Dendritic cells combined with anti-GITR antibody produce antitumor effects in osteosarcoma

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Abstract. We attempted to enhance the antitumor effects of tumor lysate-pulsed dendritic cells by eliminating regulatory T cells. The combinatorial effects of dendritic cells and agonist anti-glucocorticoid-induced tumor necrosis factor receptor (anti-GITR) antibodies were investigated with respect to enhancement of the systemic immune response, elimination of regulatory T cells, and inhibition of tumor growth. To determine whether the combination of dendritic cells and anti-GITR antibodies could enhance systemic immune responses and inhibit primary tumor growth in a murine osteosarcoma (LM8) model. We established the following 4 groups of C3H mice (20 mice in total): i), control IgG-treated mice; ii), tumor lysate-pulsed dendritic cell-treated mice; iii), agonist anti-GITR antibody-treated mice; and iv), agonist anti-GITR antibody- and tumor lysate-pulsed dendritic cell-treated mice. The mice that received the agonist anti-GITR antibodies and tumor lysate-pulsed dendritic cells displayed inhibited primary growth, prolonged life time, reduced numbers of regulatory T lymphocytes in the spleen, elevated serum interferon-γ levels, increased number of CD8+ T lymphocytes. The mice that received combined therapy had reduced level of immunosuppressive cytokines in tumor tissue and serum. Combining agonist anti-GITR antibodies with tumor lysate-pulsed dendritic cells enhanced the systemic immune response. These findings provide further support for the continued development of agonist anti-GITR antibodies as an immunotherapeutic strategy for osteosarcoma. We suggest that our proposed immunotherapy could be developed further to improve osteosarcoma treatment.

Introduction

Osteosarcoma is the most common primary malignant tumor of the bone. Remarkable advances in the treatment of osteosarcoma have been made in the past 2-3 decades. These include the introduction of adjuvant chemotherapy and appropriate surgical excision (1,2). However, there have also been advances in the field of immunotherapy for osteosarcoma that have received less attention (3,4). We developed a method using dendritic cells (DCs) to enhance tumor-specific immunoreactions based on the premise that DCs are the main antigen-presenting cells initiating cell-mediated immune responses in vivo (5). Our current strategy involves eliminating immunosuppressive factors such as regulatory T cells (Tregs) and enhancing cell-mediated immunity.

The glucocorticoid-induced tumor necrosis factor receptor (GITR) family-related protein is constitutively expressed at high levels on Tregs and presented ubiquitously at lower levels on various immune subsets including cytotoxic T lymphocytes (CTLs) (6,7). GITR ligation provides a costimulatory signal that enhances CD4+ and CD8+ T cell proliferation and effector functions, particularly in the context of suboptimal T cell receptor stimulation (8,9). Signaling through GITR, using agonist anti-GITR antibodies or GITR ligands abrogates the suppressive effects of Tregs (7,10) and enhances T cell responses (6,8,9,11). Administration of agonist anti-GITR antibodies promotes the activation of CTLs, and interferon (IFN)-γ is reportedly required for the antitumor response induced by anti-GITR antibodies (12,13). Recently, several studies showed that in vivo GITR ligation by using anti-GITR antibodies can augment antitumor T cell responses and induce tumor rejection (14-16). However, the efficacy of the combination of tumor lysate-pulsed DCs and agonist anti-GITR antibodies in an osteosarcoma model has not been evaluated. Therefore, we hypothesized that an antitumor effect may be triggered if Tregs are controlled, resulting in the activation of CTLs and inhibition of tumor growth.

We investigated how immunotherapies that target the inhibitory pathways of Tregs using anti-GITR-mAbs can potentially synergize the effects of cryotreated tumor lysate-pulsed DCs to generate systemic antitumor immunity. We verify that, in contrast to tumor lysate-pulsed DC or anti-GITR-Ab treatment alone, the combination therapy enhanced antitumor immunity and slowed the growth.

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Materials and methods

Cell line. LM8 cells, derived from Dunn osteosarcoma, were provided by the Riken BioResource Center (Saitama, Japan). The cells were maintained in complete medium consisting of RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 µg/ml streptomycin and 100 U/ml penicillin. Cells were cultured at 37°C in 5% CO₂.

A total of 1x10⁶ LM8 cells (a murine osteosarcoma cell line) was hypodermically implanted into the subcutaneous gluteal region of 20 female C3H mice 6-8 weeks old. We purchased the C3H mice from Sankyo Labo Inc. (Toyama, Japan) and housed them in a specific pathogen-free animal facility in our laboratory.

