Apoptosis and apoptosis-related proteins (Fas, Fas ligand, Blc-2, p53) in lymphoid elements of human ovarian tumors

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Summary

Different types of lymphocytes have different roles in tumor suppression. Thus, their expression of apoptosis-related proteins (ARP - Fas and Fas ligand, bcl-2, p53) in lymphocytes and their apoptosis were analyzed immunohistochemically in ovarian tumors of different grades. Ovaries without oncologic disorders had few lymphocytes, mainly T cells, and no ARP. Benign cysts presented features of weak immune reaction: small lymphoid infiltration and few lymphocytes. The ARP were present in 13.7% to 23.5% of the lymphocytes, and apoptosis was rare. In borderline tumors, expansion of lymphoid infiltrates and increased density of lymphocytes resulted in a tenfold rise in total lymphocytes, reflecting intensification of the immune response. Most lymphocytes were T cells (92%) predominated by CD8+ cells that were in direct contact with tumor epithelial cells. ARP species were found in 47% to 65% of the lymphocytes, and apoptosis in 2.2%. In carcinomas with ligh lymphoid infiltration, lymphocytes were 2.5 times more abundant, and the apoptotic index as well as the number of CD20+ and CD25+ lymphocytes rose sharply, whereas bcl-2 positive lymphocytes decreased to 8% of their number in borderline tumors. In carcinomas with low lymphoid infiltration, the total lymphocyte count decreased eightfold compared to carcinomas with high lymphoid infiltration, reflecting the deep subcompensation of the lymphoid system. Few p53-positive lymphocytes were found in the carcinomas. In conclusion, we found a positive correlation between apoptosis and the numbers of CD4+ or CD8+ lymphocytes in epithelial ovarian tumors. This correlation could reflect the antitumor activity of T cells. However, the high expression of ARP studied by immune cells at the vicinity of the tumor ARP reveals the lymphoid vulnerability to apoptosis, resulting in devastation of the lymphoid tissue, and consequently in tumor progression.

Key words: Apoptosis; bcl-2; Fas and Fas ligand; Lymphocytes; Ovarian epithelial tumors.

Introduction

Lymphocytes are a major component of the immune system and thus play an important role in the antitumor response. Indeed, different cell-types of lymphocytes participate differently in the inhibition of tumors: in breast cancer patients, the number of T cells decreases, whereas that of CD25+ B cells, expressing the interleukin (IL)-2 receptor, increases significantly [1]. Killer cells in the peripheral blood display higher proliferation and enhanced cytotoxicity than tumor infiltrating lymphocytes [2]. It has also been found that lymphocytes affect the apoptosis of epithelial cells [3, 4]. In a previous study, we have shown that human ovarian tumor cells contain high levels of Fas and Fas ligand (FasL) proteins, and probably so inhibit the antitumorigenic activity of lymphocytes [5]. In the present study we attempt to clarify and detail the relationship that may exist between the nature of the tumor and its lymphocyte population, and in particular the consequential lymphocytic expression of apoptosis-related proteins (ARP) such as bcl-2, Fas, FasL and p53.

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Material and Methods

Patients. Forty-six human ovarian epithelial tumors of different histologic grades were studied. They included benign cysts (n=10), adenocarcinomas with low malignant potential (borderline tumors, n=14) and carcinomas (n=22). Each group had both serous and mucous tumors, and the carcinoma group also included a few endometroid cancers. The carcinoma group was further divided into high and low lymphoid infiltration subgroups. Six ovaries from women with diseases other than cancer were analyzed for comparison.

Morphological studies. Histopathological studies were performed using standard procedures with uniform conditions of fixation and staining with hematoxylin and eosin (H&E) of 3-μm sections of tissue from formalin-fixed and paraffin-embedded samples.

