Journal of Pediatric Surgery xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

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

Sequential actions of immune effector cells induced by viral activation of dendritic cells to eliminate murine neuroblastoma

Naonori Kawakubo [a,*], Sakura Tanaka [b], Yoshiaki Kinoshita [a], Tatsuro Tajiri [c], Yoshikazu Yonemitsu [d], Tomoaki Taguchi [a]

[a] Department of Pediatric Surgery, Reproductive and Developmental Medicine, Faculty of Medical Sciences, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan
[b] Maternal and Child Health Division, Ministry of Health, Labour and Welfare, 1-2-2 Kasumigaseki Chiyoda-ku Tokyo, 100-8916, Japan
[c] Department of Pediatric Surgery, Kyoto Prefectural University of Medicine, Kyoto, Japan, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto, 602-8566, Japan
[d] R&D Laboratory for Innovative Biotherapeutics, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan

ARTICLE INFO

Article history:
Received 14 June 2017
Received in revised form 16 July 2017
Accepted 22 August 2017
Available online xxxx

Key words:
Dendritic cell
Neuroblastoma
Irradiation
Recombinant Sendai virus

ABSTRACT

Purpose: In preclinical trails, we reported the antitumor effect of dendritic cells activated with Sendai virus (rSeV/DC) combined with γ-irradiation against neuroblastoma. However, what kind of effector cells for the combined therapy were used to show the antitumor effect was unclear. In this study, we performed radiation and rSeV/DC therapy in vivo and examined the effector cells involved.
Methods: Dendritic cells were cultured from bone marrow cells, activated with SeV and administered intratumorally at 10^6 weekly for 3 weeks. Radiation was administered at 4 Gy/time × 3 times. During the treatment, CD4+ and CD8+ cells and natural killer (NK) cells were removed by antibodies.
Results: Complete remission of neuroblastoma was observed in 62.5% of individuals in the combined therapy group. By depleting the effector cells using antibodies, the tumor increased in size from an early stage of treatment in the CD4+ and NK cell-depleted group. In contrast, the tumor increased in size in the late stage of treatment in the CD8+ cell-depleted group.
Conclusion: The combination of radiation and rSeV/DC therapy induces different effector cells, depending on the time point during treatment.
Level of Evidence: V.

Neuroblastoma is the most common extracranial solid tumor seen in children [1] and presents in multiple manners, with varied clinical and molecular features. Neuroblastoma arising in children <1 year of age shows a relatively good prognosis; however, older patients with advanced disease exhibited a poor prognosis [2–4]. Intensive chemotherapy supported by stem cell transplantation has improved the survival of such patients [3,4]. The combination of anti-GD2 antibody, IL-2 and GM-CSF has now become a novel therapy in patients with advanced neuroblastoma [5]. However, recurrent cases in these children remain intractable [3].

Recent progress in understanding the role of the immune system in fighting cancer has resulted in the proposal of an alternative approach to a dendritic cell (DC)-based immunotherapy that has already been in use for several years. DCs are mononuclear cells that have a powerful ability to present antigens to T cells and activate other immune cells. This characteristic is encouraging recent applications to therapeutic cancer vaccines. DCs loaded with tumor antigens ex vivo and administered as cell vaccines have been found to induce protective antitumor immunity in laboratory animals. The first clinical study enrolled 15 children, including 3 individuals with intractable neuroblastoma, and demonstrated modest antitumor responses [6]. Another group subsequently reported the results of tumor RNA-loaded DC vaccination for 11 patients with advanced neuroblastoma [7]. Although these clinical studies successfully showed that DC therapy elicited specific immune reactions against neuroblastoma, the clinical outcomes are far from the level expected of standard therapies.

DC-based immunotherapy is still a developing technology, and the clinical outcomes have been limited in other malignancies; therefore, scientists and physicians should clarify 1) the most effective DC subtypes, 2) the optimal conditions and activation stimuli for generating activated DCs showing the greatest antitumor effect in vivo, 3) the optimum route of administration and 4) the optimum dose and frequency of DC vaccination [8–10].

