A phase I study of adoptive immunotherapy for recurrent non-small-cell lung cancer patients with autologous $\gamma\delta$ T cells

Jun Nakajima a,*, Tomohiro Murakawa a, Takeshi Fukami a, Shigenori Goto c, Toru Kaneko c, Yukihiro Yoshida b, Shinichi Takamoto a, Kazuhiro Kakimi b

a Department of Cardiothoracic Surgery, The University of Tokyo Graduate School of Medicine, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
b Department of Immunotherapeutics (Medinet), The University of Tokyo Graduate School of Medicine, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
c Seta Clinic Group, 3-6-5, Iidabashi, Chiyoda-ku, Tokyo 1020072 Japan

Received 28 September 2009; received in revised form 9 November 2009; accepted 16 November 2009

Abstract

Objectives: Human $\gamma\delta$ T lymphocytes can recognise and kill non-small-cell lung cancer cells by V$\gamma$9V$\delta$ T-cell receptor and/or NKG2D. We have established large-scale ex vivo expansion of $\gamma\delta$ T cells by zoledronate and interleukin-2. This pilot feasibility study evaluates the safety and potential anti-tumour effects of activated autologous $\gamma\delta$ T cells administered intravenously to patients. Methods: Patients who had measurable foci of recurrent non-small-cell lung cancer were registered to undergo $\gamma\delta$ T-cell immunotherapy, designed as a one-way, open, clinical research, after their informed consent. Mononuclear cells collected from peripheral blood of the patient were cultured with zoledronic acid and interleukin-2. After 2-week incubation, the $\gamma\delta$ T-cell fraction was proliferated and it was intravenously reinjected to the patient. Results: Ten patients had undergone the $\gamma\delta$ T-cell immunotherapy. They were administered autologous $\gamma\delta$ T cells 3–12 times (mean = 6) every 2 weeks. No patient died during the study period. Adverse events, not directly related to the immunotherapy, were observed five times in four patients (grade 3 pneumonia in two and grade 1 coldness in three). According to the Response Evaluation Criteria in Solid Tumours, neither complete nor partial response was achieved in any patient; stable disease was observed in three; and progressive disease in five at 4 weeks after six consecutive injections of during immunotherapy. The Functional Assessment of Cancer Therapy-Biologic Response Modifier scores of the patients during immunotherapy were stable or improved, except for one patient who had suffered from pneumonia. The patients were followed up after immunotherapy for 240–850 days (median = 401 days). At the end of the observation, six patients were alive. Conclusions: We suggest that $\gamma\delta$ T cell immunotherapy might be safe and feasible for patients with recurrent non-small-cell lung cancer.

Keywords: Carcinoma; Non-small-cell lung; Immunotherapy; T-lymphocyte subsets; Prospective studies

1. Introduction

Human $\gamma\delta$ T cells that express V$\gamma$9V$\delta$ T-cell receptor (TCR) account for 1–5% of circulating lymphocytes [1,2]. Because of their potent cytoxicity and the preferential expansion of $\gamma\delta$ T cells in vitro by several ligands, their adoptive transfer is counted on novel cancer immunotherapy [3]. Clinical phase I/II studies on advanced renal cell carcinoma demonstrated that adoptive immunotherapy using $\gamma\delta$ T cells was safe and feasible [4,5].

Recently, we have established large-scale ex vivo expansion of $\gamma\delta$ T cells by zoledronate [6]. $\gamma\delta$ T cells can lyse a broad range of epithelial tumour cells, including non-small- and small-cell lung cancer (NSCLC) cells [7]. Therefore, a clinical study of adoptive $\gamma\delta$ T-cell transfer therapy has been conducted for treating NSCLC patients to evaluate the safety profile and the potential anti-tumour effects of $\gamma\delta$ T cells.

In this pilot phase I study, the principal objectives were to evaluate the safety of the activated autologous $\gamma\delta$ T cells administered intravenously to patients. The secondary objective was to evaluate the potential anti-tumour effects of the $\gamma\delta$ T cells.

