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# Phase I/II study of adoptive transfer of $\gamma\delta$ T cells in combination with zoledronic acid and IL-2 to patients with advanced renal cell carcinoma

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Junji Yagi · Nagahiro Minato · Kazunari Tanabe

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**Abstract** Human V $\gamma$ 2 V $\delta$ 2-bearing T cells have recently received much attention in cancer immunotherapy. In this study, we conducted a phase I/II clinical trial of the adoptive transfer of  $\gamma\delta$  T cells to patients with advanced renal cell carcinoma. Eleven patients who had undergone nephrectomy and had lung metastasis were enrolled. Peripheral blood  $\gamma\delta$  T cells obtained from the patients were stimulated *ex vivo* with 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP), a synthetic pyrophosphomonoester antigen, and transferred in combination with zoledronic acid (Zol) and teceleukin (recombinant human interleukin-2). Expanded  $\gamma\delta$  T cells exhibited potent cytotoxic activity against tumor cells *in vitro*, and the proportion of peripheral blood  $\gamma\delta$  T cells among CD3<sup>+</sup> cells typically peaked three to 5 days after

transfer. Tumor doubling time was prolonged in all 11 patients, and the best overall responses were 1 CR, 5 SD, and 5 PD, as defined based on Response Evaluation Criteria in Solid Tumors (RECIST). Although ten patients developed adverse reactions of grade  $\geq 3$ , they were likely to have been the result of the concomitant infusion of Zol and IL-2, and most symptoms swiftly reverted to normal during the course of treatment. In conclusion, this clinical trial demonstrated that our regimen for the adoptive transfer of  $\gamma\delta$  T cells in combination with Zol and IL-2 was well tolerated and that objective clinical responses could be achieved in some patients with advanced renal cell carcinoma.

**Keywords**  $\gamma\delta$  T cell · Nitrogen-containing bisphosphonate · Pyrophosphomonoester · Isopentenyl pyrophosphate · Renal cell carcinoma · Cancer immunotherapy

Part of this study was previously published as a case report [43].

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## Introduction

Human  $\gamma\delta$  T cells that express V $\gamma$ 2 V $\delta$ 2 (also termed V $\gamma$ 9 V $\delta$ 2)-bearing TCR recognize nonpeptide antigens derived from microbial pathogens such as mycobacteria [1–4] and exhibit natural cytolytic activity against a wide array of tumor cells *in vitro* [5, 6]. It is worth noting that  $\gamma\delta$  T cells exert specific cytolysis in a TCR-dependent manner when they encounter human tumor cells pulsed with nitrogen-containing bisphosphonates (N-BPs), such as pamidronate and zoledronic acid (Zol) [7, 8]. It is demonstrated that the inhibition of farnesyl pyrophosphate synthase by N-BPs results in the accumulation of isopentenyl pyrophosphate (IPP), a prenyl pyrophosphate intermediate, in tumor cells, which leads to the activation of  $\gamma\delta$  T cells [9, 10]. Although the elevated level of IPP is

essential in the activation, it is not clear whether IPP per se is directly recognized by  $\gamma\delta$  T cells.

Based on these findings, a novel cancer immunotherapy using  $\gamma\delta$  T cells and N-BPs has been proposed [11–13]. It has recently been reported that the addition of Zol to first-line chemotherapy in the treatment of patients with multiple myeloma significantly improved disease-free survival [14]. Because Zol was administered every 3–4 weeks, the induction of tumor immunity, and especially the activation of  $\gamma\delta$  T cells, was considered to be one of the mechanisms by which Zol elicited beneficial effects on the clinical outcomes. In addition, we have previously observed that the proportion of renal cell carcinoma (RCC) patients whose peripheral blood  $\gamma\delta$  T cells were up-regulated increased as the patients progressed through the stages of the disease [15] and that the degree of the increase in  $\gamma\delta$  T cells in stage III patients correlated with their 10-year overall survival rate [16]. These clinical findings and in vitro observations encouraged us to develop a novel cancer immunotherapy.