DC generation. Bone marrow-derived DCs were generated as described by Lutz and Rössner (17) with minor modifications (5). Two weeks after tumor inoculation, we resected the primary tumor lesion and soaked the entire tumor in liquid nitrogen to kill the tumor cells. The freeze-thawed tumor lysate was added to the DC cultures on day 6 at a ratio of five DC equivalents to one tumor cell (i.e., 5:1) and incubated at 37°C in an atmosphere containing 5% CO₂. The homogenate was passed through a 0.2-µm filter to remove bacteria and tissues and mixed with the DCs for 24 h. After 24 h of incubation, non-adherent cells including DCs were harvested by gentle pipetting.

Antibody administration. Mice received 0.5 mg/mouse of agonistic affinity-purified anti-GITR monoclonal antibody (rat anti-mouse IgG; BioExpress). The control antibody is monoclonal antibody IgG (rat anti-mouse IgG, isotype control (rat anti-mouse IgG; BioExpress). The control antibody is agonistic affinity-purified anti-GITR monoclonal antibody (anti-GITR-Ab, n=5); and iv), DCs exposed to cryotreated tumor lysates were injected twice a week into the subcutaneous contralateral lateral gluteal region [DC(Ly), n=5]; iii), intraperitoneal injection of agonist anti-GITR antibody was performed twice per week (anti-GITR-Ah, n=5); and iv), DCs exposed to cryotreated tumor lysates and injected twice a week into the subcutaneous contra-lateral gluteal region and intraperitoneal injection of agonist anti-GITR antibody was performed twice per week [DC(Ly) + anti-GITR-Ab, n=5]. All experiments were performed under the guidelines for animal experiments as stipulated by the Oita University Graduate School of Medical Science. Tumor size was measured in 2 perpendicular dimensions parallel with the surface and the depth of the tumor in mice using a caliper.

Flow cytometry. The markers Foxp3 and CD4, which are expressed on the surface of Tregs, were counted with a FACSVersu™ flow cytometer (Becton-Dickinson, San Jose, CA, USA) and stained them with fluorochrome-conjugated antibody (BD Pharmingen, Tokyo, Japan) for the following markers: phycoerythrin (PE)-conjugated anti-mouse Foxp3 staining kit (eBioscience, San Diego, CA, USA) and fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 (clone, RM4-5; BD Pharmingen). Data analysis was performed with FACSuite™ software (Becton-Dickinson).

Immunohistochemistry. Immunohistochemistry was used to measure the levels of Foxp3, a marker of Tregs, and CD8, a marker of CTLs, inside primary tumor lesions. Lung specimens were fixed in frozen section. Five samples per mouse were cut into 15-µm-thick slices. Rehydrated tissue sections were incubated with primary Abs against CD8 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Foxp3 (Abcam, Cambridge, MA, USA) diluted at 1:200 in Ab Diluent (Dako ChemMate, Dako, Japan) overnight at room temperature. For CD8α staining with FITC donkey anti-rabbit IgG and Foxp3 staining with Texas red goat anti-rat IgG (Invitrogen, Carlsbad, CA, USA), secondary antibodies were diluted at 1:300 in Ab Diluent and added for 60 min at room temperature in the dark. Digital images were taken on a BIORÉVO microscope equipped with a confocal microscopy system (BZ-9000; Keyence, Japan).

ELISA. We measured murine IFN-γ and IL-10 release by enzyme-linked immunosorbent assay using Quantikine® (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions using a SkanIt for Multiskan FC microplate reader (Thermo Fisher Scientific, Tokyo, Japan).

Western blot analysis. Tumor tissue was dissected and washed briefly with chilled PBS, then cut into smaller pieces whilst keeping on ice. The tissue was placed in a homogenizer adding RIPA buffer (500 µl per 10 mg of tissue) with protease inhibitor. Tissue was homogenized thoroughly and kept on ice for 30 min. Total cellular protein (15 µg) was resolved on a precast 10% Tris-HCl Criterion 10-well gel (Bio-Rad) at 200 V (300 mAmp) for 30 min. The gel was wet-transferred to a PVDF membrane for 1 h, and blocked with PBST containing 5% instant dry non-fat milk for 30 min at room temperature. Antibodies against transforming growth factor (TGF)-β (R&D Systems) was obtained from Cell Signaling Technology (Tokyo, Japan); IL-10 (sc-7888) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA); and IL-6 (ab6672) and β-actin (ab61039) were from Abcam (Cambridge, UK). Immunocomplexes were visualized with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibodies, and developed using ECL Plus system with a ChemiDoc camera (ImageQuant LAS 4000 mini) (all from GE Healthcare, Tokyo, Japan). The quantification of western blot signals was performed by the densitometry with ImageQuant TL software (GE Healthcare). All western blot experiments were repeated at least 3 times.

