Clinical Immunology (2007) **125**, 76–87

available at www.sciencedirect.com

www.elsevier.com/locate/yclim

Established B16 tumors are rejected following treatment with GM-CSF-secreting tumor cell immunotherapy in combination with anti-4-1BB mAb

Betty Li[a,*], **Jianmin Lin**[a], **Melinda VanRoey**[a], **Maria Jure-Kunkel**[b], **Karin Jooss**[a]

[a] *Cell Genesys Inc., 500 Forbes Blvd., South San Francisco, CA 94080, USA*
[b] *Bristol-Myers Squibb, USA*

Received 19 March 2007; accepted with revision 9 July 2007
Available online 13 August 2007

KEYWORDS
GM-CSF;
Immunotherapy;
Anti-4-1BB;
CD137;
Tumor rejection;
T cell response;
TIL infiltration

Abstract Immunization with irradiated tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates potent, specific and long lasting anti-tumor immunity in clinical and preclinical settings. Efforts to further increase immunotherapy efficacy with immune-modulatory agents are under evaluation. Based on the immune-modulatory properties of 4-1BB (CD137), it has been postulated that agonistic 4-1BB antibodies may add additional anti-tumor efficacy to GM-CSF-secreting tumor cell immunotherapy. The combination of GM-CSF-secreting tumor cell immunotherapy and anti-4-1BB monoclonal antibody (mAb) treatment resulted in rejection of established tumors in the B16 melanoma model. These anti-tumor effects correlated with persistent tumor-specific $CD8^+$ T cell responses. In addition, early tumor infiltration of functional $CD8^+$ T cells and a greater expansion of antigen-specific memory T cells were found in mice treated with the combination therapy. In summary, an agonistic anti-4-1BB mAb combined with GM-CSF-secreting tumor cell immunotherapy may provide a novel and potent treatment strategy for patients with cancer.

Introduction

4-1BB (CD137) is a type I membrane protein of the TNFR family which has been shown to augment CD4 and CD8 T cell responses [1]. Its expression is upregulated on activated $CD4^+$ and $CD8^+$ T cells, NK cells and monocytes. It binds to the 4-1BB ligand (4-1BBL) expressed on antigen-presenting cells (APC) such as macrophages, dendritic cells (DC) and activated B cells. Binding of 4-1BB to 4-1BBL delivers a signal to T cells leading to their activation, survival and growth [2–4]. Ligation of 4-1BB by antibodies is known to enhance anti-tumor immunity in tumor-bearing mice [5,6]. Anti-tumor immune responses include enhancement of proliferation and CTL activity of tumor-specific T cells [2,7], prevention of activation induced cell death (AICD) [2,3,8], enhancement of T cell infiltration into the tumor [9]

* Corresponding author. Fax: +1 650 266 3080.
E-mail address: betty.li@cellgenesys.com (B. Li).

1521-6616/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.clim.2007.07.005

and generation and maintenance of antigen-specific CD8 memory cells [10,11]. Considered together, these findings suggest that 4-1BB ligation may be an effective way to further enhance an on-going immune response.

Immunization with irradiated tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates potent, specific and long lasting anti-tumor immunity in clinical and preclinical settings [12–17]. Anti-tumor protection requires both CD4 and CD8 T cells [16,18,19]. The mechanism underlying the stimulation of anti-tumor immunity involves improved tumor antigen presentation by DCs recruited to the site of immunization [20]. DCs are highly activated at the immunotherapy injection site by the GM-CSF-secreting tumor cell immunotherapy and traffic readily to the draining lymph nodes (DLN) to activate a tumor-specific immune response [20]. Tumor-specific T cells infiltrate the tumor, resulting in tumor destruction.

The studies reported here evaluated the combination of GM-CSF-secreting tumor cell immunotherapy with anti-4-1BB monoclonal antibody (mAb) in preclinical models. We demonstrate in the poorly immunogenic and highly aggressive B16 melanoma model that mice treated with the combination therapy rejected established tumors when therapy was initiated as late as 11 days after tumor inoculation. In addition, long-lived cytolytic activity and enhanced IFNγ secretion by $CD8^+$ T cells were observed in the combination therapy-treated animals compared to animals treated with either monotherapy alone. Furthermore, higher numbers of activated effector CD8 T cells were detected in lymphoid tissues and the tumor microenvironment and a greater expansion of memory T cells was found in animals treated with the combination therapy. These data show that anti-4-1BB mAb combined with a GM-CSF-secreting tumor cell immunotherapy increases the level of anti-tumor efficacy in preclinical models.