We recently demonstrated dramatically improved efficacies using DCs activated by an RNA virus that replicates cytoplasmically, the

Abbreviations: DCs, dendritic cells; rSeV, recombinant Sendai virus; Ts-rSeV/dF, temperature-sensitive mutant F-defective nontransmissible rSeV; CTLs, cytotoxic T lymphocytes; NK cells, natural killer cells; i.t., intratumoral injection; i.p., intraperitoneal injection.

* Corresponding author at: Department of Pediatric Surgery, Reproductive and Developmental Medicine, Faculty of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Tel.: +81 92 642 5573; fax: +81 92 642 5580.
E-mail address: naonori@med.kyushu-u.ac.jp (N. Kawakubo).

http://dx.doi.org/10.1016/j.jpedsurg.2017.08.019
0022-3468/

recombinant Sendai virus (rSeV) [11–17]. We have therefore proposed a new concept – 'immunostimulatory virotherapy' – and explored its utility in multiple syngenic mouse models bearing highly malignant tumors. Using this system, we showed that rSeV-activated DCs expressing the interferon (IFN) β gene, especially in combination with γ-irradiation, led to the complete elimination of less-immunogenic c1300 neuroblastoma [17]. IFN-β strongly upregulated MHC class I molecules of c1300 and the CTL activity; however, the mechanisms underlying the immune aspects of this therapy remain unclear. Moreover, in our previous reports concerning the effector cell subsets of DC therapy, CD4+ cells as well as NK cells were important to the prevention of lung metastasis of prostatic cancer [16]. We hypothesized that not only CTLs but also other effector cells are crucial to prevent neuroblastoma from progression. In particular, we aimed to reevaluate the importance of CD4+ cells and NK cells in DC cell therapy. IFN-β strongly upregulates the expression of MHC-Class1 of tumor cells, so DCs expressing the IFN β gene are not suitable to estimate the efficacy of CD4+ cells or, especially, NK cells because the cytotoxicity of NK cells is downregulated by the MHC-Class I. So, in this study, we evaluated the efficacy and examined the effector cell subsets of the combination of γ-irradiation and ts-rSeV/dF-DC immunotherapy (not expressing the IFN-β gene) in order to investigate the effect of CD4+ or NK cells more clearly.

1. Materials and methods

1.1. Mice and tumor cell lines

Female A/J mice (H-2^a) 6 to 8 weeks of age were obtained from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan) and kept under specific-pathogen-free and humane conditions. Murine neuroblastoma c1300 (syngeneic with A/J mice) and MuSS murine malignant fibrous histiocytoma (obtained with the kind permission of Dr. Itaru Watanabe, Department of Surgery, Tasuda Hospital) [18] were obtained from RIKEN Cell Bank (Tsukuba, Ibaraki, Japan). These cell lines were maintained in complete medium (RPMI 1640 medium; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal calf serum (BioWest, Nuaille, France), penicillin and streptomycin under a humidified atmosphere containing 5% CO_2 at 37 °C.

1.2. Temperature-sensitive mutant nontransmissible rSeVs

The preparation and recovery of temperature-sensitive mutant F-defective nontransmissible rSeVs were performed as previously described [19,20]. Briefly, vectors were prepared using recombinant LLC-MK_2 cells carrying the F gene (LLC-MK_2/F7). An adenovirus vector, AxCANCre, expressing Cre recombinase was used to induce the F protein in LLC-MK_2/F7 cells (referred to as LLC-MK_2/F7/A). Recombinant vaccinia virus vTF7-3 carrying T7 RNA polymerase was inactivated with psoralen and long-wave UV irradiation, and then used for the ribonucleoprotein complex recovery. The viral vectors were further amplified by several rounds of propagation. The titers of the recovered viral vectors were expressed as cell infectious units.

1.3. Generation of DCs and transfection with rSeVs

Murine bone marrow-derived DCs (mBM-DCs) were generated as previously described [11,12] and maintained under endotoxin-free conditions using endotoxin-free reagents throughout this study. Briefly, bone marrow cells from A/J mice were collected and passed through a nylon mesh, and red blood cells and lineage-positive (B220, CD5, CD11b, Gr-1, TER119, 7/4) cells were depleted using a SpinSep mouse hematopoietic progenitor enrichment kit (StemCell Technologies, Vancouver, BC). These lineage-negative cells ($5–10 \times 10^4$/5 ml/well) were cultured in 50 ng/ml GM-CSF (PeproTech, London, UK) and 25 ng/ml IL-4 (PeproTech) in endotoxin-free complete medium in 6-well plates. On day 4, half of the culture medium was replaced with fresh medium supplemented with GM-CSF and IL-4 at the same concentration. On day 7, DCs were collected and used for subsequent experiments. These DCs were not pulsed with any tumor antigen throughout the experiments. For rSeV-mediated transduction, DCs (1×10^6 cells/ml) were simply incubated with rSeVs at MOI 100 without any supplementation.