2. Patients and methods

The study was registered as a University hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) Clinical Trial (Unique trial Number: C00000336) on 1 March 2006 (UMIN-CTR URL: http://www.umin.ac.jp/cutr/index.htm).
Research Review Board at our institution examined and approved our research protocol in light of the Declaration of Helsinki. Written informed consent was obtained from each patient before enrolling for the study. The study was conducted in compliance with Good Clinical Practice (GCP).

2.1. Patient selection

Eligibility criteria include patient’s age >20 years, those who had undergone surgical resection, chemotherapy or radiotherapy for the treatment of NSCLC, with measurable foci of the recurrence by computed tomography (CT). Patients were required to have a life expectancy of at least 24 weeks, and an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1. Patients with positive adult T-cell leukaemia-associated antigen (ATLA) or anti-human immunodeficiency virus antibody were excluded from the study, because these diseases could be exacerbated after γδ T-cell transfer therapy.

2.2. Study design

A one-way open, non-randomised, phase I study was carried out. Following informed consent, the patient’s peripheral blood was drawn for γδ T-cell culture six times biweekly. Cell culture methods were described previously [6]; briefly, peripheral blood mononuclear cells (PBMCs) from the patients were harvested and stimulated in vitro in the presence of interleukin 2 (IL-2) and zoledronate. After 2-week incubation, the cultured γδ T cells were intravenously administered to each patient (Fig. 1).

The first five consecutive patients were initially administered autologous activated γδ T cells, beginning at 1 × 10⁷ per dose and escalated in 10-fold increments to a maximum of more than 1 × 10⁸ γδ T cells per dose biweekly for six times. The next five consecutive patients were administered as many γδ T cells as possible (more than 1 × 10⁸ γδ T cells) from the first injection.

Peripherical blood of the patients was assessed at every visit for the γδ T-cell injection not only to diagnose the adverse effects of the immunotherapy but also to assess the number of γδ T cells in peripheral blood analysed by fluorescence-activated cell sorting (FACS) flow cytometry.

If the patient perceived clinical benefit or evident response was observed without significant toxicity, the patient could go on to undergo further treatments up to 12 times.

2.3. Isolation of PBMCs and γδ T-cell culture

Whole blood (70 ml) was collected in BD Vacutainer blood collection tubes with sodium heparin (BD, Franklin Lakes, NJ, USA) and directly centrifuged to isolate PBMCs. According to our previous work, it is estimated that more than 10⁹ of γδ T cells will be obtained from this volume [6]. Plasma was collected into 2-ml cryovials and cryopreserved until use. PBMCs were stimulated with 5-μM zoledronic acid (Novartis, Basel, Switzerland) in A12560-γδ medium (Cell Science and Technology Institute, Sendai, Japan) containing 1000 IU ml⁻¹ recombinant IL-2 and 10% autologous serum at the beginning of the culture. The medium containing IL-2 (1000 IU ml⁻¹) was added every 2–3 days and the cultures were transferred into new flasks or culture bags as necessitated by the degree of cell growth. Cultured cells were analysed at indicated time points.

2.4. Flow cytometry and enzyme-linked immunosorbent assay

The following monoclonal antibodies (mAbs) were used for phenotypic analysis: fluorescein isothiocyanate (FITC)-labelled anti-CD3 and -TCR Vβ9; phycoerythrin (PE)-labelled anti-CD16, -CD19, -CD56, -TCRαβ, -NK2D and mouse IgG1 isotype control; phycoerythrin-Cy5 (PC5)-labelled anti-CD3, -CD8, -CD14, -CD27, -CD56 and mouse IgG1 isotype control; and phycoerythrin-Texas Red-X (ECD)-labelled anti-CD4, -CD45, -CD45RA and mouse IgG1 isotype control. All these PE- and anti-CD69 and anti-TCR Vβ62 mAb were purchased from BD Bioscience Pharmingen (San Diego, CA, USA). The PBMC absolute cell count was determined by the addition of Flow-Count Fluospheres (Beckman Coulter) and cell viability was determined by staining with 7-AAD (Beckman Coulter). The cells were stained with antibodies (Abs) and analysed using a Cytomics FC 500 (Beckman Coulter). The data were processed using CXP Analysis 2.0 software (Beckman Coulter). The concentration of plasma interferon-gamma (IFN-γ) was measured using Human IFN-γ ELISA Set (BD Bioscience, San Jose, CA, USA), according to the manufacturer’s instruction.

