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Resveratrol analog, HS-1793 enhance anti-tumor immunity by reducing the CD4+CD25+ regulatory T cells in FM3A tumor bearing mice

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ABSTRACT

Natural agents with the immunomodulating property have been gaining traction to be employed in the complementary therapy of cancer because the ineffectiveness of numerous therapeutic strategies may be related in part to the tumor-induced immunosuppressive phenotypes, especially regulatory T (Treg) cells found in the tumor microenvironment. The present study was undertaken to examine whether HS-1793, synthetic resvertrol analog free from the restriction of metabolic instability and high dose requirement of resveratrol, induces an in vivo anti-tumor effect in FM3A tumor bearing mice through the suppression of Treg cells, which contribute to an increase in tumor specific cytotoxic T cell responses. Intraperitoneal injections of HS-1793 showed not only therapeutic benefits on established tumors, but also preventive anti-tumor effects. Treg cells (CD4+CD25+Foxp3+ cells) were significantly reduced in the total splenocytes as well as tumor tissues from HS-1793-administered mice, and the production of TGF-β inducing Treg showed a similar pattern. On the contrary, the administration of HS-1793 increased IFN-γ-expressing CD8+ T cells, upregulated IFN-γ production, and enhanced the cytotoxicity of splenocytes against FM3A tumor cells both in therapeutic and preventive experimental animals. These results demonstrated the suppressive role of HS-1793 on the function of Treg cells contributing to tumor specific cytotoxic T lymphocyte responses in tumor-bearing mice, which explained the underlying mechanism of the anti-tumor immunity of HS-1793.

1. Introduction

The recent advances in the definition of the mechanisms responsible for tumor progression have suggested the possibility of controlling cancer growth not only through chemotherapy-induced cancer cell destruction but also by stimulating anti-tumor immunity. Tumor-infiltrating lymphocytes (TILs) are seen as a reflection of a tumor-related immune response and recognized as the principal effectors of the local antitumor immune response [1]. During the neoplastic process, tumor cells acquire immunotolerance and cause an accumulation of immunosuppressive infiltrates in the tumor microenvironment [2]. One of the most potent and well-studied suppressive phenotypes found in the tumor microenvironment is the regulatory subpopulation among CD4+ cells (Treg cells), constitutively expressing high levels of CD25, CTLA-4, GITR, and a transcriptional factor forkhead box P3 (Foxp3) [3–5]. Actually, higher numbers of Treg cells are associated with the progression of malignancies including various solid tumors (lung, breast and pancreas) as well as hematological malignancies, and the increased populations of CD4+CD25+ Treg cells appear to correlate with poor survival in several types of cancers [6].

Several agents with the ability to stimulate anti-tumor immunity and can be useful in complementary cancer therapy have been isolated from plants. One of these agents is resveratrol which is gaining much attention. Resveratrol has been shown to inhibit cellular events associated with tumor initiation, promotion and progression in various types of solid tumors and to enhance the immune response in mice by promoting the production of Th1 cytokines, enhancing lymphocyte proliferation and suppressing Treg cell population [7–10]. However, its biological activities require high doses and are limited by photosensitivity and metabolic instability. In our previous studies, resveratrol analog [4-(6-hydroxy-2-naphthyl)-1,3-benzenediol, HS-1793] designed to overcome these problems, displayed stronger anti-tumor effects as compared to resveratrol in most cancer cell lines and induced the modulation of tumor-derived T lymphocytes, especially in its suppressive role on Treg cell population [11–13]. As there has still been considerable uncertainty about the effect of HS-1793 on the in vivo growth of tumor cells, the present study demonstrated that anti-tumor immunity

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in tumor bearing mice could be enhanced by HS-1793 through the suppression of the Treg cell population contributing to an increase in tumor specific cytotoxic T lymphocyte responses, which make the peritumoral microenvironment unfavorable to tumors and eventually result in tumor cell growth inhibition.

2. Materials and methods

2.1. Preparation of resveratrol analog, HS-1793

To obtain HS-1793, the stilbene double bond present in resveratrol was substituted with a naphthalene ring (Fig. 1) as previously described [11–13]. A stock solution was made in absolute ethanol at 50 mM, and the working dilutions were directly made in saline. The control vehicle was saline containing amounts of ethanol equivalent to those present in HS-1793.

