Clinical Research Paper

Prognostic significance of tumor-associated macrophage infiltration in advanced epithelial ovarian carcinoma

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Key words: ovarian neoplasm, tumor associated macrophage, prognosis, immunohistochemistry

Background and Objective: Tumor-associated macrophage (TAM) infiltration promotes the progression of various malignancies. This study was to investigate the influence of TAM infiltration on the survival and prognosis of patients with ovarian cancer. Methods: The expression of CD68, a TAM-specific marker, in 67 specimens of ovarian cancer and 22 specimens of benign ovarian lesion was detected by SP immunohistochemistry to investigate the density of TAM. The correlation of TAM density to the prognosis of ovarian cancer was analyzed. Cox multivariate proportional hazard model was used to analyze prognostic factors of ovarian cancer. Results: Observing under ×400 lens, the mean density of TAM was significantly higher in ovarian cancer than in benign ovarian lesions (57.7 vs. 25.3 per vision field, p < 0.01). The 5-year survival rate was significantly higher in low-density TAM group than in high-density TAM group of ovarian cancer patients (73.3% vs. 41.2%, p = 0.01). Univariate analysis found that TAM infiltration was more obvious in serous carcinoma, moderately and poorly differentiated carcinoma and in patients aged ≥ 40 than in the counterparts. Multivariate analysis revealed that histological grade and TAM infiltration status are independent predictors for overall survival. Conclusion: TAM infiltration is obvious in advanced ovarian cancer, which indicates poor prognosis.

Studies have revealed that a substantial portion of the inflammatory cells in tumor tissue are macrophages, which might account for over 50% of all inflammatory cells.1 Currently, there is no clear definition for tumor-associated macrophages (TAMs). Generally, macrophages infiltrated in and around tumor tissue are considered as TAMs. Previously, it was suggested that higher amount of macrophages infiltrated in tumor tissue is associated with stronger activity and better antitumor effect. However, it was revealed recently that macrophage infiltration is associated with poor prognosis in numerous malignant tumors.2,5 Macrophage infiltration in ovarian cancer and its correlation to the prognosis have seldom been reported. This study was to investigate macrophage infiltration in ovarian cancer tissues and analyze its prognostic significance.

Patients and Methods

Patient selection. Patients were selected according to following criteria: epithelial ovarian cancer; first treated in Cancer Center of Sun Yat-sen University; no chemotherapy, radiotherapy, hormone therapy or anti-inflammatory treatment were given before operation; treated with cytoreductive surgery for ovarian cancer; postoperative stage was stage III or IV (FIGO); with complete clinicopathologic and follow-up records; treated with platinum-based combination chemotherapy as scheduled after operation; no radiotherapy after operation. From 1990 to 2002, 67 eligible patients were included. The specimens of ovarian cancer were routinely paraffin-embedded and sliced. Twenty-two specimens of benign ovarian lesions obtained during the same period were used as control.

Clinical features. The median age of the 67 ovarian cancer patients was 48.7 years (range, 25–72 years). With FIGO staging, 54 patients were at stage III and 13 at stage IV. The diameter of postoperative residual tumor was > 1 cm in 46 patients, and ≤ 1 cm in 21 patients. Of the 67 cases of ovarian cancer, 36 were serous carcinoma, 15 were mucinous carcinoma, one was clear cell carcinoma, and 15 were undifferentiated adenocarcinoma; 16 were well differentiated, 28 were moderately differentiated, 22 were poorly differentiated, and one was undifferentiated. After operation, 37 patients were treated with less than six courses of chemotherapy due to financial issue or poor compliance during the treatment, 17 were treated with 6–8 courses of chemotherapy, and 13 were treated with more than eight courses.

Of the 22 cases of benign ovarian lesion, 15 were simple ovarian cysts, three were luteal cysts, two were inclusion cysts, one was lutein cyst, and one was luteal hematoma.
Prognostic significance of tumor-associated macrophage infiltration in advanced epithelial ovarian carcinoma

TAM infiltration in ovarian cancer tissue and benign ovarian lesion. CD68-stained TAMs were observed in all ovarian cancer specimens; TAM infiltration was seen in interstitial tissues and cancer nests. TAM density in the 67 specimens of ovarian cancer ranged from 1/HP to 124/HP, with a median value of 57.8/HP. With the median density as a cutting point, 33 patients were divided into low TAM density group (Fig. 1A) with a mean TAM density of (35.6 ± 2.5)/HP, and 34 into high TAM density group (Fig. 1B) with a mean TAM density of (79.8 ± 3.1)/HP.

Small amount and low density of macrophages were seen in benign ovarian lesions (Fig. 1C). In the 22 specimens of benign lesion, TAM density ranged from 3/HP to 87/HP, with a mean value of 25.3/HP.

The mean TAM density was significantly higher in epithelial ovarian cancer than in benign ovarian lesions [(57.6 ± 3.4)/HP vs. (25.3 ± 4.5)/HP, p < 0.01].

