Pharmingen Ltd., San Diego, CA). Morphometry included the determination of the area of lymphoid-macrophagic infiltration. ARP-positive macrophages were counted per 10-12 50,000 μm² fields visualized at a magnification of x400 and their percentage in the total macrophage population was calculated.

SPSS-8 statistical software package was used for the analysis of the experimental data. Data is presented as mean ± SEM, and the significance level was set at $p<0.05$. All data were compared using the Student's *t*-test followed by Fisher's significant difference test. Coefficients of correlation (r) and of multiple regression (b) were evaluated.

Results

Ovaries of women without oncologic disorders had few CD68+ monocytes-macrophages (1.1±0.6/50,000 μm²). In cases where the corpus luteum was present, this number increased to 8.2±0.7 per 50,000 μm². ARP were not found in the macrophages, and isolated CD25+ macrophages were infrequently encountered near the corpus luteum.

In benign cysts, the macrophages were located in the small lymphoid-macrophagic infiltrates that occupied 3.7±0.5% of the section, mostly in the subepithelial stroma (Table 1, Figure 1). Only scant macrophages were seen in direct contact with tumor epithelial cells. Of the total macrophages, 23.4% were Fas and FasL positive (Table 2), bcl-2+ and CD25+ macrophages were not detected, and only isolated apoptotic macrophages were seen in the tumors. The number of Fas+ macrophages correlated with the rate of lymphoid infiltration (r=0.66).

In borderline tumors, the average number of macrophages was 11.9±1.8/50,000 μm². They were located mainly in the tumoral stroma, in the form of large infiltrates that occupied up to 15.6±1.7% of the total section (Figure 2, Table 1). In mucoid borderline tumors, the infiltrates were predominated by macrophages (11.9±1.2/50,000 μm²), mainly "young" non-phagocytic monocytes containing ARP, and few CD4+ and CD8+ lymphocytes. Fas+ and FasL+ macrophages were found adjacent to exfoliated tumor epithelial cells. Aggregates of macrophages having large vacuoles and no apoptotic bodies were seen near destroyed small epithelial cysts. In the stroma of serous borderline tumors, the density of macrophages was 7.5±1.1/50,000 μm², compared to 13.2±1.8/50,000 μm² in mucoid borderline tumors, their infiltrates were also smaller, and no phagocytosis of epithelial cells was observed.

The absolute number and relative abundance of Fas, FasL, bcl-2 and CD25-positive macrophages in borderline tumors increased significantly compared to cystomas (Tables 1, 2). There were scant p53-positive macrophages and few isolated apoptotically destroyed macrophages. An inverse correlation was found between the numbers of CD68+ or Fas+ cells and the area of lymphoid-macrophagic infiltration (r=–0.59 and r=–0.43, respectively). The apoptotic index was inverse to the number of FasL+ macrophages (r=–0.45).

In carcinomas with high lymphoid-macrophagic infiltration, the area occupied by the infiltrate (17.5±1.6%) was higher than in borderline tumors (Table 1). Macrophages were present in the tumoral stroma and in the parenchyma among the epithelial cells. Abundance of macrophages was noted in regions of grouping apoptosis of tumor epithelial cells, where they phagocytosed apoptotic cells (Figure 3). The number of CD25+ cells significantly increased whereas the amount of Fas and FasL - positive macrophages significantly decreased compared to those in borderline tumors (Tables 1, 2). All the ARP were similarly represented in the macrophages (Table 2), but the overall number of apoptotic macrophages increased (Figure 4, Table 1). The numbers of FasL+ macrophages correlated highly with the numbers of bcl-2+ macrophages in the stroma (r=0.71), and so did the correlation of tumoral parenchymatic bcl-2+ macrophages with CD68+ cells (r=0.8).

Carcinomas with the low lymphoid-macrophagic infiltration, where the infiltrate occupied 5.1±0.4% of the section, had lower macrophage density compared to the other carcinoma with the high lymphoid-macrophagic infiltration (8.6±0.7 and 12.1±1.3 per 50,000 μm², respectively). Macrophages were concentrated in zones of grouping apoptosis of tumor epithelial cells, and there they contained apoptotic bodies that were not always stained by the ApopTag reaction and often contained Fas and FasL proteins. Many of the macrophages in these areas were necrotized, whereas apoptosis of macrophages themselves was usually observed in the tumoral stroma.