Immunohistochemical studies. The apoptotic index (AI) was determined using an ApopTag marker (Oncor Inc., CA) and was calculated as the number of TUNEL (terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nick end labeling)-positive cells per 50,000 μm² of a slide. In parallel, ARP such as Fas and FasL, bcl-2 and p53 were studied with commercial kits (Santa Cruz Biotechnology, CA, USA, and Novocastra Labs., Newcastle, England). Distribution of different types of lymphocytes was determined using commercial markers such as CD3, CD4 and CD20 (Novocastra Labs., Newcastle, England), CD8 (DBS, Fremont, CA) and CD25 (Pharmingen Ltd., San Diego, CA).

Table 1. — *Characteristics of lymphoid components in human ovarian tumors of different grades (mean ± SE).*

Parameters studied	Grades of tumors			
	1	2	3A	3B
Area of lymphoid infiltrates[a]	3.7±0.5	15.6±1.7[c]	17.5±1.6[c]	5.1±0.4[b,c]
All T lymphocytes (CD3+)	82.4±7.7	91.8±8.2	72.8±5.8	77.8±6.4
Helper cells (CD4+)	5.9±0.6	32.1±3.4[b]	15.6±1.1[b,c]	12.9±1.5[b,c]
Suppressors, killers (CD8+)	76.4±5.3	61.9±7.1	55.9±5.4[b]	60.6±6.3[b]
All B lymphocytes (CD20+)	17.6±1.8	6.0±0.9[b]	26.1±3.8[c]	20.5±2.3[c]
CD25+ lymphocytes	–	1.5±0.4	11.5±1.2[b,c]	12.9±1.4[b,c]
Fas+ lymphocytes	23.5±1.5	47.0±2.9[b]	24.1±2.7[c]	31.1±3.1[c]
FasL+ lymphocytes	13.7±0.9	62.7±4.8[b]	20.0±2.2[b,c]	48.5±5.2[b,c,d]
bcl-2+ lymphocytes	19.6±2.3	64.9±8.0[b]	2.4±0.9[b,c]	2.3±0.6[b,c]
Apoptotic index	–	2.2±0.5	5.1±0.8[b,c]	3.3±0.3[b,d]

Grades of tumors: 1, benign cyst; 2, borderline; 3A, adenocarcinoma with high lymphoid infiltration; 3B, adenocarcinoma with low lymphoid infiltration.
[a] Percentage of whole section.
[b] Significantly different from benign cyst, p<0.01.
[c] Significantly different from borderline tumors, p<0.01.
[d] Significantly different from highly infiltrated carcinoma, p<0.01.

Table 2. — *Density of lymphoid elements in different grades of human epithelial ovarian tumors (mean cells/50,000 μm²±SE).*

Parameters studied	Grades of tumors			
	1	2	3A	3B
Total number of lymphocytes	5.1±0.9	13.4±1.2[a]	29.5±2.4[a,b]	13.2±1.3[a,c]
Parenchyma				
All T lymphocytes (CD3+)	0.9±0.2	6.6±1.1[a]	7.9±1.8[a]	3.7±0.6[a,b,c]
Suppressors, killers (CD8+)	0.9±0.2	3.5±0.9[a]	5.8±0.7[a,b]	2.9±0.8[a,c]
Stroma				
All T lymphocytes (CD3+)	3.3±0.8	5.7±1.0	13.9±3.2[a,b]	6.4±1.2[a,c]
Suppressors, killers (CD8+)	3.1±0.7	4.5±0.8	10.7±2.3[a,b]	5.1±1.2[c]
Helper cells (CD4+)	0.3±0.2	4.3±0.9[a]	4.6±1.0[a]	1.7±0.4[a,b,c]
B lymphocytes (CD20+)	0.9±0.1	0.8±0.6	7.7±2.2[a,b]	2.7±0.7[a,b,c]
CD25+ lymphocytes	–	0.2±0.1	3.4±0.9[a,b]	1.7±0.4[a,b]

Grades of tumors: 1, benign cyst; 2, borderline; 3A, adenocarcinoma with high lymphoid infiltration; 3B, adenocarcinoma with low lymphoid infiltration.
[a] Percentage of whole section.
[b] Significantly different from benign cyst, p<0.05-0.001.
[c] Significantly different from borderline tumors, p<0.01.
[d] Significantly different from highly infiltrated carcinoma, p<0.01.

