Biological characteristics of dendritic cells derived from peripheral blood of patients with epithelial ovarian cancer

Chun-Yan Lan,1,2 Ji-Hong Liu,1,2,* Jian-Chuan Xia1,3 and Li-Min Zheng1,4

1State Key Laboratory of Oncology in South China; 2Department of Gynecologic Oncology; 3Research Department; 4Biotherapy Research Center; Cancer Center; Sun Yat-sen University; Guangzhou, Guangdong P.R. China

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Background and Objective: Dendritic cells (DCs) are thought to be the most potent antigen-presenting cells (APC) and play a vital role in stimulating human immune response against cancer. At present, most data concerning the immuno-biological function of DCs are obtained from healthy donors. The information about the biological characteristics of DCs from patients is limited. In this study, the biological characteristics of monocyte-derived dendritic cells (MoDCs) from patients with ovarian cancer were investigated. Methods: Monocytes were isolated from peripheral blood mononuclear cells (PBMC) of eight epithelial ovarian cancer patients and 13 healthy women volunteers, cultured with interleukin 4 (IL-4) and granulocyte-macrophage colony stimulating factor (GM-CSF), and stimulated with tumor necrosis factor-α (TNF-α). At seven days after induction, the morphological characteristics of MoDCs were observed. The features of phenotype were analyzed using flow cytometry. The ability of MoDCs to stimulate proliferation of lymphocytes was tested by allogeneic mixed leukocytes reaction (MLR). Results: Mature MoDCs with typical morphology were obtained after seven days of culture. MoDCs from both patients and healthy women expressed high levels of HLA-ABC, HLA-DR, CD86 and large amounts of CD80. There was no significant differences between MoDCs from ovarian cancer women and healthy women in the mean fluorescence intensity (MFI) of HLA-ABC, HLA-DR, CD86 and CD80 (p > 0.05). The MLR was significantly weaker in ovarian cancer patients than in healthy women (p < 0.05). Conclusion: MoDCs from ovarian cancer patients may present lower capacity of stimulating proliferation of lymphocytes, indicating that the patients’ MoDCs may have immunological function defect at certain extent.

In recent years, biological therapy, such as immunotherapy, is the fourth antitumor therapy and has gradually attracted attention in the treatment of ovarian cancer. Dendritic cells (DCs), the most potent antigen-presenting cells (APC), are mainly characterized by being able to stimulate initial T cells and trigger antigen-specific CD8+ cytotoxic T lymphocytes to kill target cells. Therefore, DCs play an important role in antitumor immunotherapy. At present, DC-based immunotherapy shows a bright future for the treatment of ovarian cancer.1-6 However, due to major histocompatibility complex (MHC) restriction, only autologous DCs can be clinically used. Currently, most data concerning the biological characteristics of DCs are obtained from healthy donors while those from patients are limited. In this study, we induced DCs with peripheral monocytes to observe and compare the biological characteristics of monocyte-derived dendritic cells (MoDCs) obtained from ovarian cancer patients and healthy women, and provide reference for clinical immunotherapy of ovarian cancer.

Materials and Methods

Study subjects and specimens. Ovarian cancer patients. A total of eight epithelial ovarian cancer patients treated at the Sun Yat-sen University Cancer Center from December 2005 to July 2006 were included. Their median age was 49 years (range, 22–68 years). Inclusive criteria were as follows: (1) previously untreated at our hospital; (2) pathologically confirmed as primary epithelial ovarian cancer; (3) undergoing comprehensive staging operation or cytoreductive surgery for ovarian cancer; (4) undergoing no chemotherapy or radiotherapy before blood collection; (5) without a secondary malignant tumor. Of the eight cases, two were at stage Ia, one at stage Ic, three at stage IIIc and two at stage IV according to pathologic staging. Healthy women. A total of 13 healthy women were included. Their median age was 37 years (range, 27–50 years). Current infection, diabetes and autoimmune diseases were excluded in all study subjects. Informed consents were obtained from all subjects.

