Original Article

Decrease of Peripheral Blood CD8+/CD28- Suppressor T Cell Followed by Dentritic Cells Immunomodulation among Metastatic Breast Cancer Patients

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ABSTRACT

Objective: To explore the effects of dentritic cells on the peripheral blood lymphocyte subpopulations of metastatic breast cancer patients treated with chemotherapy.

Methods: The current study involved 44 metastatic breast cancer patients treated with docetaxel-based chemotherapy. Among them, 25 cases were treated with dendritic cells derived from $CD34^+$ hematopoietic stem cells enriched autologous peripheral mononuclear cells after chemotherapy, and 19 cases received chemotherapy alone. Peripheral blood samples were collected from each patient before and after treatment, and lymphocyte subpopulations including CD3+, CD3+/CD4+, CD3+/CD8+, CD3-/CD16+56+, CD3+/CD16+56+, CD4+/CD25+, CD8+/CD28-, CD8+/CD28+, CD4/CD8, DC1, DC2 and DC1/DC2 were analysed   by a 3-color flow cytometric analysis.

Results: The two treatment groups were well matched with regard to demographic and baseline disease characteristics. Comparing the changes of lymphocyte subpopulations between the two groups, it showed that the difference of the change of CD8+/CD28-lymphocyte had statistic significance. The percentage of CD8+/CD28-lymphocyte was lower in the chemotherapy+DC group, but higher in the chemotherapy-alone group.

Conclusion: As CD8+/CD28-lymphocyte represent a kind of suppressive T lymphocyte, we conclude that dentritic cell therapy can relieve immunosuppression to some extent.

Key words: Metastatic breast cancer; Dentritic cell; Lymphocyte subpopulations; Regulatory T cell

INTRODUCTION

The outcome for patients with metastatic breast cancer (MBC) is quite poor. Significant progress has been made in this field. New cytotoxic agents and target therapy have all shown enhanced therapeutic benefit for breast cancer patients[12]. Nonetheless, tumor relapse, the emergence of toxicities, and early death due to the emergence of resistant disease continue to pose great challenges for the clinical management of this disease. Hence, there is an urgent needed for novel intervention strategies capable of synergising with standard adjuvant therapies; maintaining activity against refractory tumors and avoiding further immune suppression. Immuno-suppression may contribute to the disease progression. Complex interactions between the immune system and tumor exist in patients with metastatic cancer, nonspecific and systemic immune dysfunction is prevalent in advanced and metastatic cancer[3-5]. In previous studies significant changes have been demonstrated in the numbers and functions of peripheral blood lymphocyte subsets in patients with different malignant disorders[4,6-8]. So to achieve a better outcome for patients with MBC, immuno-therapy is one potential treatment option.

Immunotherapeutic strategies that successfully activate the immune system against tumor antigens

are a prime alternative as they would probably be non-cross-resistant, specifically boost antitumor immunity and have non-overlapping toxicities. Dendritic cells (DCs) are antigen-presenting cells that are capable of stimulating antigen-specific naive and memory T cells. Ample evidences now indicate the existence of an immune response against breast cancer involving DC recruitment and activation within tumor tissue[9,10-12]. Despite the evidences, numerous studies have demonstrated severe phenotypic and functional impairment of DCs in patients with breast cancer[13,14]. Circulating DCs isolated from patients with breast cancer exhibited an impaired capacity to stimulate T lymphocyte proliferation and cytokine secretion[13]. Moreover, patients with metastatic breast cancer showed a large number of immature APCs with poor immunological function in the blood DC compartment[15]. Thus DC therapy is emerging as a crucial strategy to enhance anticancer activity for patients with this disease.

In this study we treated the metastatic breast cancer patients with mature dendritic cell derived from autologous peripheral CD34$^+$ hematopoietic stem cells enriched autologous peripheral mononuclear cells after chemotherapy, and try to explore the effects of dentritic cell on the immune function, such as the peripheral blood lymphocyte subpopulations of these patients.