All patients were followed up through telephone calls and clinic visits. The date of death was recorded for patients who died during follow-up. For those who were lost to follow-up, patient’s status and the date of the last visit were recorded.

Reagents and immunohistochemistry. Mouse anti-human CD68 monoclonal antibody (PG-M1), the specific marker of TAMs, was purchased from ZYMED Co. (USA). DAB staining kit, EDTA repairing solution, secondary antibody, goat serum, and horseradish peroxidase-labelled streptavidin were purchased from Zhongshan Goldenbridge Company (Beijing).

CD68 was detected with SP immunohistochemistry. Antigens were repaired by microwave. Dewaxed tissues slices were incubated with EDTA repairing solution (1 mmol/L, pH8.0, pre-heated to 100°C) for 15 min, then added with primary antibody and incubated at 4°C overnight. The next day, slices were stained with DAB and then hematoxyline, and were sealed with neutral gum. As negative control, PBS was added instead of primary antibody.

Result evaluation. CD68 was expressed in the cytoplasm of macrophages. Cells with well-defined tawny or yellow-brown particles in the cytoplasm were considered as positive cells (Fig. 1). TAM infiltration in hot spots in cancer tissue and surrounding interstitial tissue was observed under microscope. TAMs in five visual fields (× 400) of each slice were counted, and the mean TAM count was regarded as the density of TAM infiltration. All slices were observed by two experienced pathologists. Based on related literatures, median density of TAM infiltration in all cases of ovarian cancer was selected as the cutting point for dividing patients into high TAM density group and low TAM density group.

Statistical analysis. All data were analyzed with statistical software SPSS13.0. Differences were considered significant when p ≤ 0.05. Difference in TAM density between ovarian cancer and benign ovarian lesion groups was tested for homogeneity variance, and were then analyzed with independent sample T test. Patients’ survival were analyzed by Kaplan-Meier method. The prognosis was analyzed with Cox regression model.

Results

TAM infiltration in ovarian cancer tissue and benign ovarian lesion. CD68-stained TAMs were observed in the specimens of all ovarian cancer; TAM infiltration was seen in interstitial tissues and cancer nests. TAM density in the 67 specimens of ovarian cancer ranged from 1/HP to 124/HP, with a median value of 57.8/HP. With the median density as a cutting point, 33 patients were divided into low TAM density group (Fig. 1A) with a mean TAM density of (35.6 ± 2.5)/HP, and 34 into high TAM density group (Fig. 1B) with a mean TAM density of (79.8 ± 3.1)/HP.

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Survival comparison between low and high TAM density groups. Among the 67 ovarian cancer patients, the follow-up rate was 94%; four patients were lost to follow-up who had been followed up for more than five years. The 67 patients had been followed up for (90.4 ± 49.3) months: 31 survived more than five years, and 36 died within five years. The mean survival was (129.2 ± 12.3) months in low TAM density group, with a 5-year cumulative survival rate of 73.3%, and was (69.1 ± 11.6) months in high TAM density group, with a 5-year cumulative survival rate of 41.2% (p = 0.01) (Fig. 2).
Correlation of TAM infiltration to clinicopathologic features of ovarian cancer. TAM infiltration showed no correlation to tumor stage, whereas it was correlated to the pathologic type, histological classification and patients’ age. TAM infiltration was more obvious in serous carcinoma, moderately and poorly differentiated carcinoma, and patients aged above 40 than in the counterparts (Table 1).

Multivariate analysis on the prognosis of ovarian cancer patients. Patients’ age, FIGO stage, postoperative residual tumor volume, pathologic classification, histological classification, number of courses of postoperative chemotherapy, and density of TAM infiltration were introduced into a Cox regression model for multivariate prognostic analysis. Factors were selected with a forward stepwise method. TAM infiltration density and histological classification were independent prognostic factors for advanced epithelial ovarian cancer (Table 2). The relative risk ratio for TAM infiltration density was above 1, suggesting poorer prognosis in the patients with higher TAM density.

Discussion
Van Netten et al.2 have found that TAM infiltration was associated with local infiltration and distant metastasis of tumor, and suggested this might be related to the epithelial growth factor (EGF) secreted by TAMs. Takanami et al.3 measured TAM infiltration density in tumor tissues obtained from 113 patients with lung adenocarcinoma, and revealed that TAM infiltration was an independent prognostic factor, higher density of TAM infiltration predicted poorer prognosis. Moreover, obvious TAM infiltration have also been observed in numerous malignant tumors, including head and neck cancer, liver cancer, renal cancer, pancreatic cancer, endometrial cancer and cervical cancer, and high density of TAM infiltration predicted poor prognosis.