1.4. DC-based immunotherapy of the established c1300 tumor

The DCs used in this study were not pulsed with any tumor antigen throughout the experiments. To examine the potentials of cancer vaccines tested here to treat highly malignant phenotypes in vivo, we additionally assessed a 'later treatment regimen' when tumors were well established and vascularized (5–7 mm in diameter) [11,12]. Radiation pretreatment (4 Gy/day for 3 days, daily) was performed if necessary.

The DCs were collected as described above, and subcutaneous implantation (A/J for 5×10^5 c1300 cells) was performed at the right thigh (to avoid irradiation-induced enterocolitis) on day 0, with 1×10^6 DCs injected intratumorally on days 10, 17 and 24. The size of the tumors was assessed using microcalipers three times a week, and the volume was calculated by the following formula: (tumor volume, mm^3) = 0.5236 × (long axis) × (short axis) × (height) [11,12].

1.5. In vivo depletion of immune cell subsets

Anti-CD4 and anti-CD8 monoclonal antibodies (250 μg/dose) were derived from GK1.5 and 53-6.72 hybridoma cells, respectively [21,22]. Anti-asialo GM1 (Wako, Tokyo, Japan) was given intraperitoneally (i.p., 50 μg/dose) for NK cell depletion. These antibodies were injected on days 4, 7, 11, 14, 17 and 20 after the primary tumor inoculation. Flow cytometry confirmed >98% depletion of the target cells for at least 7 days after injection in all animals. The mice were grouped by the treatment: no treatment group (described as "untreat" in Fig. 1); n = 6, radiation + DC therapy only (described as "no Ab" in Fig. 1); n = 6, anti-asialo GM1 antibody administered group; n = 6, anti-CD4 antibody administered group; n = 5, anti-CD8 antibody group; n = 5.

1.6. Rechallenge of tumor cells

This assay was aimed to investigate whether or not the complete elimination of c1300 tumors contributed to the establishment of long-lasting protective immunity.

1.6.1. Second challenge

Thirteen animals bearing c1300 tumors were divided into 4 groups as follows: untreated, $n = 2$; 4 Gy × 3 days radiation, $n = 4$; ts-rSeV/dF-DC, $n = 3$; and 4 Gy × 3 days radiation + ts-rSeV/dF-DC, $n = 4$. The animals were then treated as described in the schematic illustration in Fig. 1. The animals that survived were used for the second challenge. On day 129, 5×10^5 cells of c1300 were inoculated into the dermis of the abdominal wall. On day 224, tumor formation was assessed.

1.6.2. Third challenge

Three animals survived after the second challenge and were used for the third challenge. On day 318, 5×10^5 cells of c1300 (left) and MuSS (right) were inoculated into the bilateral dermis of the abdominal wall.

1.7. In vivo depletion of CD8+ cells during second challenge

Twelve animals that survived after the first inoculation of c1300 were divided into 2 groups as follows: radiation + ts-rSeV/dF-DC (without Ab) n = 6; radiation + ts-rSeV/dF-DC (anti-CD8), $n = 6$. The animals were then treated as described in the schematic illustration in Fig. 2. On day 141, 5×10^5 cells of c1300 were inoculated into the dermis of the abdominal wall. The group described as "radiation + ts-rSeV/dF-DC (anti-CD8)" was administered anti-CD8 antibody twice a week from day131 to day161. The group described as "Naïve control" did

Please cite this article as: Kawakubo N, et al, Sequential actions of immune effector cells induced by viral activation of dendritic cells to eliminate murine neuroblastoma, J Pediatr Surg (2017), http://dx.doi.org/10.1016/j.jpedsurg.2017.08.019