Specimen collection. Blood samples were taken from ovarian cancer patients before treatment (surgery or chemotherapy) and...
were taken from healthy women on the day that relevant experiments were conducted. About 35 mL of blood was taken from each subject, placed at room temperature and immediately used for experiments.

Major reagents and instruments. RPMI-1640 medium was prepared by the Research Department of the Sun Yat-sen University Cancer Center. Fetal calf serum (FCS) was purchased from Hangzhou Sijing Biological Engineering Materials Co., Ltd. Lymphocyte separation medium was purchased from Tianjin Hao Yang Biological Manufacture Co., Ltd. Recombinant human interleukin-4 (rhIL-4) and recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) were purchased from Cytolab Ltd. (USA). Recombinant human tumor necrosis factor-α (rhTNF-α) was purchased from Biosource Co. (USA). Recombinant human interleukin-2 (rhIL-2) was purchased from Beijing Sihuan Bio-Pharmaceutical Co., Ltd. FACSCalibur flow cytometer was purchased from BD Co. (USA).

Cell separation and culture. Separation of monocytes and culture of DCs: Peripheral venous blood (35 mL from each subject) was overlayed onto lymphocyte separation medium for density gradient centrifugation. Cells in the white band were harvested, washed with normal saline solution and inoculated to complete medium, then cultured at 37°C with 5% CO₂ for 1.5 h. At this point, adherent cells were monocytes while non-adherent cells were lymphocytes. Non-adherent cells were harvested and transferred to another culture flask. Adherent cells were cultured in the DCs solution containing rhIL-4 (50 ng/mL) and rhGM-CSF (50 ng/mL) at 37°C with 5% CO₂. Half of the culture medium was refreshed every other day. The morphology of cells was examined under phase contrast microscope every day. On day 6 after culture, rhTNF-α was added at a final concentration of 10 ng/mL to induce cell maturation. On day 7, cells were harvested and divided into two parts: one was used for phenotyping to detect the expression of surface molecules; the other was used as stimulator for “mixed lymphocyte reaction” to determine their ability to stimulate allogeneic lymphocyte proliferation.

Lymphocyte separation and culture. Non-adherent cells separated from the peripheral blood of healthy women using the above-mentioned method were lymphocytes. Lymphocytes were cultured in the complete medium containing rhIL-2 (40 U/mL) at 37°C with 5% CO₂. Cultured lymphocytes were used as responder cells for “mixed lymphocyte reaction.” It was unnecessary to separate lymphocytes from ovarian cancer specimens.

Determination of cell phenotype. MoDCs were stained with FITC-labeled mouse anti-IgG, HLA-ABC, HLA-DR, CD86 and CD80 monoclonal antibodies. MoDCs stained with FITC-labeled mouse anti-IgG were used as negative control to exclude the interference of non-specific fluorescence. Stained cells were detected by flow cytometry. CellQuest Pro software was used to analyze data and record the expression of molecules on the surface of MoDCs. Mean fluorescence intensity (MFI) was used to reflect the expression levels of these molecules.

Mixed lymphocyte reaction. Responder cells were adjusted to a concentration of 2 × 10⁶ cells/mL and seeded to 15 wells of a 96-well plate (100 μL/well). MoDCs were adjusted to a concentration of 2 × 10⁵ cells/mL and added to lymphocyte-containing wells at responder:stimulator ratios of 1:500, 1:100, 1:50 and 1:10, respectively. Triplicate wells were run for each dilution, and a total of 12 MoDC-containing wells were used. Three wells containing no MoDCs were used as blank controls. Mixed cells were cultured at 37°C with 5% CO₂ for 96 h. The absorbance value of each well at 570 nm (A₅₇₀) was measured by MTT assay. The average value of triplicate wells was used for subsequent analysis.