Morphometric studies included determination of the areas of lymphocytic infiltration as described [6] and also the calculation of the number of different types of lymphocytes. The number of ARP-positive lymphocytes was calculated at a magnification x400 per 50,000 μm² in ten fields per slide and was evaluated finally as a percentage of the total number of lymphocytes.

Statistical analysis. The SPSS-8 computerized system was used to perform the statistical analysis of experimental data. All data were compared using the Student's t-tests followed by Fisher's significant difference test to compare the means of separate groups. Coefficients of correlation (r) and multiple regression (b), reflecting the interdependency between the parameters compared, were evaluated.

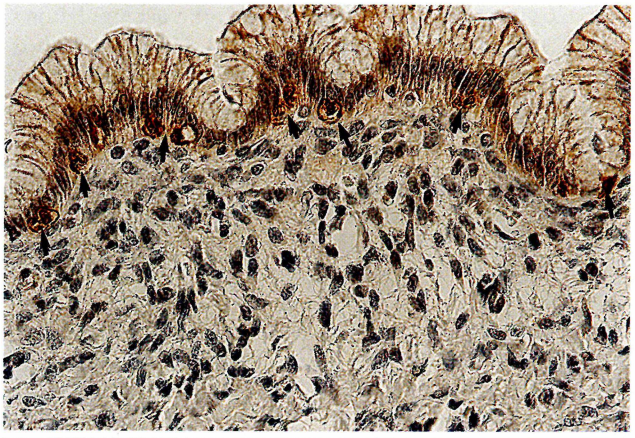
Results

Ovaries of women without oncologic disorders are characterized by the low number of lymphocytes (4.2±0.8 per 50,000 μm²) with the exception of the corpus luteum where we found a three fold increase in the number of lymphocytes (12.5±1.2) compared to the stroma. In spite of this high number, the ratio of the different cell types remains similar to the rest of the ovarian tissue. These were mostly T cells and only a few CD25+ cells were seen. No expression of ARP studied was detected in the normal tissue.

In benign cysts, areas of lymphoid infiltration and the total number of lymphocytes are not high (Fig. 1, Table 1, Group 1). T cells were in large excess to B cells (82.4% vs. 17.6%), and CD8+ lymphocytes were the predominant type of T cells (76.4%). Lymphocytes were found mainly in the stroma and very rarely among the epithelial tumor cells (0.9 cells/50,000 μm²) or between the basal membrane and the epithelium. The number of ARP-positive lymphocytes was low, and apoptosis of lymphocytes was seen very rarely (Table 1). A strong inverse correlation was found between the quantity of CD4+ cells in the stroma and of CD3+ lymphocytes in the tumoral parenchyma (r= –0.86).

In borderline tumors, a sharp expansion of the area of lymphoid infiltrates and a significant increase in the total number of lymphocytes were noticeable (Table 1). The

