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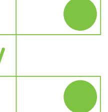

Establishment of Synergistic Chemoimmunotherapy for Head and Neck Cancer Using Peritumoral Immature Dendritic Cell Injections and Low-Dose Chemotherapies

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Abstract

The lack of available tumor antigens with strong immunogenicity, human leukocyte antigen restriction, and immunosuppression via regulatory T-cells (Tregs) and myeloid-derived suppressor cells are limitations for dendritic cell (DC)–based immunotherapy in patients with advanced head and neck cancer (HNC). We sought to overcome these limitations and induce effective antitumor immunity in the host. The effect of low-dose docetaxel (DTX) treatment on DC maturation was examined in an *ex vivo* study, and a phase I clinical trial of combination therapy with direct peritumoral immature DC (iDC) injection with OK-432 and low-dose cyclophosphamide (CTX) plus DTX was designed. Low-dose DTX did not negatively affect iDC viability and instead promoted maturation and IL-12 production. Five patients with metastatic or recurrent HNC were enrolled for the trial. All patients experienced grade 1 to 3 fevers. Intriguingly, elevated CD8+ effector T-cells and reduced Tregs were observed in four patients who completed two treatment cycles. All patients were judged to have progressive disease, but tumor regressions were observed in a subset of targeted metastatic lesions in two of five patients. Our results show that the combination of direct peritumoral iDC injection with OK-432 and low-dose CTX plus DTX is well tolerated and should give rise to changing the immune profile of T-cell subsets and improvement of immunosuppression in advanced HNC patients. Additionally, our *ex vivo* data on the effect of low-dose DTX treatment on DC maturation may contribute to developing new combination therapies with low-dose chemotherapy and immunotherapy.

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Introduction

Dendritic cell (DC)–based immunotherapy is anticipated to be an effective therapeutic strategy for advanced head and neck cancer (HNC). DCs play an important role in the initiation of antitumor immunity by increasing tumor antigen-specific cytotoxic T lymphocytes [1]. In general, *ex vivo* DC generation requires immunogenic epitopes of tumor antigens to elicit antitumor immunity. However, there are currently few well-defined tumor antigens for DC vaccines for HNC [2]. Direct injection of immature DCs (iDCs) into tumor tissue along with standard therapies, including chemotherapy and radiotherapy, has been used to treat advanced malignancies [3,4]. This strategy requires *in vivo* DC pulsing but has several advantages relative to *ex vivo* DC generation: 1) the expression of a specific tumor antigen is not required, and 2) there is no human leukocyte antigen (HLA) restriction. However, no studies to date have reported therapy using direct peritumoral iDC injection in HNC.

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HNC progression is associated with immune suppression in the host [5]. In advanced-stage HNC, tumor cells acquire immune resistance, escaping from antitumor immune responses by decreasing their immunogenicity and HLA class I expression [1]. Moreover, immunosuppressive components, including regulatory T-cells (Tregs) and myeloid-derived suppressor cells (MDSCs), support immune escape by several mechanisms: 1) inhibition of activated T cells by cell-cell contact and production of the immunosuppressive cytokines IL-10 and TGFβ and 2) functional suppression of activated DCs [6,7]. Therefore, immune resistance remains a serious problem that must be resolved to achieve clinical benefit from DC-based immunotherapy.

Increasing studies are focusing on the synergistic benefits of combining cancer immunotherapy with chemotherapy [8]. OK-432, an immunotherapeutic agent derived from penicillin-killed *Streptococcus pyogenes*, received clinical consensus for HNC treatment [9]. OK-432 enhances DC secretion of proinflammatory cytokines to stimulate CD8+ T cells, leading to enhanced antitumor immunity in the host [10]. Moreover, low-dose chemotherapy is favored in combination with DC-based immunotherapy because high-dose exposure to chemotherapeutic agents inevitably decreases the number of neutrophils and lymphocytes, leading to a deleterious effect on the host immunity [11] Cyclophosphamide (CTX) and docetaxel (DTX) exhibit immunomodulatory effects to upregulate antitumor immune responses in a dose-dependent manner [11]. Both drugs induce immunogenic cell death (ICD) in tumor cells, thereby initiating and stimulating the adaptive and innate immune responses [12,13]. Moreover, low-dose CTX depletes Tregs [14]. Unlike CTX, DTX can inhibit MDSCs, but not Tregs, in tumor-bearing hosts [15]. These observations indicate that direct iDC injection with OK-432 and low-dose chemotherapy might provide antitumor effects by attenuating immunosuppression. However, whether this strategy is a feasible treatment for advanced HNC has not been investigated.

