Cancer Letters 374 (2016) 175–185

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Dendritic cell based immunotherapy using tumor stem cells mediates potent antitumor immune responses

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ARTICLE INFO

Article history:
Received 31 October 2015
Received in revised form 8 January 2016
Accepted 11 January 2016

Keywords:
Cancer stem cell
Immunotherapy
Dendritic cell
Melanoma
Immunity

ABSTRACT

Cancer stem cells (CSCs) are demonstrated to be usually less sensitive to conventional methods of cancer therapies, resulting in tumor relapse. It is well-known that an ideal treatment would be able to selectively target and kill CSCs, so as to avoid the tumor reversion. The aim of our present study was to evaluate the effectiveness of a dendritic cell (DC) based vaccine against CSCs in a mouse model of malignant melanoma. C57BL/6 mouse bone marrow derived DCs pulsed with a murine melanoma cell line (B16F10) or CSC lysates were used as a vaccine. Immunization of mice with CSC lysate-pulsed DCs was able to induce a significant prophylactic effect by a higher increase in lifespan and obvious depression of tumor growth in tumor bearing mice. The mice vaccinated with DCs loaded with CSC-lysate were revealed to produce specific cytotoxic responses to CSCs. The proliferation assay and cytokine (IFN-γ and IL-4) secretion of mice vaccinated with CSC lysate-pulsed DCs also showed more favorable results, when compared to those receiving B16F10 lysate-pulsed DCs. These findings suggest a potential strategy to improve the efficacy of DC-based immunotherapy of cancers.

Introduction

Recent evidence suggests that tumors arise from a small subpopulation of primary cells known as cancer stem cells (CSCs) or tumor initiating cells [1], which are capable of generating heterogeneous tumor cell populations [2]. Since the first identification of cancer stem cells in acute myelogenous leukemia (AML) [3], these cells have been identified and described in several solid and non-solid malignancies including melanoma [4–11]. CSCs have been proposed to originate either from malignant transformation of normal somatic tissue stem cells or progenitor cells [12]. Although conventional methods to treat cancer including chemotherapy and radiotherapy are able to improve the quality of life and survival of most patients, cancer stem cells are usually slow cycling cells and thus less sensitive to these treatments [13]. Indeed, therapies that address the pool of differentiated tumor cells but fail to eradicate the CSC compartments might ultimately result in relapse of cancer, proliferation of therapy-resistant species and more aggressive tumor cells. An ideal treatment would kill cancerous cells in all stages of differentiation and, at the same time, selectively target and kill CSCs to avoid the tumor relapse [14]. Nowadays, a burst of research is directed towards targeting of different potentially tags of CSCs including cell surface antigens, signaling pathways related to self-renewal mechanisms, stem cell niches and transporters responsible for their drug resistance [15–21].

A wide variety of therapeutic modalities are being investigated for the treatment of malignant tumors including melanoma, in which dendritic cell-based immunotherapy with promising results have been tried in mice and humans [22–25]. Recently few studies have been shown that DC-based CSC vaccines can elicit anti-CSC humoral and cellular immune responses, leading to an efficient antitumor immunity. These studies provide supporting evidence for the idea that CSCs can be not only recognized and eradicated by the immune system, but also a rationale for designing new immunotherapeutic approaches aimed at targeting CSCs [26–29].

In the present study, we attempted to target cancer stem cells using DC-based immunotherapy in malignant melanoma as a cancer model in mice. In this light, we examined the potency of dendritic cells loaded with cancer stem cells antigen to induce cytotoxic T lymphocytes, which potentially target melanoma stem cells and increase the survival rate of tumor bearing mice.

Materials and methods

Cell lines, mice and antibodies

B16-F10 melanoma cells (Pasteur Institute of Iran, Tehran, Iran) were cultured in Dulbecco's modified Eagle's medium F12 (DMEM-F12) (Gibco, USA), containing

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http://dx.doi.org/10.1016/j.canlet.2016.01.021
0304-3835/

2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum (FBS) (Gibco, USA) and incubated at 37 °C with 5% CO2. Six- to 8-week-old female C57BL/6 mice were purchased from Pasteur Institute of Iran (Tehran, Iran). The mice were kept under optimal conditions of hygiene, temperature, humidity and light (cycles of 12 hr dark/light). All the experiments on mice were performed according to the Animal Care and Use Protocol of the Tarbiat Modares University (Tehran, Iran). Antibodies (Abs) against CD44 (FITC- conjugated), CD24 (PE- conjugated), CD11c (PE-conjugated), CD40 (FITC-conjugated), CD86 (FITC-conjugated) and I-Ab (MHC-II) (FITC-conjugated), were prepared from BD (BD Bioscience, USA).