Figure 1. — Mucous borderline tumor. Abundant lymphoid-macrophage infiltration in the stroma. Note the high number of CD8+ T cells in direct contact with epithelial tumor cells (arrows). x400
Figure 2. — Mucous borderline tumor. FasL is located in basal parts of epithelial tumor cells, in lymphocytes (narrow heads), macrophages (wide arrows) and endothelium of micro vessels (heads of arrows). x400
Figure 3. — Serous adenocarcinoma with abundant lymphoid-macrophage infiltration in the stroma. Note the high number of CD20+ B lymphocytes inside of the stroma (arrows) but not in the tumor parenchyma. x100
Figure 4. — Serous adenocarcinoma with abundant lymphoid-macrophage infiltration in the stroma. Note the high number of CD8+ T lymphocytes inside of the tumor parenchyma (arrows). x400
Figure 5. — Serous adenocarcinoma with abundant lymphoid-macrophage infiltration in the stroma. Note CD25+ lymphocytes (long narrow arrows) and macrophages (wide arrows) in the stroma (arrows). x400
Figure 6. — Serous adenocarcinoma with abundant lymphoid-macrophage infiltration in the stroma. TUNEL reaction: apoptotically destroyed lymphocytes are distinctly seen in the parenchyma and stroma of a tumor (arrows). x400
Figure 7. — Serous adenocarcinoma with abundant lymphoid-macrophage infiltration in the stroma. Fas is expressed in lymphocytes (long narrow arrows), macrophages (wide arrows), and part of epithelial cells including those in stages of mitosis (black arrowheads). x400
Figure 8. — Serous adenocarcinoma with low lymphoid-macrophage infiltration in the stroma. Note separate CD8+ T lymphocytes in the parenchyma (arrows) and rarely in lymphoid infiltrates. x200



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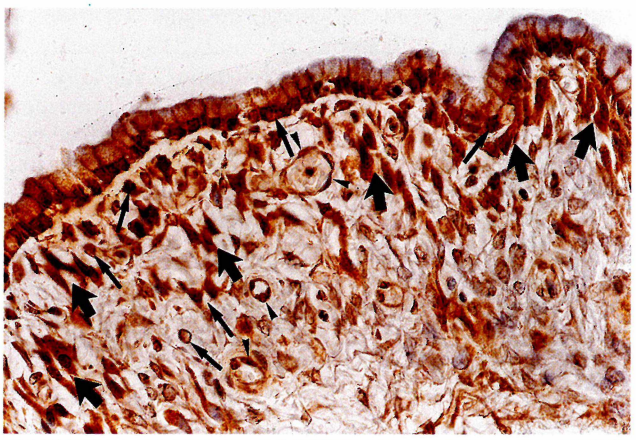

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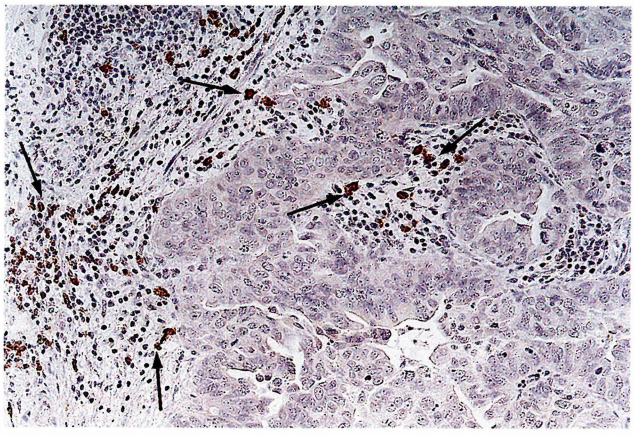

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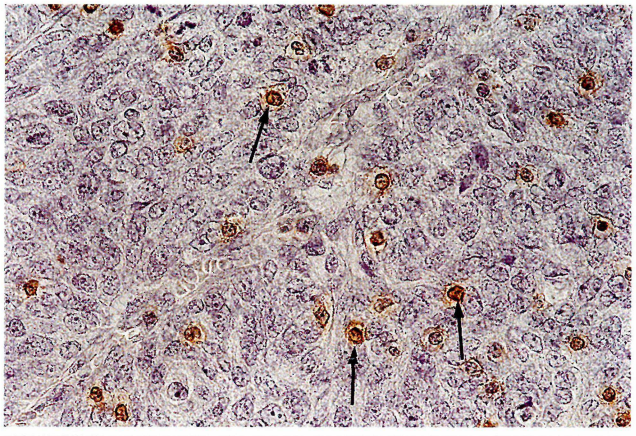