Cell isolation from fresh tumor tissues and spleen. LM8 cells were inoculated into C3H mice, and 28 days after the injection, spleen cells were prepared. The tumor tissues were dissected from the mice and minced.

Fresh tumor specimens were gently minced over a wire mesh screen to obtain a cell suspension. The cell suspension was layered over Ficoll-Hypaque (GE Healthcare) and centrifuged at 500 x g for 30 min. After density gradient centrifugation, mononuclear cells were collected and washed with RPMI-1640 medium (Gibco, Carlsbad, CA, USA) containing 5% fetal bovine serum and 1% penicillin/streptomycin. Peripheral blood mononuclear cells (PBMCs) were also isolated by Ficoll-Hypaque density gradient centrifugation.
 PBMCs were collected, washed, and analyzed immediately. Viable cell counts were obtained using trypan blue dye. For isolation of CD3+CD4+CD25high CD127low, the cells prepared from tumor tissues were stained with APC/Cy7-conjugated anti-CD3 mAb (M1/70), FITC-conjugated anti-CD4 mAb (M1/70), Pacific Blue-conjugated anti-CD25 mAb (M1/70) and Alexa Fluor 647-conjugated anti-CD127 mAb (RB6-8C5) (all from BD Biosciences, San Jose, CA, USA). The percentages of CD25high CD127low cells were determined using BD LSRFortessa™ X-20 cell analyzer and analyzed with BD FACSDiva (BD Biosciences). The population of CD25high CD127low cells was isolated by FACSAria II (BD Biosciences). The purity of the isolated cells was consistently >95%.

RNA extraction, cDNA synthesis and quantitative real-time PCR. Total RNA was extracted from prepared isolated Tregs with the TRIzol reagent (Invitrogen) and cDNA was synthesized according to the manufacturer’s instructions (Roche). Quantitative real-time PCR (qRT-PCR) was performed using a LightCycler 480 Probe Master system (Roche), and PCR-specific amplification was conducted in the LightCycler® Nano (Roche). The relative expression of genes, TGF-β, IL-6, IL-10 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were calculated with the 2−ΔΔCt method. The primer and probe kit of TGF-β, IL-6, IL-10 and GAPDH were obtained from Applied Biosystems (Nagoya, Japan).

Statistical analysis. We determined differences among the 4 groups using a non-repeated measures analysis of variance (ANOVA) and the Scheffe’s test. All analyses were conducted using SPSS® 18.0 software (SPSS Japan Inc., Tokyo, Japan). Results were expressed as the mean ± standard deviation, and P<0.01 was considered statistically significant. For survival analysis, the differences in survival rates were analyzed by log-rank test.

Results

Tumor volume of the primary tumor. Forty-two days after inoculation, the volume of the primary lesion in mice that received tumor lysate-pulsed DCs and the agonist anti-GITR antibody (271.86±139.11 mm³) was lower (P<0.01) than in the mice that received tumor lysate-pulsed DCs (627.06±119.13 mm³) or the agonist anti-GITR antibody alone (571.08±149.47 mm³). The volume of the primary lesion in mice that received tumor lysate-pulsed DCs and the agonist anti-GITR antibody (438.45±103.97 mm³) was lower (P<0.01) than that in the mice that received tumor lysate-pulsed DCs (887.87±121.19 mm³) or the agonist anti-GITR antibody alone (701.47±95.97 mm³) (Fig. 2).

Survival rate. The median survival time was in IgG control, 35.7 days (range, 28-42); tumor lysate-pulsed DCs, 45.9 days (range, 40-51); anti-GITR antibody, 47.7 days (range, 41-66);...
and the tumor lysate-pulsed DCs and the anti-GITR-4 antibody group, 67.4 days (range, 41-83). Survival was significantly prolonged but differences in tumor lysate-pulsed DCs alone and anti-GITR antibody alone group were small compared with control IgG group (P<0.01). There was no significant difference between the tumor lysate-pulsed DCs alone and anti-GITR antibody alone groups. Further lifetime prolongation was observed in tumor lysate-pulsed DCs and the anti-GITR antibody group compared with the tumor lysate-pulsed DCs alone and anti-GITR antibody alone groups (P<0.01, Fig. 3).