Statistical analysis. All data were continuous variables expressed as mean ± SD and were analyzed using SPSS10.0 software package. The homogeneity of variance between two independent sample means was tested first. If population variances were homogeneous, the t-test was used to compare two independent sample means. Multiple sample means were compared using the analysis of variance. The level of significance was set at α = 0.05.

Results

Morphology of MoDCs. When stimulated with rhIL-4 and rhGM-CSF for 24 h, monocytes obtained from the peripheral blood of ovarian cancer patients or healthy women were small and round, and exhibited adherent growth. On day 3, some cells aggregated into masses, with increased size, slightly irregular shape and few small surface processes (Fig. 1A). On day 5, the number of burr-like processes and clustered suspension cells increased (Fig. 1B). On day 7, cells grew in suspension and aggregated into masses, with irregular shape and a large number of extended burr-like processes, which were considered as typical morphologic features of DCs (Fig. 1C). During the observation, MoDCs from ovarian cancer patients showed no obvious difference in shape and size when compared with those from healthy women, but the number of MoDCs from patients was relatively small.

Cell phenotype. As shown in Figure 2, MoDCs from both ovarian cancer patients and healthy women expressed high levels of HLA-ABC (MFI: 32.32 ± 9.30 vs. 40.78 ± 11.36, p > 0.05) and HLA-DR (62.42 ± 51.83 vs. 58.83 ± 25.47, p > 0.05); MoDCs from both groups expressed large amounts of CD86 (32.13 ± 7.00 vs. 47.37 ± 22.32, p > 0.05) and CD80 (34.94 ± 29.11 vs. 30.79 ± 15.42, p > 0.05).

Mixed lymphocyte reaction. Using MoDCs from either ovarian cancer patients or healthy women, A₅₇₀ was rising along with the rise of responder:stimulator ratio. Using the same responder:stimulator ratios, A₅₇₀ was significantly lower in ovarian cancer group than in healthy group (p < 0.05, Table 1).

Discussion

In vitro induction of dendritic cells from monocytes obtained from ovarian cancer patients. Although DCs are widely distributed throughout the body, they constitute only 0.1–1% of peripheral blood monocytes, thus limiting their applications in basic and clinical research. Hence, it is essential to induce and amplify DCs in vitro to meet the needs of basic research and clinical applications. At present, approaches for in vitro induction and amplification of DCs from precursor cells are mature. Induction of DCs from peripheral blood monocytes is generally recognized as the best approach for obtaining mature DCs since it has advantages of simplicity, little invasiveness, and high homogeneity of cultured DCs.
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Figure 1. Morphology of monocyte-derived dendritic cells (MoDCs) from patients with ovarian cancer and healthy women (× 100). After 24-hour culture, the adherent cells are small and round-shape. On day 3, the adherent cells are enlarged. On day 5, the cells display a veiled or dendritic appearance, and adhere loosely to nests. On day 7, the enlarged cells suspend and aggregate to nests. The cells, which display spikes on surface, are classified as typical MoDCs. There is no obvious difference in the morphology of MoDCs between ovarian cancer patients and healthy women.

However, no specific antigens are available so far to detect and identify whether typical mature DCs are obtained through in vitro induction from peripheral blood monocytes; mature DCs are evaluated mainly through observing their morphologic characteristics, expression of surface molecules and ability to stimulate the proliferation of initial T cells. Our study showed that, on Day 7 after culture, monocytes obtained from either ovarian cancer patients or healthy women exhibited irregular shape and a large number of extended burr-like processes, expressed high levels of HLA-ABC (MHC-I), HLA-DR (MHC-II) and large amounts of CD86 and CD80, and can potently stimulate allogeneic lymphocyte proliferation. These results indicated that typical mature MoDCs were successfully induced in vitro from monocytes obtained from either ovarian cancer patients or healthy women. Moreover, no morphologic difference was found between MoDCs obtained from ovarian cancer patients and healthy women.

