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The dysfunction and abnormal signaling pathway of dendritic cells loaded by tumor antigen can be overcome by neutralizing VEGF in multiple myeloma

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ABSTRACT

We investigated whether dendritic cells (DCs) from multiple myeloma (MM) patients were affected by loading tumor antigens and whether the defective DC function associated with MM could be overcome by the neutralization of VEGF. MM-specific DCs were generated by loading tumor lysates from myeloma cells at diagnosis or relapsed/progressive state, respectively. DCs loaded with tumor lysates showed lower phenotypic maturation, less T cell stimulatory capacity, less cytotoxic T lymphocyte activities, and highly abnormal cytokine secretions of IL-6 and IL-12, compared to myeloma lysate-unloaded DCs. The levels of VEGF, phospho-STAT3 and phospho-ERK1/2 in DCs were significantly higher with loading myeloma lysates. After the neutralization of VEGF activity, the DC function, signal transduction and cytokine production returned to normal. The defective function of DC in patients with MM is significantly affected by loading tumor antigens, correlating with abnormal STAT3 and the NF-κB signaling pathway, and neutralization of VEGF can overcome this DC dysfunction through the elimination of abnormal signal transduction.

1. Introduction

Multiple myeloma (MM) is a clonal B-cell hematologic malignancy described as the latent accumulation in bone marrow (BM) of secretory plasma cells with a low proliferative index and a prolonged life span, and remains essentially incurable with conventional or high-dose chemotherapy [1,2]. Myeloma cells can produce immunological cytokines, such as transforming growth factor beta (TGF-β), interleukin-10 (IL-10), IL-6 and vascular endothelial growth factor (VEGF), which play major roles in the pathogenesis of MM [1,2]. Among them, IL-6 has an essential role in the malignant progression of MM by regulating the growth and survival of tumor cells. These tumor-derived factors can also modulate anti-tumor host immune responses, including the abrogation of dendritic cell (DC) function, by constitutive activation of the signal transducer and activator of transcription 3 (STAT3) [3].

VEGF is one of the most potent endothelial mitogens that induce angiogenesis by stimulating endothelial proliferation and reducing endothelial death in cancer cells. VEGF is well known to induce the defective maturation of DCs from hematopoietic progenitors via inhibition of the transcription factor NF-κB [4,5]. In addition, the defective function of DCs in MM patients is closely related with an increased plasma level of VEGF [6]. However, the responsible receptors and molecular transduction mechanisms of VEGF in differentiation and maturation of DCs are still unclear. Recently, differential roles of VEGF receptors have been reported, such as the type of receptor which is more specific for inhibiting the maturation and function of DCs. Additionally, the blockade of VEGF by anti-VEGF neutralizing antibody or anti-VEGF receptor antibodies overcomes the functional inhibition of DCs [6–8].

Patients with MM have DCs that are functionally defective, evidenced by the decreased number of circulating precursors of myeloid and plasmacytoid DCs, as well as impaired T cell stimulatory capacities compared with normal controls [9]. The defective functions of DCs in patients with MM are partially attributed to the production of IL-6 and other tumor-derived factors [3,9]. Effective

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adoptive cellular therapy in MM patients has attempted to establish potent DCs for the generation of autologous myeloma-specific CTLs [10–13]. In addition to generation of potent and specific DCs using better cytokine combinations or effective tumor antigens, alternative methods are tried to restore defective DC function and to enhance DC function in MM. The methods target specific signaling pathways, such as STAT3 or mitogen-activated protein kinase (MAPK) p38, attempt to inhibit tumor-derived factors directly [6,14–17]. We evaluated effects of tumor-derived inhibitory factors on the ex vivo maturation and induction of potent DCs by pulsing tumor antigen. Using this strategy, we have identified means capable of reverting the immune suppression in MM patients. Prevention of myeloma cells from escaping host immune surveillance should be crucially accounted when immunotherapy regimens, especially DC-based approaches, designed.

In this study, we investigated whether the activity of DCs were affected by myeloma tumor antigens. The defective generation of DCs was associated with the abnormal signaling pathways, and could be overcome by a VEGF blockade.