We and others previously identified IPP-relating pyrophosphomonoesters and their nucleoside triphosphate  $\gamma$ -ester derivatives as antigens for human V $\gamma$ 2 V $\delta$ 2-bearing T cells [2, 4]. Subsequently, it was demonstrated that (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate, a microbial metabolite, was the most potent stimulant of natural origin [17]. Because of the structural similarity between pyrophosphomonoesters and bisphosphonates, several N-BPs were examined for antigenicity in stimulating  $\gamma\delta$  T cells, leading to the discovery that N-BPs of the second and third generations effectively induced the expansion of human  $\gamma\delta$  T cells both in vitro and in vivo [7]. There are several differences between pyrophosphomonoesters and N-BPs. Whereas pyrophosphomonoesters can stimulate both unprimed and primed  $\gamma\delta$  T cells without accessory cells, N-BPs require monocyte lineage cells for efficient stimulation in primary  $\gamma\delta$  T-cell responses and human tumor cells for primed  $\gamma\delta$  T cells [8]. In terms of stability, pyrophosphomonoesters can be readily hydrolyzed by serum alkaline phosphatases, but N-BPs are generally resistant to serum enzymes.

Taking these immunological and pharmacological properties of nonpeptide antigens into account, we employed a strategy consisting of the adoptive transfer of  $\gamma\delta$  T cells to yield effector cells, followed by N-BP infusion to sensitize tumor cells in the present phase III clinical trial. During the course of this study, we evaluated the safety of  $\gamma\delta$  T-cell transfer concomitant with Zol and IL-2 infusion, the kinetics of  $\gamma\delta$  T cells in the peripheral blood, the prolongation of tumor doubling time, and the clinical outcomes based on RECIST.

## Materials and methods

### Patients and patient eligibility

Patients with histologically confirmed renal cell carcinoma (any T, any N, and M1, stage IV), who had undergone nephrectomy, with PS of 0, who had evaluable lung metastasis on computed tomography (CT) 3 months before the start of treatment, whose tumor doubling time before treatment was evaluable, whose age ranged from 20 to 80 years old, whose life expectancy was at least 6 months, whose major organs maintained function, whose lung metastatic lesions progressed even after treatment with IFN- $\alpha$  for at least 3 months, who met the laboratory test standards of our institution, and who voluntarily provided written consent to participate in this trial after having been thoroughly briefed and informed of its nature, were eligible for enrollment. Major exclusion criteria were a history of cancer other than RCC within 2 years, treatment with anticancer drugs, treatment with steroids, and serious complications.

### Study design

The protocol was designed and written by the authors, in collaboration with staff from the Translational Research Informatics Center (TRI), Kobe, Hyogo, Japan, and reviewed and approved by the Tokyo Women's Medical University Hospital Ethics Committee. This trial was a nonrandomized, uncontrolled, open-label, single-institutional study and is registered at <http://www.clinicaltrials.gov> as TRIC-CTR-GU-05-01. RCC patients who met the inclusion criteria were enrolled in this study.

Peripheral blood mononuclear cells were collected from each patient using an apheresis machine and then manually purified further with Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The cells were resuspended in 350 ml of the serum-free medium ALyS505 N (Cell Science & Technology Institute, Sendai, Miyagi, Japan) containing recombinant human interleukin-2 (rIL-2, Proleukin, Chiron, CA), 100 international units (IU)/ml, and stimulated with 2-methyl-3-butenyl-1-pyrophosphate (2M3B1PP), a pyrophosphomonoester, prepared at our institution as previously described [2] at a final concentration of 100  $\mu$ M in an air-permeable culture bag (Nipro Corp., Kita-ku, Osaka, Japan) for 11 days at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> at the Cell Processing Center of Tokyo Women's Medical University Hospital, which is based upon GMP. The expanded  $\gamma\delta$  T cells were transferred into a sterile infusion bag (Nipro Corp.). The patients were infused with 4 mg of Zol in 100 ml saline over a period of 30 min. Then,  $\gamma\delta$  T cells were administered for 5 min starting 2 h after the completion of Zol infusion.