In our study, we found significant difference in TAM infiltration between benign ovarian lesion and ovarian cancer, with more obvious TAM infiltration in advanced epithelial ovarian cancer. Wang et al.6 discovered that macrophage infiltration significantly increased in the peritoneum of ovarian cancer patients as compared with that in benign ovarian lesion patients. Studies on other malignant tumors yielded similar findings. Peng et al.4 compared macrophage infiltration in nasopharyngeal cancer tissue and healthy nasopharyngeal mucosa, and found no macrophages in healthy nasopharyngeal mucosa, while found large amount of macrophages in nasopharyngeal cancer tissue. Song et al.7 also found significantly increased macrophage infiltration in oral squamous cell carcinoma when compared with that in healthy tissue. Currently, the role of increased macrophage infiltration in tumor tissue is unclear yet. Melichar et al.8 compared the macrophages in ascitic fluid and peripheral blood of patients with epithelial ovarian cancer, and revealed substantial differences in phenotype and function of these cells. Gordon et al.9 explored the functions of macrophages in peripheral blood of patients with epithelial ovarian cancer, and found no antibody-dependent cell-mediated cytotoxicity (ADCC) in these macrophages, that is, these cells could not kill or ingest tumor cells. Thereby, it was assumed that the phenotypes and functions of macrophages in ovarian cancer tissue might differ from those in benign ovarian lesion. Researchers further realize that macrophages can be activated in different manners in different circumstances, and thus become subsets that express different molecules and functional features. Currently, at least two subsets were identified:10 (1) macrophages activated in a classical way, also known as M1 macrophages, are mainly involved in Th1 immune response and cytotoxicity targeting infectious pathogens and tumor cells; (2) macrophages activated in an alternative way, also known as M2 macrophages, are mainly involved in Th2 immune regulation, tissue repairing and remodeling, and vascularization. Mantovani et al.11 reviewed recent studies regarding TAMs, and concluded that TAMs were M2 macrophages and TAMs could promote tumor growth and progression. Sica et al.12 further confirmed that TAMs were M2 macrophages, and suggested that during the differentiation from monocytes toward macrophages, monocytes would develop into M1 macrophages in a microenvironment where cytokines such as M-CSF, LPS, IFN-γ or bacterial products predominated, while differentiate into M2 macrophages in a microenvironment where cytokines such as M-CSF, IL-4, IL-13, IL-10 and PGE predominated. Whether TAMs in ovarian cancer tissue are M2 macrophages need to be further investigated.

Our study reveals that high density of TAM infiltration predicts poor prognosis of advanced epithelial ovarian cancer, which is in line with the findings of numerous studies on varied solid tumors. The prognostic significance of TAMs might be explained...
by the following facts: (1) TAMs promote vascularity. Tumor vascularity is an important process during progression and metastasis of tumors. Extensive microvasculatization has been proved to be a marker for poor prognosis in numerous solid tumors. Macrophage infiltration is related to local vascularity in various malignant tumors. Leek et al.13 revealed intensive macrophage infiltration in breast cancer tissues, and found that microvasculatization level was significantly correlated to increased macrophages, increased macrophage count was related to decreased recurrence-free survival and decreased overall survival. (2) Proteases and cytokines produced by TAMs promote local infiltration and metastasis of tumors directly.14 When tumor cells begin to infiltrate outward, a large amount of inflammatory cells, of which nearly 50% are TAMs, tend to cluster around the tumor. Proteases produced by TAMs degrade the basal membrane and thus let tumor cells into the intercellular matrix,15 which is a critical step in tumor metastasis. (3) Macrophage infiltration leads to carcinogenesis directly.15 As a major regulator in many types of inflammatory responses, macrophages produce large quantity of reactive oxygen species and nitric oxides during inflammatory responses, which promote the synthesis of nitrites and thus lead to DNA mutation in epithelia and adjacent cells.

Currently, fundamental investigations are rare regarding how TAM infiltration in ovarian cancer influences tumor progression. Saccani et al.16 found that overexpression of P50 NF-κB in TAMs inhibited the M1 inflammatory response and antitumor effect of macrophages on ovarian cancer. Kryczek et al.17 revealed B7-H4 expression in ovarian cancer cells, while the expression of B7-H4 on the surface of TAMs inhibited specific T-cell immune response against tumor-associated antigen and suppressed normal macrophages; in vivo blockade of B7-H4 restored the antitumor effect of T cells. Shah et al.18 revealed significant correlation of TAMs to T cell count in ovarian cancer tissue, while excessive T cells in the tumor was related to P53 mutation in tumor cells. Therefore, it is reasonable to assume that inhibition on antitumor effect by TAMs might be related to suppression on T cells and mutation of certain genes. Nonetheless, the roles of TAMs in ovarian cancer has to be confirmed in future fundamental studies.

How to improve treatment efficacy on advanced epithelial ovarian cancer has been a hot issue. Since the traditional trident of surgery, chemotherapy and radiotherapy has failed to significantly improve the survival for these patients, some oncologists have turned their focus onto biotherapy. Intensive TAM infiltration can be seen in advanced epithelial ovarian cancer, and is negatively related to the prognosis. This finding might lead to a new pathway for exploring immunotherapy for advanced epithelial ovarian cancer.