Phenotypic integrity of MoDCs from ovarian cancer patients. Many studies showed that tumor microenvironment could
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impair the infiltration, migration and maturation of DCs, prevent the entry of DCs into tumor tissue and impede their effective recognition of tumor antigens, thereby suppressing the generation of immune response. Moreover, Gabrilovich et al.15 further pointed out that functional defects of DCs from tumor patients were not only confined to tumor tissue, but were found throughout the body. Although culture supernatant of tumor cells could inhibit normal differentiation of DC precursor cells, DC precursor cells separated from tumor-bearing patients could undergo normal differentiation when cultured in the absence of tumor cells. In the present study, MoDCs from the peripheral blood of both ovarian cancer patients and healthy women expressed surface molecules of similar high levels, suggesting that monocytes from ovarian cancer patients can undergo normal differentiation when cultured in the absence of tumor cells, and induced MoDCs possessed phenotypes to fulfill their immune function, which is very important for triggering antitumor immunity. This result provides a basis for using normally differentiated DCs from ovarian cancer patients to conduct clinical immunotherapy.

In this study, MoDCs from both ovarian cancer patients and healthy women expressed high levels of HLA-ABC and HLA-DR as well as costimulatory molecules CD86 and CD80, which were consistent with the results of many reports in China,16-20 but the expression levels of these surface molecules were lower than those reported in other countries.8,21,22 The following two reasons may explain this observation. First, the serum used in this study was FCS which contains many allogeneic proteins and may lead to potential infection.23 Moreover, FCS contains large amounts of active substances such as cytokines and enzymes. Since the contents and activities of these active substances vary among different batches of FCS, the quality of DCs may be affected when cultured with different batches of FCS. Second, some studies23-27 indicated that stimulating immature DCs with monocyte-conditioned medium (MCM) or MCM mimic (IL-1β, IL-6, TNF-α and PGE2 in combination) could induce DCs with more stable characteristics and higher maturity. Mature DCs induced with MCM or MCM mimic could maintain their characteristics for 1–3 days when cultured in the absence of cytokines. In this study, DCs were stimulated with TNF-α. Thurner et al.28 believed that the potency of TNF-α was lower than that of MCM and MCM mimic in the stimulation of DCs. This reason may explain the impaired maturation of DCs and relatively low expression of phenotypic molecules observed in our study.

Changes in immune function of MoDCs from ovarian cancer patients. Current evidences indicate that the abilities of DCs to present antigens and induce specific antitumor immune response depend on normal expression of various immune molecules on

![Figure 2. Phenotypes of MoDCs from patients with ovarian cancer and healthy women on day 7 after culture detected by flow cytometry.](image)

Table 1 Mixed lymphocyte reaction (MLR) in cervical cancer patients and healthy women