Subsequently, rIL-2,  $1.4 \times 10^6$  JRU (Teceleukin, Shionogi & Co., Ltd., Japan, 1 JRU: Japan reference unit = 1 IU), was administered every day for 5 days. This procedure was repeated six times, once every 4 weeks. The target lung lesions were measured through standard CT imaging at -3, 0, 3, and 7 months after the start of treatment. Changes in laboratory test values were monitored to assess patient safety throughout the study, and immunological properties of the expanded  $\gamma\delta$  T cells and peripheral blood T cells were examined by means of flow cytometry. The standard cytotoxic assay was performed as described below.

The primary endpoints were the incidence of adverse events (AEs) and the increase in the proportion of peripheral blood  $\gamma\delta$  T cells. All AEs occurred during and within 1 month after completion of the treatment and were classified according to the NCI-Common Terminology Criteria for Adverse Events (NCI-CTCAE) ver. 3.0. The proportion of V $\delta$ 2-bearing  $\gamma\delta$  T cells among peripheral blood T cells was determined as described below.

The secondary endpoints were the prolongation of tumor doubling time and the best overall responses as defined by the RECIST criteria [18]. The target lesions were scanned using helical CT with a slice thickness of 5 mm. The tumor volume was calculated as  $ab(a+b)\pi/12$ , where  $a$  represents a major axis of the tumor ellipse and  $b$  a minor axis. The tumor doubling time was defined as  $\log_2(T_1 - T_0)/(\log V_1 - \log V_0)$ , where  $V_0$  and  $V_1$  represent the tumor volumes at time  $T_0$  and  $T_1$ , respectively. When two or more lesions were present in the lungs, the tumor volume was defined as the sum of the individual tumor volumes.

#### Immunological monitoring

The profiling of peripheral lymphocytes and cultured cells was examined by means of flow cytometry. Cells were treated with PC5-conjugated anti-CD3 mAb (SK7, Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA), or phycoerythrin (PE)-conjugated anti-CD4, anti-CD8, or anti-CD25 mAbs (BD Pharmingen Inc., San Diego, CA, USA), and fluorescein isothiocyanate (FITC)-conjugated anti-V $\delta$ 2 mAb (Immunotech, Marseilles, France) at  $2 \times 10^5$  cells/50  $\mu$ l of phosphate buffered saline (PBS)/2% fetal calf serum (FCS) on ice for 30 min. After being washed three times with 200  $\mu$ l of PBS/2% FCS, the cells were subjected to flow cytometry (EPICS XL, Coulter Electronics, Hialeah, FL, USA). The flow cytometric data were processed and analyzed using EXPO32 software (Coulter Electronics).

The serum cytokines, IL-2, IFN- $\gamma$ , IL-4, IL-5, IL-10, and TNF- $\alpha$ , were measured using Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kits (Becton-Dickinson) according to the manufacturer's instructions.

To determine cytotoxic activity, the  $\gamma\delta$  T cell-sensitive Daudi (Burkitt's lymphoma) line and the Caki-1 and VMRC-RCW (renal cell carcinomas) lines were treated with [ $^{51}$ Cr]-sodium chromate solution (3.7 MBq) at 37°C for 1 h. After being washed with medium, the tumor cells were resuspended in the medium and placed in a round-bottom 96-well plate. To the suspension was added the expanded  $\gamma\delta$  T cells at the effector/target ratio of 40:1. The plate was briefly centrifuged and incubated at 37°C and 5% CO $_2$ . After 4 h of incubation, the supernatants were examined for [ $^{51}$ Cr]-sodium chromate release using a  $\gamma$ -counter.

Data were collected by the investigators at Tokyo Women's Medical University Hospital and processed and analyzed by the staff of TRI.

#### Statistical analysis

Statistical analyses were conducted to test the differences between two items using the log-rank test, using the Stat View 5.0 J software package (Abacus Concepts, Inc, CA, USA).