Characterization of distinct subsets of CD25+Foxp3+ T cells in the spleen. Agonist anti-GITR antibodies markedly reduced the CD4+Foxp3+ Treg population in the spleen. The groups that received agonist anti-GITR antibodies alone or in combination with tumor lysate-pulsed DCs displayed marked decreases in the proportion of CD4+Foxp3+ cells compared to the control IgG or tumor lysate-pulsed DC-treated groups (Fig. 4A).

Cytokine release. Mice treated with tumor lysate-pulsed DCs and the agonist anti-GITR antibody displayed higher serum IFN-γ levels (278.33±18.64 pg/ml, P<0.01) than those that received tumor lysate-pulsed DCs (129.6±13.28 pg/ml) or the anti-GITR antibody alone (144.98±20.37 pg/ml, Fig. 4B). Serum IL-10 levels were lower (P<0.01) in mice that received the anti-GITR antibody alone (53.24±21.29 pg/ml) than in those that received tumor lysate-pulsed DCs alone (145.43±16.38 pg/ml). Serum IL-10 levels were lower (P<0.01) in mice that received tumor lysate-pulsed DCs and the anti-GITR antibody (15.38±9.26 pg/ml) than in those that received the anti-GITR antibody alone (53.24±21.29 pg/ml, Fig. 4C).
Infiltration of CD8⁺ T lymphocyte and Tregs in the tumor.

Foxp3 levels were significantly decreased, whereas CD8⁺ T cell numbers were significantly increased in the primary tumor lesions in the agonist anti-GITR antibody-treated group. Foxp3⁺ cells were not recruited to the primary area in the agonist anti-GITR antibody-treated group, but were recruited in the control IgG-treated group (Fig. 5A). The number of CD8⁺ T lymphocytes per unit area was higher (P<0.01) in mice that received tumor lysate-pulsed DCs and the agonist anti-GITR antibody (26.99±5.03 cells/mm²) than in those that received tumor lysate-pulsed DCs (12.34±4.22 cells/mm²) or agonist anti-GITR antibody alone (11.18±4.32 cells/mm², Fig. 5B). The number of Foxp3⁺ T lymphocytes per unit area was lower (P<0.01) in mice that received the agonist anti-GITR antibody (12.49±4.59 cells/mm²) than in those that received tumor lysate-pulsed DCs (27.38±4.31 cells/mm²). The number of Foxp3⁺ T lymphocytes per unit area was lower (P<0.01) in mice that received tumor lysate-pulsed DCs and the agonist anti-GITR antibody (4.31±3.29 cells/mm²) than in those that received the agonist anti-GITR antibody alone (Fig. 5C).

Immunosuppressive activity of tumor-infiltrating Tregs and tumor tissue.

To confirm the immunosuppressive activity of Tregs which gathered in the tumor, we analyzed the expression levels of TGF-β, IL-6 and IL-10 on Tregs sorted from tumor tissues. The expression of TGF-β (0.59-fold), IL-6 (0.61-fold), and IL-10 (0.59-fold) were significantly lower in DC with anti-GITR-Ab group compared with the control-IgG, DC alone and anti-GITR-Ab alone group as determined by real-time quantitative RT-PCR (Fig. 6A). We also performed immunoblot analysis to evaluate the protein levels of those immunosuppressive molecules using tumor tissue lysate. Western blot analysis showed that the expression levels of TGF-β, IL-10 and IL-6 of tumor tissue dramatically decreased in DC and anti-GITR-Ab compared with control-IgG, DC alone, and anti-GITR-Ab alone group (Fig. 6B).