2. Patients, materials and methods

2.1. Patient samples and DC generation

BM samples were collected continually from 6 MM patients at times for initial diagnosis and assessing the relapse/progressive state during the therapeutic course. Peripheral blood stem cell (PBSC) products were collected from the same patients mobilized with cyclophosphamide and G-CSF after obtaining the informed consent for this study and the approval from the Chonnam National University Hwasun Hospital institutional review board. After the isolation by density gradient centrifugation with Ficoll-Hypaque (LymphorepTM, Nycomed, Oslo, Norway), mononuclear cells (MNCs) were cryopreserved at a temperature of −170 °C until use. The clinical characteristics of patients are shown in Table 1. All patients were treated with same induction regimens and preceded to autologous PBSC collections after the achievement of clinical response. Induction chemotherapy consisted of thalidomide (400 mg, orally D1–5, D15–19), cyclophosphamide (150 mg/m^2, orally D1–4) and dexamethasone (20 mg/m^2, orally D1–5, D15–19) every 28 days.

In order to generate DCs, CD14$^+$ cells were isolated from MNCs of PBSC products using the magnetic-activated cell sorting (MACS) system (Miltenyi Biotec, Auburn, CA, USA). The cells were cultured in RPMI 1640 (Gibco-BRL, grand Island, NY, USA) supplemented with 10% FBS (Hyclone, Logan, UT, USA) and 1% penicillin-streptomycin (Gibco-BRL), in the presence of 50 ng/ml GM-CSF (LG Biochemical, Daejon, Korea) and 10 ng/ml IL-4 (R&D Systems, Minneapolis, MN, USA) for 6 days. Freshly prepared cytokines were added every 2 days, by replacing the half volume of the culture medium. On day 6, immature DCs were pulsed with myeloma cell lysates at 10 μg/ml for 2 h at 37 °C in the presence or absence of a neutralizing anti-VEGF Ab (50 ng/ml). The DCs were matured by the addition of conventional cytokine cocktail combination with 20 ng/ml of TNF-α, 10 ng/ml of IL-1β, 10 ng/ml of IL-6, and 1 μg/ml of prostaglandin E_2 for additional 2 days.

After staining with mouse monoclonal antibodies anti-CD14-fluorescein isothiocyanate (FITC), anti-CD86-FITC, anti-HLA-DR-FITC, anti-CD80-phycoerythrin (PE) and anti-CD83-PE (PharMingen, San Diego, CA), the samples were acquired on a FACSCalibur cell sorter (Becton Dickinson, San Jose, CA) and the data were analyzed with CellQuest software (Becton Dickinson). Isotype controls were run in parallel and cell debris was eliminated from the analysis by forward and side scatter gating.

2.2. Generation of myeloma cell lysates

In order to enrich the purity of myeloma cells, CD138$^+$ cells were isolated using the MACS system (Miltenyi Biotec) from the cryopreserved BM MNCs, which were obtained at diagnosis (plasma cells: 40–50%) or relapsed/progressive state (plasma cells >20%). The purity of the isolated CD38$^+$CD138$^+$ cells was >90%. The purified myeloma cell lysates (CD138$^+$ cells) were prepared by five cycles of freezing (liquid nitrogen) and thawing (37 °C), confirmed by SDS-PAGE, and analyzed with a BCA protein assay kit.

Table 1
Patient's characteristics

Patient no.	Age/sex	Paraprotein	Durie-Salmon/ISS*	Chromosome
1	57/female	Ig A/Lambda	IA/II	46,XX
2	63/female	Ig G/Kappa	IIIA/II	47,XX, +9
3	65/female	IgG/Kappa	IIIA/III	46,XX
4	40/male	IgG/Lambda	IIIA/II	46,XY
5	59/female	IgG/Lambda	IIIA/III	46,XX
6	70/female	IgG/Kappa	IIIA/III	46,XX

* ISS: International staging system.

2.3. Allogeneic mixed lymphocyte reaction (MLR) assay

The allogeneic CD3$^+$ T cells (40,000/well) obtained by positive selection using MACS (Miltenyi Biotec) from the PB of healthy donors were co-incubated with graded doses (2500–40,000) of irradiated (30 Gy) myeloma lysate-pulsed DCs in 96-well U-bottom plates for 5 days. After pulsing with 1 μCi per well of [^{3}H]-methylthymidine during the last 16–18 h of culture, the proliferation of T cells was analyzed with a liquid scintillation counter (Beckman, Fullerton, CA, USA).