## Results

#### Patients' profiles

A total of 11 patients who were diagnosed with metastatic renal cell carcinoma were recruited between January 2006 and March 2008. All patients underwent nephrectomy for RCC before enrollment. The final outcomes of all patients were assessed in October 2008, and the data were compiled and analyzed in December 2008. The patients' profiles are presented in Table 1. Eight of the patients were men and three were women; at enrollment, the median age was 59.4 years and PS was 0. Memorial Sloan-Kettering Cancer Center (MSKCC) Risk status was assessed at the time of enrollment. Based on histological examination, 9 patients had been diagnosed with RCC of clear cell type, 1 with RCC of clear cell with sarcomatoid, and 1 with RCC of papillary cell type. All patients had lung metastasis and/or other site of distant metastasis and received IFN- $\alpha$  and/or IL-2 after the surgery.

After obtaining written informed consent, patients were treated for metastatic renal cell carcinoma in the Study Design section. Five patients completed the whole treatment schedule, whereas six eventually discontinued for various reasons: one patient due to a brain tumor after four cycles of the treatment, one patient due to a lack of efficacy after three cycles of treatment, two patients due to an investigator's decision in response to the patients' apprehension, and two patients through a withdrawal of informed consent.

**Table 1** Patients' profile and clinical outcome

Patient	Age/sex	MSKCC <sup>a</sup> risk group	Type of cell <sup>b</sup>	Metastatic lesion	Previous treatment	Treatment cycles	Overall response	Clinical Outcome
TR1	68/M	Intermediate	Clear	Lung	IFN- $\alpha^c$	4	SD	Death
TR2	52/M	Poor	Clear with salcomatoid	Lung/bone/lymph node	IFN- $\alpha$ /IL-2 <sup>d</sup>	2	PD	Death
TR3	65/M	Intermediate	Clear	Lung	IFN- $\alpha$	6	SD	Survival
TR4	56/F	Intermediate	Clear	Lung/liver	IFN- $\alpha$ /IL-2/metastasectomy	1	PD	Survival
TR5	61/M	Intermediate	Clear	Lung	IFN- $\alpha$	6	CR	Survival
TR6	63/M	Poor	Clear	Lung/bone/pleura	IFN- $\alpha$ /metastasectomy	1	PD	Survival
TR7	59/F	Good	Clear	Lung/pleura	IFN- $\alpha$ /metastasectomy	5	PD	Survival
TR8	39/M	Intermediate	Clear	Lung/pleura	IFN- $\alpha$	3	PD	Survival
TR9	61/M	Intermediate	Clear	Lung	IFN- $\alpha$	6	SD	Survival
TR10	66/F	Intermediate	Clear	Lung/lymph nodes/retro peritoneal cavity/muscle/ascending colon	IFN- $\alpha$	6	SD	Survival
TR11	63/M	Intermediate	Papillary	Lung/lymph nodes	IFN- $\alpha$	6	SD	Survival

<sup>a</sup> MSKCC Memorial Sloan-Kettering Cancer Center

<sup>b</sup> World Health Organization Classification of Tumors [44]

<sup>c</sup> IFN- $\alpha$  interferon-alpha

<sup>d</sup> IL-2 interleukin-2

### Immunological responses

PBMC were stimulated with 2M3B1PP, and the resulting  $\gamma\delta$  T cells were collected and examined for immunological properties. As shown in Fig. 1a, there was no intrinsic difference in the proportion of CD3<sup>+</sup> cells (lozenge) in each cycle of expansion. By contrast, the proportions of V $\delta$ 2<sup>+</sup> cells among the CD3<sup>+</sup> cells (rectangle) decreased over the course of the treatment, whereas those of CD4<sup>+</sup> cells (cross) and CD8<sup>+</sup> cells (triangle) gradually increased. The absolute numbers of V $\delta$ 2<sup>+</sup> cells in transferred cells are measured in each cycle of the treatment in each patient. Whereas the number of V $\delta$ 2<sup>+</sup> cells in the sixth cycle was higher than that in the first cycle in patients who achieved SD/CR, the number significantly decreased as the cycle progressed in 3 of 5 PD patients as shown in Fig. 3b, c.