2.2. Animals and cells

Female C3H/He mice, 6 weeks of age, were obtained from a Central Lab. Animal Inc. (Seoul, Korea) and housed at the animal maintenance facility of Clinical Research Center of Dong-A University Hospital. All animals were maintained in Specific Pathogen Free conditions according to the Good Laboratory Practices OECD guidelines. All animal procedures were performed according to approved protocols (Approval number; DIACUC-09-24) from the Institutional Animal Care and Use Committee (IACUC) of Dong-A University and in accordance with recommendations for the proper use and care of laboratory animals. FM3A murine breast cancer cells originated from the mammary gland of the C3H/He mouse and cells were cultured in complete RPMI 1640 medium as previously described [13]. For the evaluation of the therapeutic effect of HS-1793, FM3A cells in the logarithmic growth phase (2×10^6cells/50 μl saline) were inoculated subcutaneously on the right flank of mice. Mice were monitored daily for signs of toxicity and survival, and tumor volumes were calculated weekly as $(\text{width})^2\times\text{length}\times0.52$. When the tumor grew to a size of about 40 mm^3, mice were treated with an intraperitoneal injection of HS-1793 twice a week for 30 days. For the evaluation of the preventive effect, HS-1793 was injected simultaneously with the inoculation of tumor cells, and the injections of HS-1793 were continued twice a week for 30 days. When sacrificing the tumor-bearing mice following the last injection of HS-1793, splenocytes were prepared from an aseptically removed spleen, and paraffin-embedded sections (5 μm thick) were prepared from tumor tissue and major organs.

2.3. Lymphocyte subpopulation analysis

Lymphocyte subpopulations among splenocytes were analyzed with a FACS Calibur flow cytometer (Beckman Coulter) following standard surface staining procedures using the appropriately diluted labeled rat anti-mouse antibodies (anti-CD4 FITC, anti-CD8 PE and anti-CD25 PERCP-Cy5.5; BD Pharmingen). For the confirmation of Treg cells and the identification of IFN-γ expressing CD8+ T cells, standard surface staining procedures were combined with an intracellular staining method using anti-IFN-γ FITC (BD Pharmingen) and anti-FoxP3 PE (eBioscience) antibodies, respectively.

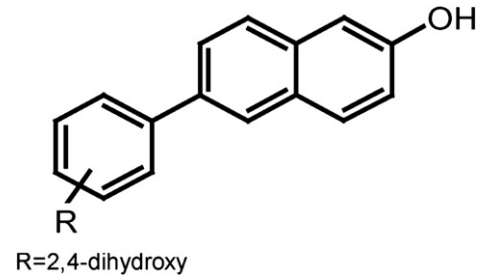

Fig. 1. Chemical structure of resveratrol analogs, HS-1793.

2.4. Cytokine production assay

Splenocytes were cultured with concanavalin A (5 μg/ml) for 24 h at 10^7 cells/ml in serum-free RPMI medium containing 200 μg/ml BSA. The TGF-β and IFN-γ concentrations in the culture supernatants were determined with enzyme-linked immunosorbent assay kits (R&D Systems, USA) according to the manufacturer's instructions.

2.5. Histopathology

Tumor infiltrating lymphocytes were evaluated with immunohistochemistry using deparaffinized sections (5 μm thick) that were pretreated for antigen retrieval with 1 M Tris–EDTA buffer for 15 min. CD8+ T cells were stained using a the streptavidin–biotin complex (ABC) method. The sections were successively incubated with primary rat anti-mouse CD8 (eBioscience) antibody, secondary anti-rat immunoglobulin (Dako) antibody, and an ABC reagent (Vectastain Elite kit, Vector Labs.), following the manufacturer's instructions. After the development with 3-3′-diaminobenzidine-H_2O_2, the sections were counterstained with Harris' hematoxylin and mounted. The slides were observed and photographed using a Zeiss Axiophot microscope with differential interference contrast equipment. Treg cells and IFN-γ expressing CD8+ T cells were stained by immunofluorescent staining. The sections were incubated with a pair of antibodies (anti-CD25 FITC/anti-FoxP3 PE; BD Pharmingen or anti-CD8 PE/anti-IFN-γ FITC; eBioscience). The slides were observed and photographed using a Nikon ECLIPSE 80i fluorescence microscope.

2.6. Cytotoxicity assay

Splenocytes (3×10^7 cells/5 ml) were restimulated with mitomycin C (10 μg/ml for 20 min)-treated FM3A cells for 3 days. The restimulated splenocytes (effector cells) were incubated with target cells (2×10^4 FM3A cells) at various effector/target ratios in 96-well round-bottom microplates (200 μl) for 6 h. After centrifugation, 100 μl of supernatants was collected, and the lactate dehydrogenase (LDH) released from the target cells was measured with a LDH release assaying kit (Roche-Applied) according to the instruction of the manufacturer. The percentage of cytotoxicity was calculated by the following formula:

$$\%\ \text{Cytotoxicity} = \left(OD_{\text{experiment}} - OD_{\text{effector spontaneous}} - OD_{\text{target spontaneous}}\right) / OD_{\text{target maximum}} - OD_{\text{target spontaneous}} \times 100.$$

The $OD_{spontaneous}$ of effector and target cells was controlled by separate incubation of the respective populations. $OD_{maximum}$ was measured after lysis of the target cells with 2% Triton X 100.

2.7. Statistical analysis

All data are expressed as a mean standard deviation (S.D.). The evaluation of statistical significance was performed using the Student *t*-test or one-way analysis of variance (ANOVA) with the Statistical Package for the Social Sciences (SPSS) statistical software for Windows, Version 18.0 (Chicago, IL, USA). For all analyses, a difference of $P<0.05$ was considered to be significant.