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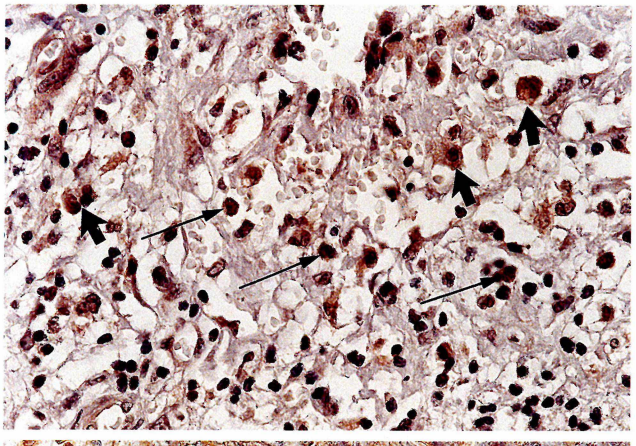

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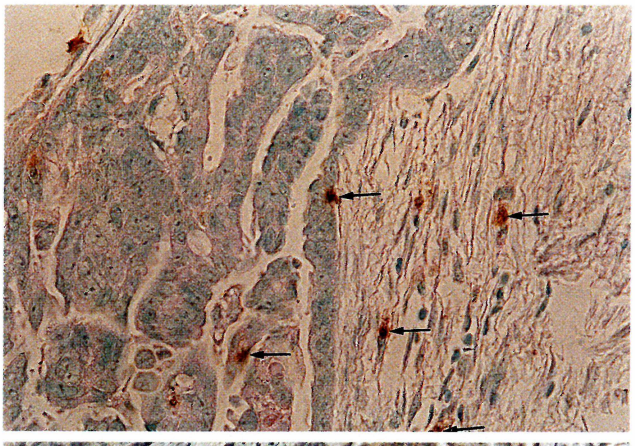

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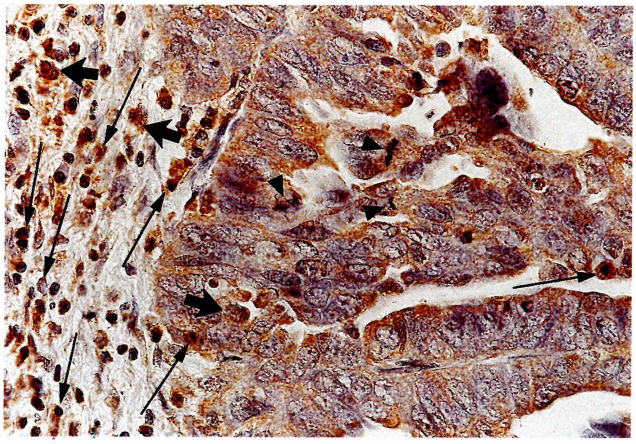

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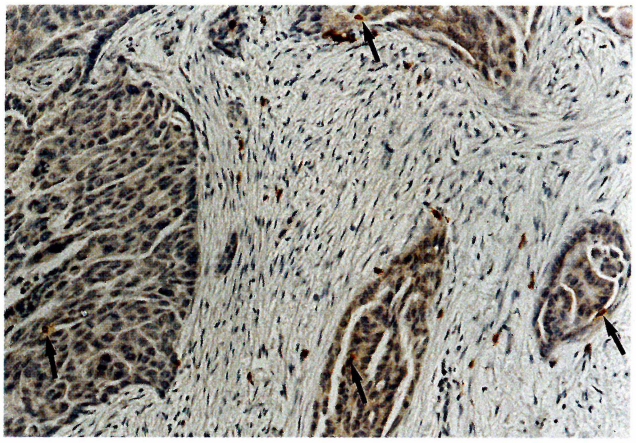

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main types were T cells and among the latter –CD8+ cells (Table 2). A high number of CD3+ and CD8+ T cells was found to be in direct contact with tumor cells (Fig. 1). The extremely high amount of CD4+ T cells was seen only in the stroma of tumors. Up to 60% of lymphocytes were ARP positive. FasL was expressed by epithelial cells, by lymphocytes and macrophages (Fig. 2) whereas bcl-2+ cells were found almost only among CD3+ and CD8+ T cells; p53-positive lymphocytes were not observed at all. Apoptosis of lymphocytes was detected in a small number of lymphocytes (Tables 1, 2). The number of different types of T cells (CD3, CD4, CD8) was correlated with the AI (r= 0.47 to 0.73).

