Cytotherapy, 2015; ■■: ■■–■■

International Society for Cellular Therapy ISCT®

Ex vivo expansion of natural killer cells from human peripheral blood mononuclear cells co-stimulated with anti-CD3 and anti-CD52 monoclonal antibodies

JUN-ICHI MASUYAMA[1,2], TAKASHI MURAKAMI[3], SANJU IWAMOTO[4] & SANEHIKO FUJITA[1]

[1]New City Osaki Clinic, Tokyo, Japan, [2]Division of Inflammation Research, Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan, [3]Laboratory of Tumor Biology, Takasaki University of Health and Welfare, Takasaki, Gunma, Japan, and [4]Department of Biochemistry, School of Medicine, Showa University, Tokyo, Japan

Abstract

Background aims. This study developed a new method to expand $CD3^-CD56^+$ natural killer (NK) cells from human peripheral blood mononuclear cells (PBMCs) without feeder cells for clinical trials. *Methods.* PBMCs from healthy subjects were co-stimulated with anti-CD3 and anti-CD52 monoclonal antibodies and cultured for14 days in newly developed NKGM-1 medium containing autologous plasma and interleukin-2. Expanded NK cells were examined for cell number, phenotype, *in vitro* and *in vivo* cytotoxicity and interferon (IFN)-γ secretion. We also evaluated the proliferative ability of NK cells after cryopreservation. A patient with advanced pancreatic cancer was treated with autologous-expanded NK cells through the use of this method in combination with chemotherapy. *Results.* Expanded NK cells expressed higher levels of activating molecules compared with resting NK cells and exhibited potent cytotoxicity against K562 cells and IFN-γ secretion by cytokine stimulation. Significant anti-tumor activity was observed in immunodeficient mice injected with the human pancreatic cancer cell line BxPC-3. Large-scale cultures generated a median 5.7×10^9 NK cells from 20 mL of peripheral blood ($n = 38$) after 14 days of culture and 8.4×10^9 NK cells after 18 days of culture through the use of a cryopreservation procedure. The number of NK cells and cytotoxic activity in the peripheral blood of the patient with pancreatic cancer greatly increased, and successful clinical responses were observed after multiple infusions of expanded NK cells. *Conclusions.* These data demonstrate that this simple and safe methodology for the *ex vivo* expansion of NK cells can be used for cancer immunotherapy.

Key Words: *CD52, co-stimulation, immunotherapy, NK cell expansion*

Introduction

Natural killer (NK) cells, which are defined as $CD3^-CD56^+$ lymphocytes, are a major component of the innate immune system and exert cytotoxic effects on malignant and virus-infected cells without the recognition of specific tumor antigens on target cells in a major histocompatibility complex (MHC)-restricted manner, similar to T cells [1]. The net balance between activating and inhibitory signals exquisitely regulates the NK cell–mediated cytolytic response [2,3]. Killer cell immunoglobulin-like receptors (KIRs) on NK cells primarily provide inhibitory signals through interactions with MHC class I molecules that are expressed on target cells. In contrast, a variety of activating receptors, such as NK group 2D (NKG2D) and the natural cytotoxicity receptors (NCRs) NKp30, NKp44, NKp46 on NK cells, mediate activating signals [4,5]. Therefore, the lost or decreased expression of MHC on malignant cells or the enhanced expression of NK cell–activating receptors would promote NK cell–mediated killing [5–7]. NK cell–based immunotherapy is a promising candidate for immunotherapeutic approaches against malignant tumors because of the killing mechanisms of NK cells [8–10].

A variety of protocols were proposed to obtain large numbers of NK cells *ex vivo* for cellular immunotherapy [10,11]. Generally, it is difficult to expand the large numbers of NK cells that are needed for clinical applications in *ex vivo* cultures without feeder cells. A

Correspondence: **Jun-ichi Masuyama**, MD, PhD, New City Osaki Clinic, TOC Osaki Building 2F, 1–6–1 Osaki, Shinagawa 141-0032, Tokyo, Japan. E-mail: jmas@nco-clinic.jp

(Received 27 January 2015; accepted 24 September 2015)

ISSN 1465-3249
http://dx.doi.org/10.1016/j.jcyt.2015.09.011

recent study reported that the expansion of NK cells is greatly enhanced by the presence of feeder cells, such as irradiated peripheral blood mononuclear cells (PBMCs) [12], Epstein-Barr virus–transformed lymphoblastoid cell lines (EBV-LCL) [13] and gene-modified K562 cells, which act as artificial antigen-presenting cells (aAPCs) [14,15]. Particularly, K562-based aAPCs that express membrane-bound interleukin (IL)-21 (mbIL-21) induce a log-phase expansion of NK cells from unseparated PBMCs to reach a median 31,747-fold increase after 21 days in culture without evidence of senescence [15]. However, some investigators suggest that expanded NK cells with feeder cells include problematic issues that must be resolved for use in immunotherapy [11]. For example, there are concerns that the extensive expansion of NK cells beyond several weeks may reduce their cytotoxic function and *in vivo* persistence in the body after adoptive infusion because optimum culturing conditions provided by feeder cells are removed.