Table 1. — *Apoptosis-related proteins in macrophages and distribution of macrophages in human ovarian epithelial tumors of different grades.*

Parameters studied	Grades of tumors			
	1	2	3A	3B
Areas of lymphoid infiltrates[a]	3.7±0.5	15.6±1.7[b]	17.5±1.6[b]	5.1±0.4[c,d]
Fas+ macrophages[e]	1.1±0.5	9.9±1.6[b]	5.3±1.5[b,c]	7.1±1.5[b]
FasL+ macrophages[e]	1.1±0.5	10.5±1.4[b]	5.2±1.7[b,c]	8.6±1.7[b]
bcl-2+ macrophages[e]	–	3.7±0.8[b]	4.6±1.1[b]	3.0±0.7[b]
CD25+ macrophages[e]	–	0.2±0.1	5.4±1.5[b,c]	4.4±2.1[b,c]
Macrophages in tumor parenchyma[e]	–	2.1±0.5	5.5±1.5[b,c]	7.0±1.0[b,c]
Macrophages in stroma[e]	4.9±1.2	12.0±1.1[b]	8.5±1.3[c]	7.6±1.5[c]
AI of macrophages[e]	–	0.1±0.2	0.6±0.4	0.4±0.2

[a] % of area of a slide.
[b] Significantly different from group 1, $p<0.01$.
[c] Significantly different from group 2, $p<0.01$.
[d] Significantly different from group 3A, $p<0.01$.
[e] The number of cells in 50,000 μm², mean ± SE.
Grades of tumors: 1, benign cyst; 2, borderline; 3A, carcinoma with a high lymphoid-macrophageal infiltration; 3B, carcinoma with a low lymphoid-macrophageal infiltration.

Table 2. — *Amount of ARP-positive macrophages in ovarian tumors of different histologic grades (% of total number of macrophages).*

Parameters studied in macrophages	Grades of tumors			
	1	2	3A	3B
Fas[a]	23.4±1.9[b]	83.2±6.4[c]	43.9±3.1[c,d]	79.8±7.3[c,e]
FasL[a]	23.4±2.0	88.2±7.2[c]	43.0±2.9[c,d]	96.6±8.9[c,e]
bcl-2[a]	–	31.1±2.8[c]	38.0±2.7[c]	33.7±3.1[c]
CD25[a]	–	1.7±0.4[c]	44.6±3.2[c,d]	49.4±3.8[c,d]
AI[a]	–	0.8±0.2[c]	4.6±1.3[c,d]	4.5±0.9[c,d]

[a] % of total number of macrophages.
[b] mean ± SD.
[c] Significantly different from group 1, $p<0.01$.
[d] Significantly different from group 2, $p<0.01$.
[e] Significantly different from group 3, $p<0.01$.
Grades of tumors: 1, benign cyst; 2, borderline; 3A, carcinoma with a high lymphoid-macrophageal infiltration; 3B, carcinoma with a low lymphoid-macrophageal infiltration.