Materials and methods

Mice and cell lines

Female C57BL/6 mice and BALB/c mice (Taconic, Oxnard, CA) were purchased and maintained according to AAALAC guidelines. Efficacy and mechanism studies were initiated with mice between 8 and 12 weeks of age. Study designs were approved and performed according to the guidelines of the Cell Genesys Animal Use and Care Committee.

The B16F10 melanoma and CT26 colon carcinoma cells were purchased from ATCC (Manassas, VA). The generation of the retrovirally transduced GM-CSF-secreting cell lines have been previously described [17]. These GM-CSF-secreting cell lines, termed B16.GM and CT26.GM, generate approximately 150 and 80 ng/10^6cells/24 h of murine GM-CSF, respectively. Cells were maintained in Dulbecco's Minimum Essential Medium (Hyclone, Logan, UT) supplemented with 10% heat-inactivated FBS (Hyclone), 2 mM L-glutamine and 1× penicillin/streptomycin (JRH, Lenexa, KS) in a humidified incubator with 5% CO_2 at 37 °C.

To generate OVA-expressing cell lines, F10.ova and GM.ova, a CD16 Ovalbumin LAMP-1 fusion protein [21] was excised and cloned into a third generation lentiviral transfer vector [22]. Vectors were generated by transient transfection of 293T cells as described previously [22]. The resulting population was stained using an OVA-specific antibody conjugated to biotin (United States Biological, Swampscott, MA), detected with a streptavidin-conjugated PE secondary antibody (BD Pharmingen, San Diego, CA) and sorted for OVA expression using a MoFlo (Cytomation, Inc., Fort Collins, CO, USA).

Anti-4-1BB antibody

A rat IgG2a mAb against mouse CD137 (BMS-469492, clone 1D8) was produced and purified by Bristol-Myers Squibb. Anti-4-1BB mAb was certified to have <0.5 EU/mg endotoxin level, >95% purity and <5% high molecular weight species. Stock solutions of anti-4-1BB mAb were kept at −80 °C and were thawed out on ice prior to use.

Survival efficacy

In the B16 model, mice were inoculated with 2×10^5 live B16F10 cells by subcutaneous injection at a dorsal site and immunized with 1×10^6 irradiated GM-CSF-secreting B16 cells (B16.GM) by subcutaneous injection at a ventral site at designated time points. Anti-4-1BB mAb (100 μg/injection) was administered 1 and 4 days post-immunotherapy. Animals were monitored for the formation of palpable tumors twice weekly and sacrificed if tumors became necrotic or estimated to exceed 1500 mm^3 in size.

In the CT26 model, mice were inoculated with 5×10^5 live CT26 cells and immunized 7 days later with 1×10^6 irradiated GM-CSF secreting CT26 cells (CT26.GM) by subcutaneous injection at a ventral site. Anti-4-1BB mAb (100 μg/injection) was administered on days 8 and 11. Animals were monitored for the formation of palpable tumors twice weekly and were sacrificed if tumors became necrotic or estimated to exceed 1500 mm^3 in size.

Cell characterization and flow cytometry

Cells from the draining lymph nodes (axillary, lateral axillary and superficial inguinal) and spleens were collected and mechanically dissociated using glass slides. Cells were counted and stained with conjugated antibodies purchased from BD Pharmingen (San Diego, CA) or K^b-ova (SIINFEKL) tetramer purchased from Immunomics (San Diego, CA). Flow cytometry acquisition and analysis was performed on a FACScan apparatus using CellQuest Pro obtained from BD Biosciences (San Diego, CA).

ELISPOT assay

Antigen-specific responses were enumerated by an IFNγ Elispot assay (R&D Biosystems, Minneapolis MN) according to the manufacturer's instructions. Briefly, 96-well filtration Elispot plates (Millipore, Bedford, MA) were coated with the specified amount of capture antibody in 100 μl of reagent diluent for 2 h at 37 °C. Plates were washed twice with wash buffer (PBS with 0.5% Tween 20) and blocked for 2 h at room temperature in blocking buffer containing PBS supplemented with 1% BSA and 5% Sucrose. Erythrocyte-depleted splenocytes (5×10^5) were plated and incubated for 48 h at 37 °C,