The practical use of expanded NK cells requires critical evidence that the above-mentioned concerns have been overcome. The methods used should also be simple, safe and economical. We developed a novel method for extensive *ex vivo* expansion of human NK cells through CD3- and CD52-mediated co-stimulation of PBMCs with the use of autologous plasma and IL-2 without feeder cells to meet these requirements. We also treated a patient with advanced pancreatic cancer with large numbers of NK cells that were expanded with the use of this method and demonstrated a rapid increase in the numbers of NK cells and $NKG2D^+$ T cells in blood and the cytotoxic activity of PBMCs after NK cell infusions.

Methods

Ethics

All experiments, including the use of mice, were approved by the animal ethics review board of Jichi Medical University and performed in accordance with the Jichi Medical University Guide for Laboratory Animals and following the principles of laboratory animal care formulated by the National Society for Medical Research. The ethics committee of New City Osaki Clinic approved the clinical protocol for NK cell therapy. Signed informed consent was obtained from healthy subjects and the patient with cancer according to the Helsinki Declaration.

Antibodies, cytokines and culture media

The following reagents were used for the stimulation and culture of PBMCs: muromonab/Orthoclone OKT3 (anti-CD3 monoclonal antibodies [mAb], mouse immunoglobulin IgG2a; Janssen Biotech), alemtuzumab/Campath-1H (anti-CD52 mAb, humanized IgG1;Gynzyme Corporation), anti-CD28 mAb (CD28-pure, Miltenyi Biotec GmbH), trastuzumab/Herceptin (anti-Her2 mAb, humanized IgG1; Chugai Pharmaceutical Co. Ltd) and cetuximab/Erbitax (anti-EGFR mAb, chimeric IgG1; Merck KGaA). Aldesleukin/Proleukin (recombinant human IL-2, Novartis Pharmaceuticals UK Ltd), IL-12 and IL-18 (PeproTech) were used. NKGM-1 medium (Kohjin Bio) was used for the expansion of NK cells unless otherwise indicated. AIM-V (Life Technologies), X-VIVO 10 (Lonza Walkersville, Inc) and CellGro SCGM (CellGenix) media were purchased for cell culture.

Flow cytometry

Cells were stained with fluorochrome-conjugated mAbs against the following antigens: CD3, CD16, CD56, CD158a, CD159a (NKG2A), CD244 (2B4) and CD314 (NKG2D) (BD Biosciences); CD158e (NKB1), CD335 (NKp46), CD336 (NKp44) and CD337 (NKp30) (Beckman Coulter, Inc); CD19 (Miltenyi Biotec); control IgG (BioLegend, Inc). Flow cytometry was performed with the use of a MACSQuant Analyzer (Miltenyi Biotec) as described previously [16]. Samples were analyzed with the use of MACSQuantify Software by the gating of viable cells and collection of 10,000 cells gated events per sample.

Cell preparation and animals

Human PBMCs were separated from the peripheral blood of healthy subjects by use of Ficoll-Hypaque (GE Healthcare Bio-Sciences AB) density-gradient centrifugation in a LeucoSep tube (No. 227290, Greiner Bio-One). Plasma was removed from the tube and heat-inactivated for 30 min at 56°C for use in culture. Purified $CD3^-CD56^+$ NK cells and $CD3^+$ T cells were obtained with the use of a BD FACSAria II Cell Sorter (Becton Dickinson). The purity was >97% as defined by flow cytometry, and the viability was >99% by use of trypan blue exclusion. Human leukemia cell lines K562 and Jurkat cells were obtained from The American Type Culture Collection (ATCC), and the human pancreatic cancer cell lines BxPC-3 and KP-3L were obtained from ATCC and the Japanese Collection of Research Bioresources Cell Bank, respectively. Cells were cultured in Roswell Park Memorial Institute-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Firefly (*Photinus pyralis*) luciferase (luc)-expressing pancreatic cancer cells were generated as previously described [17]. Generated cells were propagated in medium containing puromycin