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Zoledronate facilitates large-scale *ex vivo* expansion of functional γδ T cells from cancer patients for use in adoptive immunotherapy*

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Background

Human γδ T cells can be activated by phospho-antigens and aminobisphosphonates such as zoledronate. Because they can kill tumor cells in a major histocompatibility complex (MHC)-unrestricted manner, adoptive transfer of activated γδ T cells may represent a novel cancer immunotherapy. We tested whether γδ T cells from advanced cancer patients can be expanded by zoledronate.

Methods

Peripheral blood mononuclear cells from healthy donors and patients with advanced non-small cell lung cancer, bone metastatic breast or prostate cancer, or lung metastatic colorectal cancer, were stimulated with zoledronate (5 μM) and interleukin (IL)-2 (1000 IU/mL) for 14 days. The phenotype and function of the expanded γδ T-cell populations from healthy donors and cancer patients were compared.

Results

γδ T cells from cancer patients and healthy donors responded to zoledronate equally well in terms of both phenotype and function. γδ T cells grew rapidly in vitro *and expression of effector molecules, such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, perforin, granzyme B, FasL and TRAIL, increased over time. Cytotoxicity peaked on days 12–14, and proliferation continued up to 14 days, during which time* $> 1 \times 10^9$ *γδ T cells could be obtained from a starting sample of 45–70 mL peripheral blood.*

Discussion

Using the agent zoledronate, already widely used in the clinic, we have established that efficient large-scale ex vivo *expansion of γδ T cells from cancer patients is possible. These cells exert potent cytotoxicity and may be used for autologous cellular immunotherapy of cancer.*

Keywords

γδ T cell, adoptive transfer, immunotherapy, bisphosphonate, zoledronate.

Introduction

γδ T cells are a minor T-cell population that constitutes 1–5% of circulating lymphocytes [1,2]. The vast majority of γδ T cells in humans express T-cell receptor (TCR) Vγ9 and Vδ2 chains that directly recognize non-peptide ligands without the necessity of presentation by major histocompatibility complex (MHC) molecules [3]. It is well established that human γδ T cells can recognize ligands expressed by tumor cells and that they exert strong cytotoxic activity against different tumor cell lines [4–8]. More recently, *in vivo* activation of γδ T cells and adoptive transfer of *ex vivo*-expanded autologous γδ T cells are being considered for deployment as a new immunotherapeutic approach [9,10]. Thus, based on the initially unexpected finding that γδ T cells are increased in some patients with multiple myeloma receiving intravenous pamidronate, a clinical trial was performed to assess the applicability of *in vivo* activation of γδ T cells for the

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treatment of malignancies. Wilhelm *et al.* [11] reported that administration of pamidronate together with interleukin (IL)-2 resulted in the activation and expansion of circulating γδ T cells in some patients with lymphoid malignancies and that this was associated with objective responses. Efficient induction of γδ effector cell function has also been achieved by *in vivo* treatment with the third-generation aminobisphosphonate, zoledronate [12]. The results of a recent phase I clinical trial in metastatic hormone-refractory prostate cancer support the concept that *in vivo* activation of γδ T cells by zoledronate and IL-2 can represent a novel immunotherapeutic strategy [13].

Alternatively, adoptive transfer of γδ T cells first expanded *ex vivo* and then re-infused into patients has been tested in several different ways [14–17]. The identification of several phospho-antigens that activate γδ T cells has facilitated the application of this approach. The synthetic phospho-antigens bromophydrin pyrophosphate (BrHPP) and 2-methyl-3-butenyl-1- pyrophosphate (2M3B1PP) were successful in expanding γδ T cells for use in clinical trials in patients with renal cell carcinoma [18,19]. These phase I clinical studies suggested that adoptive immunotherapy using *in vitro*-expanded γδ T cells is well-tolerated and worthwhile developing as a novel immunotherapy.

In the present study, we selected zoledronate to activate γδ T cells *in vitro* because this agent is known to stimulate these cells potently and is already licensed for clinical applications [12]. Autologous cells derived from cancer patients must be processed in accordance with all current regulations and ethical obligations; for this reason, it is advantageous to prepare cellular therapeutic materials using only clinically approved drugs. We investigated whether zoledronate can stimulate large-scale expansion of γδ T cells from advanced cancer patients as well as healthy donors and tested their anti-tumor activity *in vitro*.

Methods

Isolation of peripheral blood mononuclear cells and γδ T-cell culture

Informed consent was obtained from all healthy donors and patients prior to blood collection. Six healthy donors (H), 10 patients with advanced non-small cell lung cancer (LC), five patients with bone metastases from breast or prostate cancer (BM) and four with lung metastases from colorectal cancer (LM) were enrolled in the study (Table 1). All procedures were approved by the Ethical Committee of the University of Tokyo (Tokyo, Japan). Whole blood (7.5 mL) was collected in BD Vacutainer Blood Collection Tubes with sodium heparin (BD, Franklin Lakes, NJ, USA) and directly centrifuged to isolate peripheral blood mononuclear cells (PBMC). PBMC were stimulated with 5 μM zoledronic acid (Novartis, Basel, Switzerland) in AlyS203-γδ medium (Cell Science and Technology Institute, Sendai, Japan) containing 1000 IU/mL human recombinant IL-2 and 10% autologous serum. The medium containing IL-2 (1000 IU/mL) was added every 2–3 days and the cultures were transferred into the new flasks or culture bags as necessitated by the degree of cell growth. Cultured cells were analyzed at the indicated time points.

Flow cytometry

The following monoclonal antibodies (mAb) were used for phenotypic analysis: fluorescein isothiocyanate (FITC)-labeled anti-CD3 and -TCR Vγ9; phycoerythrin (PE)-labeled anti-CD16, -CD19, -CD56, -TCRαβ, -NKG2D and anti-mouse IgG1; phycoerythrin-Cy5 (PC5)-labeled anti-CD3, -CD8, -CD14, -CD27, -CD56 and anti-mouse IgG1; and phycoerythrin-Texas Red-X (ECD)-labeled anti-CD4, -CD45, -CD45RA and anti-mouse IgG1. They were all purchased from Beckman Coulter (Immunotech, Marseille, France). PE-labeled anti-CD69 and anti-TCR Vδ2 mAb were purchased from BD Bioscience Pharmingen (San Diego, CA, USA). The PBMC absolute cell count was determined by the addition of flow-count fluorospheres (Beckman Coulter) and cell viability was determined by staining with 7-AAD (Beckman Coulter). The cells were stained with antibodies (Ab) and analyzed using a Cytomics FC 500 (Beckman Coulter). The data were processed using CXP Analysis 2.0 software (Beckman Coulter).

Gene expression analysis

Total RNA was isolated from PBMC or γδ T cells using TRIZOL (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed into cDNA by SuperScript III (Invitrogen). The expression of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, perforin, granzyme B, FasL, TRAIL and GAPDH was determined by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) using a Thermal Cycler Dice (TAKARA BIO, Otsu, Japan) with EXPRESS SYBR GreenER qPCR SuperMix Universal (Invitrogen). Standard curves for the quantification of corresponding genes were constructed from the results of serial dilutions of the cDNA from the pooled activated γδ