MATERIALS AND METHODS

Patients and Treatment

The current study involved 44 patients with metastatic breast cancer who were admitted to the Department of Medical Oncology, Peking University Cancer Hospital & Institute between January 2008 and May 2009. All these patients were treated with docetaxel-based chemotherapy. Among these patients 25 cases were treated with dendritic cells derived from CD34$^+$ hematopoietic stem cells enriched autologous peripheral mononuclear cells after chemotherapy and 19 cases received chemotherapy alone. As it was a retrospective study, the case number of the two groups was not equal. DCs immunotherapy was approved by Beijing Health Administration and the Local Ethics Committee of Peking University Cancer Hospital & Institute. Written informed consent from each patient was obtained prior to study entry.

Hematopoietic Stem Cells (HSC) Mobilization and Collection

CD34+ HSCs were mobilized by docetaxel-based chemotherapy and G-CSF. After receiving chemotherapy, when the WBC count of peripherial blood decreased to ≤1.5×10^9/L, granulocyte colony stimulating factor (G-CSF) at a dose of 5μg/kg/day was given subcutaneously. Autologous peripheral blood mononuclear cells (PBMC) were isolated by leukapheresis when the WBC count increased to ≥10.0×10^9/L. Leukapheresis was performed by the CBS200 Spectra cell separator and we analyzed the numbers of PBMC and CD34$^+$ HSC in the collections by flow cytometry.

Culture and Transfusion of Dendritic Cells

DCs were generated by *in vitro* stimulation with IL-4, GM-CSF and TNF-α. Human peripheral blood mononuclear cells (PBMC) were separated by Ficoll , and the adherent cells were grown in X-VIVO 15 (Cambrex Co, USA) with 100 μg/L of GM-CSF (Tebao bio Co., Xiamen China), 10μg/L of IL-4 (R&D Co, USA), and 2.5μg/L of TNF-α (R&D Co, USA). Cell counts and phenotype of DCs were analyzed 10 to14 days after culture. Molecules of CD1a, CD11c, CD80, CD86 and anti-HLA-DR were analyzed by flow cytometer. If the cells differentiated into typical matured dendritic cells with classical phenotype, venous transfusion were conducted immediately. DCs transfusion was conducted every three or four days for altogether 3 times, each time including 10^7~10^9 dendritic cells.

Blood Samples

Heparinized peripheral blood samples were collected from each patient before chemotherapy. For the DC+chemotherapy group, blood samples were collected again after chemotherapy and the third DC infusion(about four weeks after the chemotherapy) and for the chemotherapy alone group, blood samples were collected again three or four weeks after the chemotherapy or just before the beginning of the next cycle of chemotherapy. All these fresh blood samples were analysed for T cell subpopulations, including CD3+, CD3+/CD4+, CD3+/CD8+, CD3-/CD16+56+, CD3+/CD16+56+, CD4+/CD25+, CD8+/CD28-, CD8+/CD28+, CD4/CD8, DC1, and DC2, DC1/DC2.

Cell Isolation

Heparinized peripheral blood was obtained, 100μl whole blood was used per tube and they were stained with antibodies, and incubated in the dark at 4°C for 15 min. After hemolysis 10 and gradients Centrifugation, the cells were washed twice in PBS

and subjected to flow cytometric analysis. The following antibodies, anti-CD4(FITC/CD8(PE) /CD3(PerCP)(Becton-Dickinson, BD), anti-CD4-fluorescein isothiocyanate (FITC) (Beckman-Coulter), anti-CD25-phycoerythrin (PE) (Beckman-Coulter), anti-CD28-fluorescein (Beckman-Coulter), anti-CD8-phycoerythrin (PE) (Beckman-Coulter), CD3(FITC/CD16+56(PE) (Becton-Dickinson, BD), anti-CD19-PC5(Beckman-Coulter) were used.