2.5. Assessment of anti-tumour effect and quality of life

Anti-tumour effect of γδ T cells was assessed thrice by CT (at the time of registration, at the fifth γδ T-cell injection and at 4 weeks after the sixth γδ T-cell injection). According to the Response Evaluation Criteria in Solid Tumours (RECIST), the anti-tumour effect was classified as complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD) [8]. Quality of life (QOL) of the patients was assessed by the FACT-BRM score [9]. The patients answered the questionnaire of FACT-BRM score at the time of registration, every time at γδ T-cell injection as well as during follow-up.

3. Results

3.1. Safety assessment

Out of the 12 patients enrolled in this clinical study, two were not eligible because their γδ T cells did not sufficiently proliferate in vitro. The remaining 10 patients, eight men
and two women, underwent γδ T-cell immunotherapy (Table 1); the age range was 40—83 years. The performance status was zero in eight patients and one in two patients. Eight patients had adenocarcinoma, one had squamous cell carcinoma and one had large-cell carcinoma. All the patients except one had undergone surgery earlier. Eight patients had undergone multimodality therapies (surgery, radiotherapy, chemotherapy and molecular targeted therapy). All the patients except one had measurable foci of the cancer recurrence in the lung (nine patients), the regional lymph nodes (four patients) and in the liver (one patient). The number of intravenous γδ T-cell administration ranged from 3 to 12 (median = 6). Two patients extended the treatments to 12 injections. The cumulative number of transferred γδ T cells ranged from 2.6 to 31.4 \times 10^9 (Table 3). No patient died during the study period. Adverse events during the administration period were observed five times in four patients (Table 2); flu-like syndrome (grade 1) was observed three times in two patients, bacterial pneumonia (grade 3) in one and radiation pneumonia (grade 3) in one. All of these adverse events were not directly related to the administration of γδ T cells.

### 3.2. Immunological monitoring

γδ T cells in fresh peripheral blood were cells with CD45RA⁺ CD27⁺ naive phenotype or CD45RA⁻ CD27⁺ memory phenotype (Fig. 2A). By contrast, the transferred γδ T cells displayed CD45RA⁻ CD27⁺ effector memory phenotype (Fig. 2B). Based on this phenotypic difference, we monitored the survival of the transferred ex vivo expanded γδ T cells in patients during and after the course of treatments. γδ T cells consist of 1.1% (median, ranging 0.5—4.8%) of PBMCs before treatment, while 4.1% (median,
ranging 1.8—7.8%) of PBMC were γδ T cells after the treatment (Table 3). Majority of them were CD45RA CD27−, suggesting that transferred γδ T cells were well engrafted and some of them survived more than 2 weeks in vivo after transfer. However, there was no correlation observed between the accumulation of γδ T cells in patients’ peripheral blood and their clinical outcomes.

3.3. Potential anti-tumour effect of γδ T cells

According to RECIST, CR and PR were not achieved in any patient, SD was observed in four and PD in four, at the time of the fifth injection of γδ T cells. At 4 weeks after the last γδ T-cell injection, CR and PR were not achieved in any patient, SD was observed in three and PD in five (Table 3, Fig. 3). The patients were followed up after the immunotherapy for 240—850 days (median = 401 days). At the end of the observation, six patients were alive (Table 3).