Tumor induction, tumor size measurement and single cell preparation

The melanoma tumors were induced subcutaneously in C57BL/6 mice by inoculating 5×10^5 B16F10 cells suspended in phosphate buffer saline (PBS). Tumors were detected approximately 2 weeks after injection. Tumors sizes were measured with digital calipers and approximated by multiplication of the measured dimensions. Tumor dimensions were measured every other day after their appearance and mice with tumor volume more than 4000 mm^3 were sacrificed. Tumor volumes were calculated by the following formula: $V = (L \times W \times D) \times 0.5$; V = tumor volume, L = length, W = width and D = depth.

Single cell suspensions were obtained by tumor digestion using mechanical and enzymatic methods. Briefly, tumors were removed and minced into small pieces followed by washing with DMEM-F12 medium. Tumor dissociation was carried out at 37 °C for 20 min with shaking in PBS containing 1 mg/mL collagenase D (Roche, Germany) and 30 μg/mL DNase (Roche, Germany). The cell suspension was then filtered through a 70 μm mesh to remove undigested tissue fragments and washed twice with DMEM-F12 medium. Cells were counted and their viability was determined using the trypan blue dye exclusion assay.

Mouse melanoma tumor cells immunophenotyping and sorting of tumor cell subpopulations by FACScan

To stain the melanoma tumor cells, for each 10^6 cells which were suspended in staining buffer (PBS + FBS 2%), 1 μg of (both anti- CD24 and anti- CD44) antibodies was added to the cells and the cells were incubated on ice for 20 minutes, then washed and suspended in sorting buffer (PBS, 10% FBS and 2 mM EDTA). In order to sort the melanoma cells, a density of 10×10^6 stained cells suspended in 10 mL sorting buffer was isolated using FACS Aria II (BD Bioscience, USA). The tumor cells were subtyped according to their reaction with anti-CD24 and CD44 antibodies and categorized as $CD24^+/CD44^+$, $CD24^-/CD44^+$, $CD24^+/CD44^-$ and $CD24^-/CD44^-$ subpopulations. The obtained subpopulations were maintained for less than 12 hours at 4–8 °C until further use.

Sphere formation assay

To examine the ability of the original B16-F10 cells and sorted tumor cell subpopulations to produce tumor spheres, the cells were suspended in a serum-free medium (SFM) consisting of DMEM/F-12 supplemented with 20 ng/mL EGF (Epidermal Growth Factor) (PeproTech, Germany), or 20 ng/mL bFGF (basic Fibroblast Growth Factor) (PeproTech, Germany), or both with and without B27(1X) (Invitrogen, USA). In all experiments, cells were incubated at 37 °C in a humidified 5% CO2 atmosphere for a maximum of 12 days [30]. Total number of produced spheres in all groups were determined and compared.

Tumor genesis assay

The limiting dilution assay was carried out to compare the tumor induction potency of melanoma cells subpopulations. Briefly, 8-week-old C57BL/6 female mice (n = 6 per group) were challenged with 5 dilutions of all sorted tumor cells subpopulations (the $CD24^+/CD44^+$ double-positive, $CD24^-/CD44^-$ double-negative, $CD24^+/CD44^-$ single-positive and $CD44^+/CD24^-$ single-positive profiles) and B16F10 melanoma cell line. The number of injected cells was 100,000, 50,000, 25,000, 10,000, and 5000 cells/100 μL PBS for each mouse in the groups. The tumorigenic potential of each subpopulation was followed daily for 150 days or when the tumor size reached 4000 mm^3. The frequency of the melanoma-repopulating unit was calculated for each subpopulation.

Generation of tumor lysate from B16F10 cell line and melanoma cancer stem cells

B16F10 cells were detached from culture plate using EDTA (Merck-Germany), and resuspended in RPMI 1640 (Gibco, USA) or DMEM-F12 at a concentration of 0.5×10^7 cells/mL. The $CD24^+/CD44^+$ double-positive mouse melanoma sorted cells were also collected and re-suspended in RPMI 1640 or DMEM-F12 at the same concentration. The cell suspensions were subsequently frozen and thawed 5 times and centrifuged. The supernatants were passed through a 0.22 μm filter and protein concentration was assayed by Bradford method [31].