Flow Cytometric Analysis

A 3-color flow cytometric analysis protocol was performed to determine cell phenotype. Flow cytometry was performed on Epics XL (Beckman-Coulter), and Expo32 ADC software (Beckman-Coulter) was used for analysis. Lymphocytes were gated by forward scatter versus side scatter. T lymphocytes were further gated on anti-CD3(PerCP) positive cells in the lymphocyte gate. B lymphocytes were further gated on anti-CD19(PerCP) positive cells in the lymphocyte gate. NK lymphocytes were further gated on anti-CD16+56(PE) positive cells in the lymphocyte gate. Analysis was set to stop when 5000 events had been analyzed.

Statistical Methods

Statistical analyses of data were carried out using SPSS software Version 15.0. A P value of less than 0.05 was regarded as statistically significant. Quantitative factors were compared by Pcarson's χ^2 contingency Table analysis. Results of the relative numbers of all lymphocyte subpopulations were presented as $\bar{x}\pm s$ deviations. Statistical analyses were performed using the Student's t-test to compare the changes of lymphocyte subpopulations between chemotherapy+DC group and chemotherapy alone group.

RESULTS

Study Population

A total of 44 patients were enrolled, 25 in chemotherapy DC+ group, 19 in chemotherapy alone group. The treatment groups were well matched with regard to demographic and baseline disease characteristics. The detailed results are shown in Table 1.

Table 1. *Demographic and Baseline Disease Characteristics of Patients*

characteristics	age	Menopausal		Prior-chemo for MBC	
		pre	post	Yes	no
Chemo+DC(n=25)	50.6	10(40%)	15(60%)	17(68%)	8(32%)
Chemo-only(n=19)	51.2	7(36.8%)	12(63.2)	12(63.2)	7(36.8)
P value	0.802	0.831		0.737	

Chemo: chemotherapy; DC: dendritic cell; MBC：metastatic breast cancer

Comparison of the Changes of Lymphocyte Sub-populations between Chemotherapy+DC Group and Chemotherapy-alone Group

Only the difference of the change of CD8+/CD28- lymphocyte between the two groups had

statistic significance(P=0.018). It showed that the percentage of CD8+/CD28- lymphocyte was lower in the chemotherapy+DC group, and higher in the chemotherapy-alone group. The results are shown in Table 2.

Table 2. *Comparison of the changes of lymphocyte subpopulations between the chemotherapy+DC group and chemotherapy-alone group (t-test,α=0.05)*

Lymphocyte subpopulations	Chemo+DC	Chemo-alone	T value	P value
CD3+	3.01±11.39	3.03±6.75	-0.008	0.993
CD3+/CD4+	2.47±9.03	-0.10±6.10	1.067	0.292
CD3+/CD8+	1.88±4.96	3.83±5.37	-1.247	0.219
CD3-/CD16+56+	1.03±9.41	-0.46±6.39	0.594	0.556
CD3+/CD16+56+	0.04±1.81	-0.01±1.19	0.086	0.932

CD4+/CD25+	-0.72±5.20	-1.16±4.23	-0.384	0.703
CD8+/CD28-	-2.71±8.19	4.24±10.62	-2.452	0.018
CD8+/CD28+	0.64±7.00	0.72±4.86	-0.045	0.964
CD4/CD8	0.02±0.43	-0.20±0.33	1.905	0.064
DC1	0.05±1.62	0.27±0.97	-0.528	0.6
DC2	-0.35±0.85	-0.05±0.88	-1.133	0.263
DC1/DC2	0.79±3.88	0.42±2.11	0.399	0.692