Fig. 1

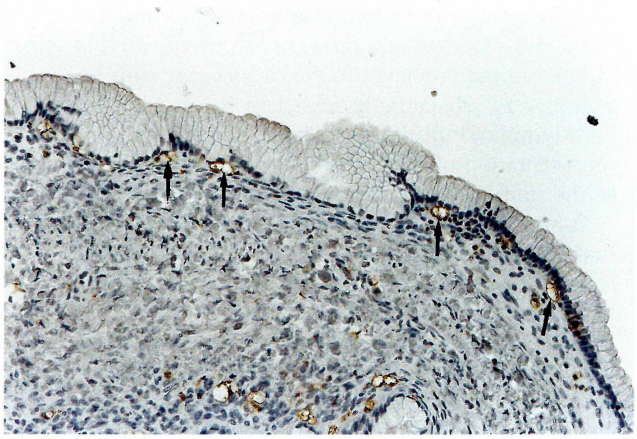

Fig. 2

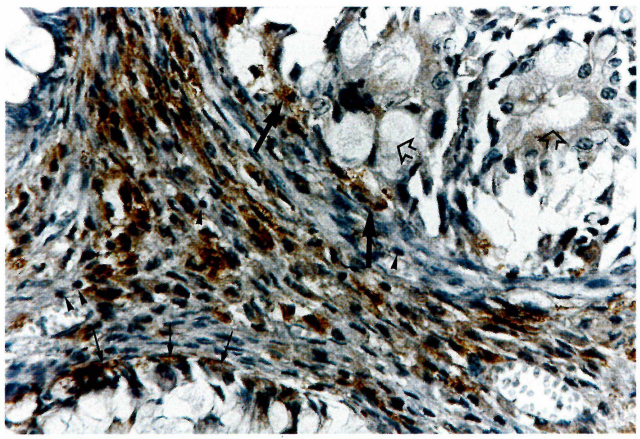

Fig. 3

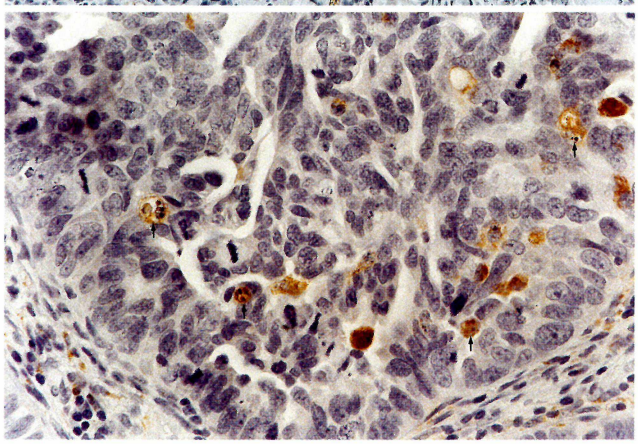

Fig. 4

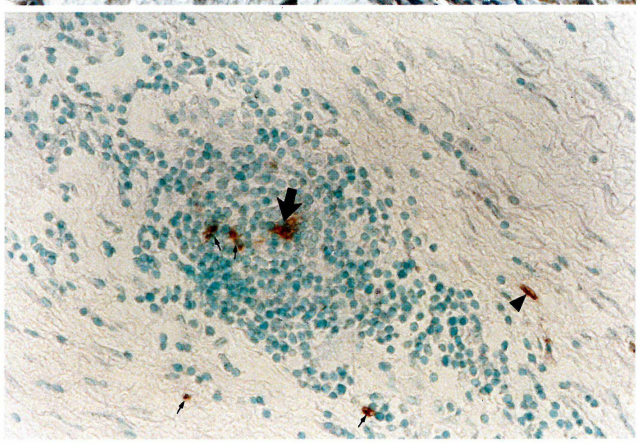

Figure 1. — A serous cyst with low lymphoid-macrophage infiltrate in the stroma with a few macrophages (arrows). Lymphoid-macrophagic infiltration is weak. Reaction to CD68. x200.
Figure 2. — A mucous borderline tumor. Large aggregations of macrophages are seen in the tumor stroma, below the tumor cells (narrow arrows) and in destroyed cysts (large arrows). Note single lymphocytes among macrophages (heads of arrows) and mucous vacuoles in the part of macrophages (white arrows). Reaction to CD68. x400.
Figure 3. — A serous adenocarcinoma. Macrophages are seen in the tumor parenchyma with phagocytotic apoptotic bodies inside (arrows). Reaction to CD68. x400.
Figure 4. — A serous adenocarcinoma. Apoptosis of macrophages (wide arrows), lymphocytes (narrow arrows), and fibroblasts (heads of arrows) inside of lymphoid-macrophage infiltrate in the tumor stroma. TUNEL. x400.

In the necrotic zones, the macrophages were situated around the borders and had large fatty and mucous vacuoles but no apoptotic bodies. The increase in the numbers of Fas and FasL-positive macrophages was not as significant as in the highly infiltrated carcinomas (Table 1), and p53-bearing macrophages were not as common.

Discussion

Our study confirmed the active participation of macrophages in the tumorigenesis of human ovaries. In benign cysts, a low lymphoid-macrophagic reaction was reflecting the weak immune response. This reaction was stronger in borderline tumors where the lymphoid-macrophagic infiltrates quadrupled compared to benign cysts and the density of macrophages more than doubled. Consequently, the total number of macrophages was 10 times higher than in benign tumors. Further intensification of this process was seen in carcinomas with high lymphoid-macrophagic infiltration, especially noticeable was the additional number of macrophages in the parenchyma of a tumor. All this amplification of macrophage reaction resulted from the intensity of the antigenic effects in advanced tumors, similar to the early local immune responses that were reported in breast cancer [9].