Carcinomas with high lymphoid infiltration had even larger expansion of lymphoid infiltration: 17.5% more than borderline tumors (Table 1, Group 3A). The total number of lymphocytes increased significantly as compared to cysts and borderline tumors due to the sharp rise in the number of T and B cells in the stroma of tumors (Figs. 3, 4). The number of CD25+ lymphocytes sharply increased (Fig. 5, Tables 1, 2). There were also significantly more apoptotic lymphocytes, most of them T cells located in the stroma and parenchyma of the tumors. The ratio of T killer cells to epithelial cells was 1:15. CD8+ lymphocytes were very rarely in contact with apoptotic tumor cells. The quantity of Fas, FasL and bcl-2 positive lymphocytes decreased significantly (Fig. 7), and p53 positive lymphocytes were very scarce in the tumors. The number of CD20+ lymphocytes correlated with those of CD3+ and CD8+ cells (r= 0.8 and r=0.53, respectively). The quantity of CD25+ T cells was inverse to the area of lymphoid infiltration and to the number of infiltrating lymphocytes (r= –0.8 and r= –0.73, respectively) and correlated positively with the expression of Fas and FasL (r=0.73 and r=0.63, respectively).

In carcinomas with low lymphoid infiltration, the area of infiltration and the total number of lymphocytes decreased significantly when compared to carcinomas with a high rate of infiltration (Tables 1, 2). The number of all types of lymphocytes decreased (Fig. 8), but their relative proportions were unchanged relative to the highly infiltrated carcinoma, and the ratio of lymphocytes to tumor epithelial cells was 1:40. The expression of ARP was also similar to that of the high infiltration carcinomas, while the AI decreased (Table 1). Positive and high correlations were found between the expression of Fas and FasL in the same type of lymphocytes (r=0.71), between the quantities of CD20+ and CD8 cells (r=0.81), and between the numbers of CD25+ and CD3+ cells (r=0.88). The AI was inversely correlated with the numbers of Fas+ and FasL+ lymphocytes (r= –0.66 and r= –0.65, respectively).

In all the carcinomas, the accumulation of CD8+ lymphocytes in the tumoral parenchyma was related to the expression of FasL+ lymphocytes (b=0.9±0.7) and inversely related to the presence of bcl-2+ lymphocytes (b= –0.7±0.5). An inverse relationship was also recorded between the accumulation of CD4+ lymphocytes in the tumoral stroma and the number of Fas+ and bcl-2+ lymphocytes (b= –0.5±0.1 and –0.8±0.3). The number of CD20+ lymphocytes increased with the accumulation of bcl-2+ lymphocytes (b=0.5±0.4) and decreased with the rising number of FasL+ lymphocytes (b= –0.8±0.5).

Discussion

Our observations show that different types of human ovarian tumors are characterized by different responses of the lymphoid system. In benign cysts, both serous and mucous, there is little lymphoid infiltration, the number of lymphocytes is low, and suppressor and killer T cells predominate. These data agree with other observations that T cells are the major constituent of the lymphocyte population in tumor tissues [7]. We found very few T killer cells to be in direct contact with tumor cells. Only a few lymphocytes expressed Fas, FasL and bcl-2 in benign cysts, and this suggests that the weak immune reaction to benign ovarian tumors is manifested mainly by stromal T cell accumulation. Similarly, the reduced number of CD4+ lymphocytes was found in the peripheral blood of patients with advanced colorectal cancer [8].