To overcome the limitations of the performance of DC-based immunotherapy in HNC, we initiated combination therapy with direct peritumoral iDC injections with OK-432 and low-dose CTX plus DTX as a clinical trial. This combination was shown to be acceptably safe and should alter the immune profile of T-cell subsets with improvement of immunosuppression in patients with advanced HNC. In addition, our *ex vivo* data on the effect of low-dose DTX treatment on DC maturation may contribute to developing new combination therapies with low-dose chemotherapy and immunotherapy.

Materials and Methods

Cell Lines, Cell Culture, and Cell Viability Assay

The HSC4 and Ca9-22 human oral squamous cell carcinoma cell lines, which expressed HLA-A*2402 by HLA typing, were cultured as previously described [16,17]. Cells were treated with DMSO or DTX (3.125 μM or 12.5 μM) for 48 hours. The culture supernatant (CS) was collected for *ex vivo* co-culture assays. Cell viability was determined by scoring Trypan blue uptake.

Preparation of iDCs, DC Maturation, and DC Activation

Peripheral blood mononuclear cells (PBMCs) from HLA-typed healthy donors were separated by centrifugation over Ficoll-Paque Plus (GE Healthcare), and monocytes were enriched by adherence to a plastic culture flask for 60 minutes at 37°C. For induction of iDCs, adherent cells were cultured for 5 days with 1000 U/ml GM-CSF (CellGenix) and IL-4 (COSMO BIO) in AIM-V (Thermo Fisher Scientific). Thereafter, iDCs were incubated in the CS of low-dose DTX–treated tumor cells for 24 hours to promote maturation. DC activation was induced by culture in AIM-V with 0.1 KE/ml OK-432 (Chugai Pharmaceutical).

For generation of iDCs under GMP conditions, blood samples were collected by leukapheresis (Fresenius Kabi) 1 month after the termination of conventional therapy. All subsequent operations were performed under GMP conditions in the Cell Processing Center. Mononuclear cells from 50 ml of the leukapheresis products were separated by Ficoll-Paque Premium gradient centrifugation (GE Health Care), washed in PBS (Thermo Fisher Scientific), and plated in flasks. After 1 hour, nonadherent cells were removed by PBS washing. Adherent monocytes were cultured for 5 days in AIM-V medium with 5 ng/ml of GM-CSF and 5 ng/ml of IL-4 at 37°C in a humidified incubator containing 5% CO_2. The iDCs were harvested, washed in PBS, and resuspended in AIM-V medium. Inspection of endotoxin contamination was also performed in each collected sample.

Flow Cytometry

Cells were analyzed for the expression of cell-surface markers on a BD Accuri C6 flow cytometer or Attune Acoustic Focusing Cytometer. After Fc blocking, PBMCs were labeled with specific antihuman monoclonal antibodies against the following molecules: 1) CD11c, CD80, CD86, CD83, and HLA-DR or 2) CD3, CD4, CD8, CD11b, CD14, CD45RO, CD25, CD62L, and CD127. All antibodies were directly conjugated to FITC, PE, PE-Cy7, or APC. We used 7-amino-actinomycin D (7-AAD) for exclusion of nonviable cells. Appropriate isotype control antibodies, such as IgG1κ, IgG2a, and IgG2b, were included in all assays. Data were analyzed using the FlowJo software V9.

Real-Time RT-PCR

Quantitative PCR analysis was performed as previously described [17]. The primers used were as follows: human *GAPDH* (sense, 5′-GCACCGTCAAGGCTGAGAAC-3′; antisense, 5′-ATGGTGGTGAAGACGCCAGT-3′), human *IL12A* (sense, 5′-GCACAGTGGAGGCCTGTTTA-3′; antisense, 5′-GCCAGG CAACTCCCATTAGT-3′), and human *IL12B* (sense, 5′-AGAACTTGCAGCTGAAGCCA-3′; antisense, 5′-CCTGGACCTGAACGCAGAAT-3′). mRNA levels were normalized to GADPH mRNA levels in the same sample. The relative expression levels of target genes were determined by the $2^{-\Delta\Delta CT}$ method.

Enzyme-Linked Immunosorbent Assay (ELISA)

CS was collected from iDCs cultured in CS of DMSO or low-dose DTX–treated head and neck squamous cell carcinoma (HNSCC) cells for 24 hours and then used for ELISA. IL-12 was measured using the Human IL-12 p70 Quantikine ELISA kit (R&D System, Minneapolis, MN). All ELISA samples were read on the Multi-Microplated Reader (CORONA, Ibaragi, Japan).

Study Design of the Phase I Trial

A phase I trial study was performed at the University of Yamanashi Hospital, Japan. The primary endpoint was adverse events graded according to the NCI-Common Toxicity Criteria version 3.0. The secondary endpoint was immune induction after chemoimmunotherapy.

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