Generation of bone marrow derived DCs, antigen loading, phenotypic analysis of DCs and mice immunization

Dendritic cells were generated from murine bone marrow cells as described by Janga et al. [32]. Briefly, bone marrow was flushed from the tibiae and femurs of C57BL/6 mice, and depleted of red blood cells by ammonium chloride. Cells were plated in 6 cm microbial culture plates (3×10^5 cells/mL) in RPMI1640 medium supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 50 μM 2-mercaptoethanol, 100 IU/mL penicillin, 100 μg/mL streptomycin, 10% FBS, 20 ng/mL recombinant mouse GM-CSF (PeproTech, Germany) and 10 ng/mL recombinant mouse IL-4 (PeproTech, Germany) at 37 °C and 5% CO2 for 9 days. Half of the culture medium was replaced to replenish the cytokines milieu on days 3, 5 and 7. The immature DCs were harvested on day 9, and loaded with either B16F10 or CSC lysate (100 μg/mL). DC maturation was performed using 5 ng/mL of ODN CpG 1826 (Invivogen, USA). The cells were then passed through 12.2% nycodenz (Axis-shield, Norway) and low density cells were harvested, washed and re-suspended in FACS buffer (PBS containing 2% FCS). The cells were then stained with antibodies to CD11c, CD40, CD86, and I-Ab, for 20 min at 4 °C and analyzed for the expression of the above mentioned phenotypic markers, using the flow cytometer. For animal immunization, matured DCs loaded with CSCs or B16F10 lysates were washed three times with PBS and re-suspended in PBS at a final concentration of 1×10^7 cells/mL. 100 μl of DCs suspension was injected subcutaneously into mouse. In order to assess the in vivo cytotoxicity and survival assessment after tumor challenge, the mice were boosted twice after 7 and 14 days with the same dose of the vaccine.

In vitro functional assay of DCs

CpG matured DCs were tested for their T cells stimulation potential by the MLR assay. For this purpose, allogenic lymph node cells from BALB/c mice were labeled with 2.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, USA) at 37 °C for 15 min. Various numbers of matured DCs were co-cultured with 3×10^4 CFSE-labeled lymph node cells. The cells were then incubated at 37 °C in a humidified 5% CO2 incubator for 5 days before flow cytometric analysis. The proliferation percentage was determined using the FlowJo software (Treestar, USA). Co-culture of syngenic DCs and T cells from C57BL/6 mice were used as a control.

In vivo migration of DCs

In order to investigate the ability of dendritic cells to migrate into lymph nodes, DCs were labeled with 5 μM CFSE (Invitrogen, USA). Labeled cells (1×10^6) were injected subcutaneously into mouse right palm. Axillary lymph nodes (LNs) were removed 72 hours later, minced and digested for 30 minutes with 1 mg/mL of collagenase D (Roche, Germany) and 0.4 mg/mL of DNase (Roche, Germany). The individual cell suspensions were evaluated for the presence of $CFSE^+$ DCs by FACScan.

Lymphocyte transformation test (LTT)

In order to determine the potency of Ag pulsed DCs for the induction of Ag specific T cell responses, C57BL/6 mice (7 mice per each group) were injected subcutaneously with either unpulsed DCs, DCs pulsed with B16F10 lysate or DCs pulsed with CSC lysate. Seven days after immunization, regional lymph node cells from vaccinated mice were labeled with 2.5 μM CFSE at 37 °C for 15 min. The lymph node cells (3×10^5 cells/mL) were cultured in DMEM-F12 supplemented with 0.5% mouse serum in 96 well plates (Corning, USA) in the presence or absence of B16F10 or CSC lysate (100 μg/mL), and incubated at 37 °C in 5% CO2 for 96 hours. Phytohemagglotinin (PHA) was used as a positive control. Cell proliferation was assessed by FACScan and the division index for CFSE-labeled lymphocytes was calculated using FlowJo software version 7.6.1.

T helper cytokine (IFN-γ and IL-4) induction potentials of antigen-pulsed DCs

C57BL/6 mice were immunized with either antigen unpulsed DCs, DCs pulsed with B16F10 lysate (DC-TL) or DCs-pulsed with CSC Lysate (DC-CSCL). Seven days following the DC immunization, regional lymph node cells (3×10^5 per well) were cultured for 72 hours in the presence or absence of 100 μg/mL B16F10 lysate or CSC lysate. Culture supernatants were assayed for IFN-γ and IL-4 secretion using the corresponding ELISA kits (PeproTech, Germany).

DC-based vaccination

The efficacy of melanoma antigen pulsed DCs as a tumor vaccine was determined in two experimental designs. In prophylactic design the mice were immunized before tumor challenge and, in treatment group immunization, was done after tumor induction and appearance. Syngeneic C57BL/6 mice received subcutaneous injections of 1×10^6 DCs loaded with either melanoma cell line B16F10 lysate (DC-TL) or CSC lysate (DC-CSCL) in a volume of 100 μL at weekly intervals for three times. PBS or antigen unpulsed DCs were injected into 2 separated control groups (7 mice per group). The melanoma tumor was induced in immunized mice one week after the final boost in prophylactic experiments. In treatment groups of mice, animals were immunized with DCs on days 0, 3 and 7 after tumor establishment. Animals