In carcinomas with low lymphoid-macrophagic infiltration, the extent of the infiltration and the number of macrophages was one quarter that of the highly infiltrated carcinoma. This decrease corresponds to changes in lymphoid elements [10] and should be considered the morphological manifestation of the deep inhibition of the immune response in highly malignant tumors.

Indeed, this process is accompanied by wide functional and morphological changes in the macrophages themsel-

ves. Macrophages of ovaries without malignancy do not express ARP, even if a tumor is present in an adjacent organ such as the uterus (Gurevich, unpublished observation). In benign cysts, a quarter of the macrophages were Fas and FasL positive while in borderline tumors they amounted to 83-88% of the macrophage population, and about one-third were positive for bcl-2. In carcinomas with a high lymphoid-macrophagic infiltration, the proportion of Fas and FasL positive macrophages decreased whereas that of bcl-2-positive macrophages and especially of CD25-positive macrophages increased, the latter by 27-fold. The elevated synthesis of IL-2 in the macrophages, as compared to borderline tumors and to CD25-positive lymphocytes [10], should be considered additional evidence of the macrophagic intense activity in carcinomas.

In carcinomas with low lymphoid-macrophagic infiltration, the number of Fas-positive macrophages was very high, and almost all macrophages (80% to 97%) were Fas and FasL-positive. Macrophage expansion was already associated with deficiency of FasL in CD4+ and CD8+ lymphocytes [11], and this suggests that in human ovarian epithelial tumors the macrophages participate in the exchange of ARP.

Phagocytosing macrophages were rarely seen in benign cysts. In borderline tumors, apoptotically destroyed tumor cells are phagocytosed. Especially intensive macrophagic phagocytosis was seen in carcinomas with a low lymphoid infiltration, where macrophages swallowed not only apoptotic bodies but also necrotic tissues.

Some peculiarities in reactions of macrophages were found in mucoid borderline tumors. Large aggregations of young monocytes-macrophages are located very close to the layer of tumor epithelial cells destroying them without apoptosis. Small cysts were seen destroyed in such a manner. The small quantity of lymphocytes together with an increase in the number of macrophages reflect the non-immune character of phagocytosis in these places. We suggest that the irritation of mucus might be the reason for the enhanced non-immune activity of macrophages in mucous tumors. In serous carcinomas, the synthesis of mucus is weak and the mucus remains in the intercellular spaces and does not create cysts. In mucous carcinomas, the number of macrophages almost triple compared to mucous borderline tumors. Such macrophages are considered tumor proximal macrophages, the quantity of which increases parallel to progress of tumorigenesis [12].

The pool of macrophages is most significant in carcinomas. Since some tumor cells increase their production of cytoactive proteins, the killing of macrophages, through several mechanisms, is enhanced and can be seen from the appearance of necrotic macrophages, especially in zones of grouping apoptosis of tumor cells. Apoptosis of macrophages was another example of the destruction of macrophages. It was rarely seen in borderline tumors, but in carcinomas about 4.5% of macrophages in the stroma were apoptotic. This decline in the number of macrophages, with a similar decrease in the number of lymphocytes [10], caused also a decrease in the areas of lymphoid-macrophagic infiltrate. Consequently, the total number of macrophages in the carcinomas with low infiltration was substantially less than in carcinomas with a high lymphoid infiltration. This is another morphological representation of the insufficiency and subcompensation of the immune system in cancer patients.

Tumor-associated macrophages have several functions that are related to the tumor's growth, proliferative rate, stroma formation and dissolution [2]. Macrophages participate in the immune response by receiving and transferring information about changes in the antigenic content of the tumor tissue. This already occurs in benign tumors, and increases sharply in borderline tumors and in carcinomas with a high lymphoid-macrophagic infiltration, but declines in carcinomas with a low lymphoid infiltration as a result of insufficiency of the immune system.

The large infiltration of macrophages into tumor parenchyma that was found in the present study is one of the ways to destroy tumors by the host lymphatic system [13]. Infiltrating macrophages are also associated with production of several biologically active substances such as monocyte chemoattractant protein, vascular endothelial growth factor and transforming growth factor [14-16]. They are also a part of the tumor-host interaction that determines the progress of the tumor. This interaction can be influenced by the release of soluble factors, not only from tumor cells but also from tumor-infiltrating lymphocytes and macrophages [17, 18]. The release of substances such as ARP and IL-2 during tumor-host interaction is a main reason for the destruction of tumor epithelial cells [19], lymphocytes [10], macrophages and other structures that participate in tumor progression.

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