Lymphocyte infiltration is often seen in different cancers, and is believed to represent the host's in-vivo immune reaction to a tumor [9-12]. In ovarian borderline tumors, the local intensification of the immune response featured a tenfold increase of the lymphocyte content relative to benign cysts. This was affected through the increase of both the infiltrated area and the quantity of lymphocytes. The lymphoid infiltration was mainly in regions with high proliferation of epithelial tumor cells, and the T killer cells were abundant in the parenchyma and many were in direct contact with tumor epithelial cells. There was also a tenfold increase in T helper cells in the stroma; lymphocytes containing IL-2 receptor appeared.

The appearance of many Fas, FasL and bcl-2-positive lymphocytes, and few apoptotic cells, is similar to that observed in human breast carcinoma where the AI varied between 0.2% to 6.2% [13]. We also found a high correlation between AI and the number of CD4+ and CD8+ cells in the stroma of the tumors.

Malignant human ovarian tumors differ in the extent of lymphoid infiltration and can be sorted thereby. The lymphoid elements and the character of lymphoid response in these carcinoma subgroups differed, and the highly infiltrated tumors had extreme numbers of lymphocytes and an intense immune response. This was underscored by the increased presence of T cells, and especially CD20+ and CD25+ lymphocytes. The number of CD8+ cells also increased in the tumoral parenchyma, setting a high ratio of T killer cells to epithelial cells. Although the number of Fas system lymphocytes did not change, the number of bcl-2 positive lymphocytes decreased sharply along with their capability to inhibit apoptosis [14]. As a result, the number of apoptotic lymphocytes was five times higher than in borderline tumors. In some areas of tumors, grouping apoptosis of lymphocytes was detected.

In carcinoma with a low lymphoid infiltration, the inhibition of the lymphoid tissue is evident from the signifi-

cant decrease in the area and the number of infiltrating lymphocytes. Because different lymphocytes decreased in a similar fashion, their ratio was practically unchanged, but then the ratio of lymphocytes to tumor cells was much smaller and there were less apoptotic lymphocytes. All this indicated the intense subcompensation of the host's T and B lymphoid systems.

It is accepted that FasL induces apoptotic death in cells that express the Fas receptor [15]. Because Fas is also expressed by lymphocytes, FasL-bearing epithelial cells can kill infiltrating lymphocytes and thereby promote tumor development [16]. We observed many Fas-bearing epithelial cells in the tumor that are able to kill the FasL bearing lymphocytes by induction of apoptosis [17, 18]. This finding might explain the large quantity of apoptotic lymphocytes that was seen in the ovarian carcinomas with high lymphoid infiltration. Indeed, many of the lymphocytes that infiltrated the stroma of the tumor expressed Fas, and we suggest that they become apoptotic upon contact with epithelial tumor cells that express FasL, in a fashion similar to other types of human tumors [16, 19, 20].

The CD4+ and CD8+ proportions were significantly depressed in patients with ovarian cancer, but not with breast cancer [1]. We demonstrated a high expression of Fas and FasL proteins in ovarian tumor cells [5] and in this work in lymphocytes. The high correlation between the apoptotic index and the appearance of CD4+ and CD8+ lymphocytes in ovarian carcinomas indicates the high ability of T cells to suppress tumor progression. This is supported by the notion that T lymphocytes were the major component of the lymphoid infiltrates in ovarian tumors (this work) and in breast carcinomas [21], and that their cytotoxic effects on tumor cells are mediated through the induction of apoptosis [22].

We conclude that tumor cells and lymphocytes interact. In non-progressive tumors, lymphocytes inhibit tumorigenesis by inducing apoptosis of tumor cells. In advanced tumors, many more lymphocytes undergo apoptosis which restricts their capacity to suppress the tumor, and thus promotes tumorigenesis.

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