The patients who received more γδ T cells (cumulative number of transferred γδ T cells in six injections) tended to result in SD than those in PD (no statistical difference). Both patients who received less than 10^{10} γδ T cells in six injections resulted in PD, while three out of five patients who received more than 10^{10} γδ T cells achieved SD (Table 3).

Plasma IFN-γ concentration was monitored during the study. IFN-γ was not detected before γδ T-cell injection and IFN-γ was never detected in 5 out of 10 patients (Table 4). Although elevation of plasma IFN-γ concentration was minimal, 5 out of 10 patients displayed elevation of IFN-γ. Importantly, IFN-γ was detected in all the three SD patients. Alternatively, all the five patients who did not demonstrate the elevation of IFN-γ resulted in PD.

3.4. Quality of life

To evaluate QOL in patients receiving treatment, their FACT-BRM score was monitored and was found stable or improved during immunotherapy except for a patient who had suffered from pneumonia (Fig. 4, #6 and #8) and bone metastasis (Fig. 4, #9).
4. Discussion

Lung cancer is one of the most common malignant neoplasms in the Western countries and Japan. In Japan, over 60,000 people died of lung cancer in 2006 (http://www.mhlw.go.jp/toukei/saikin/hw/jinkou/kakutei06/hyo7.html, written in Japanese), and the number of lung cancer deaths is increasing. As lung cancer is a highly malignant disease, surgery, radiotherapy and chemotherapy have limited effectiveness on patients with advanced NSCLC.

Recently, successful cancer regression in patients after transfer of highly reactive melanoma-specific cytotoxic T lymphocytes (CTLs) has been reported [10]. Adoptive cell-transfer therapy using \textit{ex vivo} expanded tumour-infiltrating lymphocytes (TILs) has been recognised as the most effective treatment for patients with advanced metastatic melanoma and can mediate objective cancer regression in \(\leq 50\%\) of patients. However, TILs with high avidity for tumour antigens can only be generated from some patients with melanoma and the generation of tumour-specific CTLs from patients with other solid tumours, including lung cancer, was extremely difficult. So far, no effective CTL therapy was available for the treatment of lung cancer [11].

Furthermore, loss or down-regulation of human leucocyte antigen (HLA) expression has been reported among various types of cancer, probably due to the selection pressure by the immunosurveillance mechanisms [12]. Importantly, down-regulation of HLA was observed in \(\leq 69\%\) of NSCLC patients [13]. If so, even though the generation of lung cancer-specific CTLs is successful and CTLs were transferred to the patient, these lymphocytes cannot recognise tumour cells and no clinical benefit can be expected.

Therefore, we focussed on \(\gamma\delta\) T cells. Ontogenically, \(\gamma\delta\) T cells emerge in early gestation than \(\alpha\beta\) T cell. The repertoires of \(\gamma\delta\) T cells are so limited that \(\gamma\delta\) T cells are regarded as innate immunity. \(V_\gamma V_\delta\) fraction of \(\gamma\delta\) T cells is mainly located in peripheral blood. Most \(V_\gamma V_\delta\) cells show memory cell characters, which implies that they have experienced repeat exposure of the specific antigens [1]. They are also known to be stimulated by major histocompatibility complex (MHC)-class I chain-related genes (MICA/B), which was
strongly expressed at the presence of heat-shock proteins. MICA/B are constitutively expressed on NSCLC cells as well as in breast cancer, renal cell cancer, ovarian cancer, prostate cancer and in colorectal cancer [14]. γδ T cells can kill a broad range of epithelial tumour cells by T-cell receptor and/or NKG2D-dependent recognition [7]. Even though tumour cells lost HLA expression, they were still susceptible to γδ T cells since γδ T cells can kill tumour cells by recognition of MICA/B and UL16-binding proteins (ULBPs) through NKG2D.