2.4. Generation of CTLs and IFN-γ release ELISPOT assay

CTL generation and IFN-γ release ELISPOT assay were performed as described previously [18]. Briefly, autologous CD3$^+$ T cells from the PB of MM patients were isolated using the MACS system (Miltenyi Biotec). The CD3$^+$ T cells (1×10^6 cells) were primed by autologous DCs (2×10^5 cells) that were pulsed with myeloma lysates. On day 3, IL-2 (50 U/ml) (R&D Systems) and IL-7 (5 ng/ml) (R&D Systems) were added. The effector cells were re-stimulated with the same DCs after 7 days and 14 days in culture. On the 21st day, the effector cells were co-cultured with target cells, including autologous myeloma cells (CD138$^+$) or K562, at various effector-to-target ratios, and then subjected to the IFN-γ release ELISPOT assay, as described previously [18]. ELISPOT data show the mean number of spots (±S.D.) per 0.5–2 × 10^5 T cells.

2.5. IL-6, IL-10, and IL-12 assay by ELISA

IL-6, IL-10 and IL-12 were measured in the supernatant collected at day 6 or 8 during DC culture using the Quantikine Immunoassay Kits (R&D Systems) in accordance with the manufacturer's instructions. Cytokine levels were quantified using a microplate reader at 450–490 nm, plotted against standard curves (with the cytokines), and expressed in pg/ml.

2.6. Protein extraction and Western blot analysis

Myeloma lysate-pulsed DCs were lysed with protein lysis buffer [20 mM sodium phosphate (pH 7.4), 150 mM sodium chloride, 1% Triton X-100, 5 mM EDTA, 200 μM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μg/ml pepstatin and 500 μM Na_3VO_4]. The protein extracts were electrophoresed on a SDS-polyacrylamide gel and transferred onto a nylon membrane (Millipore Corp., Bedford, MA, USA). After blocking with 5% skim-milk in 0.1% Tween 20 in PBS, membranes were probed with primary antibodies. Anti-VEGF (Santa Cruz, CA, USA), anti-phospho-STAT3 (Santa Cruz), anti-phospho-ERK1/2 (Cell Signaling Technology, MA, USA) and anti-ERK1/2 (Cell Signaling Technology) were diluted in blocking buffer, and reacted with the blots overnight at 4 °C. The bound primary antibodies were probed by a 1:2000 diluted secondary antibody and visualized by the ECL kit (Amersham, NJ).

2.7. Extraction of nuclear proteins and electrophoretic mobility shift assay (EMSA)

EMSA was performed with the Gel Shift assay system (Promega, WI, USA). Briefly, oligonucleotide with the consensus sequence for NF-κB (5′-AGTTGAGGGGACTTTCCCAGG-3′) was end-labeled with [γ-^{32}P] adenosine triphosphate (3 mCi/mmol) (Amersham Pharmacia Biotech, Buckinghamshire, UK) using the T4 polynucleotide kinase. The labeled oligonucleotide was then purified in microspin G-25 columns (Sigma) and used as a probe for EMSA. Nuclear extract protein (10 μg) was pre-incubated with the binding buffer for 5 min, followed by incubation with the labeled probe for 15 min at room temperature. Each sample was electrophoresed in a 5% nondenaturing polyacrylamide gel in 0.5× Tris–borate–EDTA buffer (pH 7.4) at 120 V for 5 h. The gel was dried and subjected to autoradiography. In competitive studies, a 100-fold excess of unlabeled oligonucleotide included in the reaction mixture along with the radiolabeled probes.

2.8. Statistical analysis

Statistical significance was determined using Mann–Whitney *U* test for nonparametric comparisons between groups. *P*-values <0.05 were considered statistically significant and the results were expressed as the mean ± S.D.

3. Results

3.1. The generation of DCs loaded with tumor antigens from MM patients

CD14$^+$ cells isolated from MNCs of PBSC products were induced to develop immature DCs in the presence of GM-CSF and IL-4 for