Discussion

Most osteosarcoma patients are treated with some combination of surgery, radiation and chemotherapy. Despite recent advances in local therapies with curative intent, chemotherapeutic treatments for primary disease are unsatisfactory owing to severe adverse effects and incomplete long-term remission. Therefore, the development of novel therapeutic options is of great interest. Several immunotherapies have been investigated as new methods to overcome progressive cancers (18-21). We focused on the inability to control immunosuppressive factors such as Tregs, which inhibit attacker cells, such as DCs and CD8⁺ T lymphocytes; as a result, Tregs are a major cause of insufficient antitumor effects. Since the initial discovery that GITR stimulation drives T cell immunity (10), agonistic anti-GITR has been used extensively for tumor immunotherapy, and GITR stimulation has been shown to drive potent CD8⁺ T cell-mediated tumor protection (13,22). The proportion of Tregs in tumor tissues is dramatically reduced, which appears to be a direct consequence of depletion (23-25). Our aims were to evaluate Tregs by using the Foxp3 and CD4, in the spleens of mice; measure the levels of Foxp3 and determine the numbers of CD8⁺ T lymphocytes inside the primary and
primary tumor lesions; determine the changes in the primary and tumor volumes; measure the levels of IFN-γ and IL-10; and measure the expression levels of immune suppression factors, TGF-β, IL-10 and IL-6 from Tregs in the tumor tissue.

The group treated with the combination of tumor lysate-pulsed DCs and the anti-GITR antibody displayed smaller tumor lesions and the lifetime was prolonged. Importantly, the result of tumor rejection in the combined therapy group correlated with the intratumor ratio of CD8+ T cells to Tregs (26). This suggests that controlling immunosuppressive factors may facilitate the activity of DCs and CTLs in the tumor. The Treg depletion using anti-GITR antibody treatment combined with tumor lysate-pulsed DCs treatment showed significantly improved survival in comparison to the tumor lysate-pulsed DCs or anti-GITR antibody monotherapy groups.

The agonist anti-GITR antibody inhibited the proliferation of Tregs in the spleen. Inhibition of Treg accumulation in the spleen can enhance systemic cell-mediated immunity through the activation of DCs or CTLs. We believe that this result could reduce Treg accumulation and CD8+ T lymphocyte proliferation in the tumor tissues.

The agonist anti-GITR antibody inhibited the accumulation of Tregs and induced the infiltration of CD8+ T cells in the primary lesions. GITR signaling in CD4+Foxp3+ Tregs is required for their immunosuppressive capacity (6,8,27). We demonstrated that stimulation of GITR led to the reduction of Foxp3+ T cells in the tumor tissues.

The group treated with the combination of tumor lysate-pulsed DCs and agonist anti-GITR antibodies also displayed smaller primary lesions. Tregs comprise one of the major components of the immunosuppressive microenvironment of tumor lesions (28). This is consistent with our results that tumor lesion volumes were significantly reduced in the combined therapy group, suggesting that controlling immunosuppressive factors may facilitate the activity of DCs and CTLs in the tumor microenvironment.

Stimulation of GITR induced the activation of cell-mediated immunity by increasing serum IFN-γ levels and decreasing serum IL-10 levels. Tregs are among the major factors that cause potent cytokine-mediated immunosuppression in tumor cells, and GITR stimulation may be useful for enhancing the efficacy of cancer therapy or vaccines (9). Our results revealed that stimulating GITR using agonist anti-GITR antibody enhanced cell-mediated immunity.

Tregs can inhibit immune cell functions either directly through cell-cell contact or indirectly through the secretion of immunosuppressive mediators, such as IL-10 and TGF-β (29). Hence, it is possible that by removing tumor-specific Tregs, antitumor immunity could be enhanced. Many studies in mice have shown that removal or inhibition of this subset of cells can enhance antitumor immune responses (30,31). Decreasing immunosuppressive cytokines by depleting Tregs in the tumor can facilitate the activity of DCs and anti-GITR Abs could represent an important adjunct to cancer immunotherapy.

As a clinical application, GITR stimulatory therapy should involve the induction of antitumor immunity without adverse effects such as autoimmunity or cytokine storms. Continuous GITR stimulation has been linked to the exacerbation of autoimmune conditions (7,8). Several studies showed that melanocyte-specific autoimmunity could be avoided entirely via limited therapeutic administration of agonistic anti-GITR (14,32,33).

Taken together, our findings clearly support the therapeutic potential of agonist anti-GITR antibodies in osteosarcoma treatment. The effectiveness of GITR stimulation in humans has yet to be demonstrated. The present studies of concomitant and post-surgical immunity demonstrate that GITR stimulation during primary tumor growth could be sufficient for treating minimal residual disease or preventing tumor metastasis and recurrence. The synergistic effect of this combined chemoimmunotherapy using anticancer agents and immunotherapy with agonist anti-GITR antibodies could enhance self-reactive CTL responses, overcome self-tolerance, and induce long-lasting antitumor immunity (24). Future studies should be directed toward translating anti-GITR antibody therapy into clinical trials for evaluation of osteosarcoma treatments.

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References