Recently, in vitro γδ T-cell culture has been feasible in the presence of zoledronate and IL-2. Zoledronate is a type of bisphosphonate, chelate of calcium ion, which inhibits farnesyl diphosphate (FPP) synthetase. Osteolytic reaction of the osteoclasts is decreased if the production of FPP is inhibited. On the contrary, isopentenyl pyrophosphate (IPP), which makes clonal expansion and activation of γδ T cells, is increased in the presence of zoledronate [15]. In patients with prostate cancer, direct administration of zoledronate and IL-2 was reported to be effective to decrease serum level of prostate-specific antigen (PSA) [16]. Bennouna et al. performed γδ T-cell transfer therapy on patients with metastatic renal cell carcinoma [5]. They described that repeated infusions of γδ T cells up to 8 × 10⁹ total cells, either alone or with IL-2, were well tolerated. Dose-limiting toxicity occurred in one patient at the dose of 8 × 10⁹ cells (disseminated intravascular coagulation). Mainly, gastrointestinal disorders and flu-like symptoms, such as fatigue, pyrexia and rigors, were the other treatment-related adverse events. In our study, γδ T cells were administered 1 × 10⁷ to 7.2 × 10⁷ at each injection (data not shown) and patients received 2.6 × 10⁸ up to 31.4 × 10⁸ in total (Table 3). No severe irreversible complications related to the therapy were observed. QOL assessed by FACT-BRM score had been kept well in all the patients during the administration of γδ T cells except for patients who had suffered from grade 3 adverse events not related to the γδ T-cell therapy.

As we were not allowed to access the tumours during the course of the study in the current protocol, we could not examine whether transferred γδ T cells reached the tumour. Although clinical results did not correlate to the engraftment of γδ T cells (frequency of γδ T cells in peripheral blood) or the cumulative number of γδ T cells transferred, IFN-γ production was only detected in patients whose clinical outcome resulted in SD, and not in PD. These results might suggest that the transferred γδ T cells recognised tumour cells and exert their effector function (IFN-γ production).

In conclusion, we suggest that γδ T-cell immunotherapy might be safe and feasible for patients with recurrent NSCLC. We also suggest that stabilisation of tumour could be observed during γδ T-cell immunotherapy.

Acknowledgements

We are grateful to Makoto Kondo and Keisuke Sato for γδ T-cell culture; Atsutaka Noguchi, Naoko Ariyoshi, Kazuko Sakuta, Akihiro Hosoi and Miki Sugiu for immunological monitoring and laboratory assistance; Takashige Kondo, Yoko Yamashita, Youichi Wada, Yukiko Katayose and Tomoko Ishida for clinical data management.

References

Appendix A. Conference discussion

Dr R. Schmid (Berne, Switzerland): This is an elegant possibility to individualise cancer therapy in these patients. My big concern is that you basically apply a drug by treating the patient with cultured cells. Are you doing viability testing after culture? Before injecting them into the patient, you have to know that the cells you are giving are active.

Dr Nakajima: Yes. Before the injection we performed a FACS analysis, trypan-blue staining and IL-2 productivity, to test the viability of the gammadelta T-cell fraction.

Dr Schmid: But can you prove that they are active in vivo then? You just test the survival of the cells, of course, but the anti-cancer activity is not proven.

Dr Nakajima: Yes. At the time of each injection, we also assessed the patient’s peripheral blood, and we found an increased number of gammadelta T cells in the peripheral blood, and so I think they are viable cells in vivo.

Dr L. Lang-Lazdunski (London, United Kingdom) I just have a brief question. Are these gammadelta T cells natural killer cells?

Dr Nakajima: No, the gammadelta T cells are not natural killer cells. Gammadelta T cells are a fraction of T cells. T cells are divided into the alphabeta TCR and gammadelta TCR exclusively, and about 5% of the peripheral T lymphocytes are gammadelta T cells.

Dr Lang-Lazdunski: And these gammadelta T cells, are they a class of cell you can boost when you use immunomodulation with low-dose cyclophosphamide, for example?

Dr Nakajima: We have no data about the immunomodulation on gammadelta T cells to date.