<table>
<thead>
<tr>
<th>MoDCs:lymphocytes</th>
<th>Healthy women</th>
<th>Ovarian cancer patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty control</td>
<td>0.44±0.08</td>
<td>0.40±0.06</td>
</tr>
<tr>
<td>1:500</td>
<td>0.58±0.07</td>
<td>0.45±0.08</td>
</tr>
<tr>
<td>1:100</td>
<td>0.62±0.06</td>
<td>0.49±0.05</td>
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<tr>
<td>1:50</td>
<td>0.67±0.06</td>
<td>0.55±0.09</td>
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<tr>
<td>1:10</td>
<td>0.81±0.15</td>
<td>0.61±0.09</td>
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All values are presented as mean ± SD of relevant groups. *p < 0.05, among all groups of healthy women; **p < 0.05, among all groups of ovarian cancer patients; ***p < 0.05, vs. healthy women.
that, besides CD86 and CD80, other molecules on DCs also play
geneic lymphocyte proliferation significantly declined, suggesting
ability of MoDCs from ovarian cancer patients to stimulate allo-
the expression levels of CD86 and CD80 was observed between
ability and antitumor immune response.
Gabrilovich et al. 32 found that peripheral DCs from breast cancer patients were inferior to those from healthy donors in stimulating allogeneic T cell proliferation, while peripheral T cells from breast cancer patients could generate normal immune response when stimulated by normal allogeneic DCs. Thus, they concluded that a major cause of cellular immune defects in breast cancer patients was due to the hypofunction of DCs, not due to functional defects of T cells. Satthaporn et al. 33 drew a similar conclusion. In fact, T cells from cancer patients are similar to those from healthy human in inducing immune response. Therefore, although the results of MLR test performed in this study were based on in vitro stimulation of allogeneic lymphocytes by MoDCs, it is reasonable to speculate that the proliferation level of T cells from ovarian cancer patients should be lower than those from healthy women. This difference is mainly due to the hypofunction of DCs from ovarian cancer patients as proved in the above-mentioned studies.
Onishi et al. 21 found that, after seven days of culture, the abilities of MoDCs from advanced cancer patients to capture antigens, express surface molecules and stimulate allogeneic lymphocyte proliferation were significantly weaker than those from healthy donors. Thus, they concluded that MoDCs from tumor patients contained short-lived MoDC subsets which may impair immune function of MoDCs. Peng et al. 19 proved that the average expression levels of costimulatory molecules CD80 and CD40 in MoDCs from cervical carcinoma patients were only 1/3 and 1/2 of those in MoDCs from healthy women, and the ability of these MoDCs to stimulate allogeneic lymphocyte proliferation was weaker than that of control cells, suggesting that the antigen-presenting ability of MoDCs from cervical carcinoma patients may be impaired. Similarly, we observed that the ability of MoDCs from ovarian cancer patients to stimulate allogeneic lymphocyte proliferation declined, suggesting defects in the immune function of these cells.
The defects in immune function of DCs may be due to signaling abnormalities of costimulatory molecules. Many molecule pairs can transmit costimulatory signals that are delivered by DCs to T cells, of which B7/CD28 and CTLA-4 are important ones. B7/CD28 family members include B7-1 (CD80), B7-2 (CD86) and B7-3. B7-1 and B7-2 play important roles in the activation of T cells. 34-37 In this study, no significant difference in the expression levels of CD86 and CD80 was observed between MoDCs from ovarian cancer patients and healthy women, but the ability of MoDCs from ovarian cancer patients to stimulate allogeneic lymphocyte proliferation significantly declined, suggesting that, besides CD86 and CD80, other molecules on DCs also play important roles in delivering costimulatory signals to T cells. The abnormalities of these molecules may impair the immune function of DCs. For example, CD40, another important costimulatory molecule that has been extensively studied in recent years, plays important roles in cellular and hormonal immunity. The binding of CD40 to its ligand could trigger the maturation of DCs, for example, upregulating the expression of costimulatory molecules (CD80, CD86, etc.) on the surface of DCs and enhancing the ability of DCs to stimulate T cell proliferation. 38 Abnormal expression of CD40 may directly result in a decline in the ability of DCs to stimulate allogeneic lymphocyte proliferation. Therefore, detecting and comparing the expression levels of CD40 in MoDCs from ovarian cancer patients and healthy women can help to elucidate the mechanism underlying defects in immune function of MoDCs from ovarian cancer patients.
Additionally, Onishi et al. 21 discovered that the dysfunctional short-lived MoDC subsets from advanced cancer patients might be derived from dysfunctional peripheral blood monocytes in cancer patients. They also observed that, besides shorter lifetime, the phagocytic capacity and IL-12 secretion of these MoDCs were also weaker than those of monocytes from healthy donors, further proving that functional defects of MoDCs may result from the dysfunction of peripheral blood monocytes in cancer patients. 21

The biological mechanisms behind immunosuppression are extremely complicated. Although defects in immune function were found in MoDCs derived from ovarian cancer patients in this study, the mechanism underlying such defects and their impact on the induction of immune response remain to be further studied.

References
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