DC: dendritic cell;   Chemo: chemotherapy

DISCUSSION

Successful treatment of advanced breast cancer is a significant clinical problem. Metastatic breast cancer patients have impaired immune functions. Immunosuppression may contribute to the progression of disease. Complex interactions between the immune system and tumor exist in patients with metastatic cancer. Multiple cellular and molecular layers of the suppressive network have been imposed in the tumor microenvironment in patients with cancers[16]. Chemotherapy is the most actively used modality in the treatment of advanced-stage cancer. It is well established that it is associated with significant immunosuppression[17,18]. Defectis in DC in cancer patients have also been considered as a reason for tumor-induced immune suppression in hosts. There is evidence to suggest that cancer patients may have DC that do not express co-stimulatory molecules and remain immature; thus, directly influencing the tumor-induced immune response and contributing to immune suppression[12,19]. Moreover a number of studies have demonstrated that tumor-infiltrating lymphocytes in 45-60% of breast cancers produce immunoregulatory cytokines, such as IL-4, IL-10 and/or transforming growth factor (TGF)-β[20,1], thus negatively influencing the generation of protective antitumor responses. Regulatory T cell (Treg)-mediated immune suppression has also been suggested[22]. The frequency of Tregs in peripheral blood of patients with breast cancer has been found to be significantly higher than in healthy volunteers, and such accumulation appears to have an impact on the efficiency of the immune response[22,23].

Regulatory T cells exist both within the CD4 (CD4$^+$CD25$^+$ T cells)[24,25] and CD8 subsets. CD8+CD28- T cells represent a unique subset of regulatory T cells which is major histocompatibility complex (MHC) class I restricted, T suppressor (Ts) cells. They inhibit both proliferative and cytotoxic T cell responses[26-29]. CD8+CD28- Ts cells acquire and exert their functions via direct interaction with APC, recognize MHC peptide complexes on the cell surface of APC and trigger the upregulation of inhibitory receptors and down- regulation of costimulatory molecules, rendering the APC tolerogenic[30,31].

It becomes increasingly clear that neither cytotoxic therapy nor immunotherapy alone would be able to successfully solve the problem to acquire a satisfactory result. Chemotherapy frequently fails because of the development of drug resistance[32], and immunotherapy alone is not able to maintain effective antitumor immune response in the presence of bulky tumor because of the effects of tumor-derived immunosuppressive factors[33]. It appears that only a combination of different modalities may provide the necessary breakthrough in the treatment of this group of patients.

In this study we treated the metastatic breast cancer patients with mature dendritic cells derived from autologous peripheral CD34$^+$ hematopoietic stem cells enriched autologous peripheral mono-nuclear cells after chemotherapy, and performed a detailed analysis of peripheral blood lymphocyte sub-populations during chemo-immune therapy. It showed that the percentage of CD8+/CD28- lymphocytes was lower in the chemotherapy+DC group, but higher in the chemotherapy-alone group. The difference of the change of CD8+/CD28- lymphocyte between the two groups has statistic significance. This means that chemotherapy has immunosuppressive effect, while combined with DC the immunosuppression can be somewhat relieved.

Our approach is based on the findings that DCs are able to take up apoptotic cells and to process and present antigens associated with these cells[34,35]. A number of studies show that dendritic cells are initiators of immune responses, it can induce T-cell responses, enhance T-cell reactivity and stimulate tumor-specific CTLs, capable of lysing autologous tumor cells[36-39]. Also some reports show that DC therapy can induce an increase of tumor-specific cytotoxic T lymphocytes in the peripheral blood[37,40,41]. But there was few reports about the effect of DC on the peripherial blood regulatory T cells, especially the CD8+/CD28- suppressor T cells. Ahn's study[42] suggests for the first time that Dendritic cells partially abrogate the regulatory activity of CD4$^+$CD25$^+$ T cells present in the human peripheral blood.

In our study, it showed that chemotherapy is associated with immunosuppression (higher percentage of CD8+/CD28- Ts cells), the CD8+/CD28- suppressor T cells decrease when combined therapy. It seems that DC downregulates the suppressor T cells, enhances the immune reconstruction. This effect may be critical for the success of combined treatment.

Thus, we can conclude that besides the activation of T cell response, the relief of immunosuppression via the downregulation of CD8+/CD28- suppressor T cells also plays an important role in the DC immunotherapy.

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