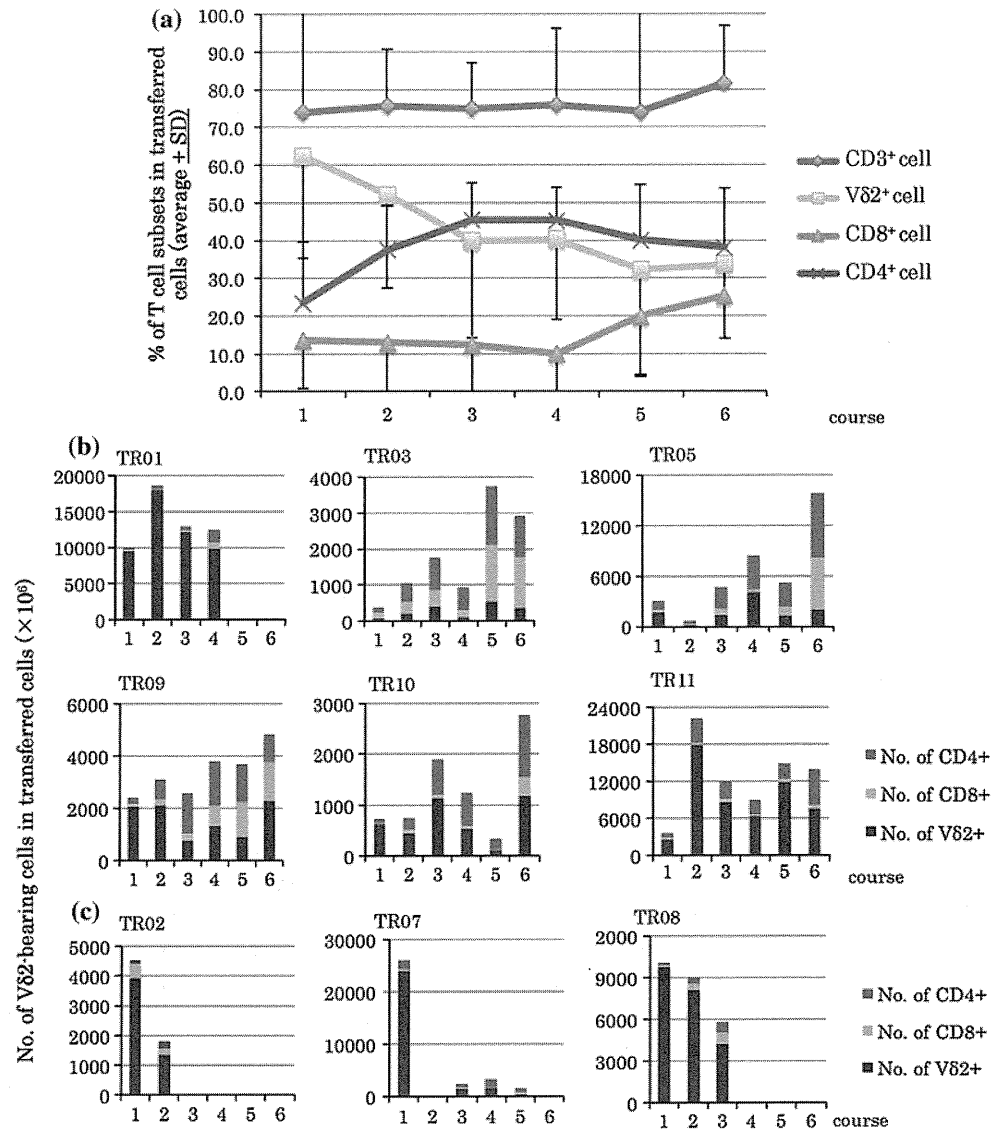
We then analyzed the proportion of V $\delta$ 2-bearing T cells among CD3<sup>+</sup> cells of peripheral blood before and after infusion of  $\gamma\delta$  T cells. Peripheral blood samples were examined for the expression of CD3, CD4, CD8, CD25, and V $\delta$ 2-TCR. In the first cycle of treatment, the proportions of peripheral blood V $\delta$ 2-bearing  $\gamma\delta$  T cells among CD3<sup>+</sup> T cells in all patients increased with time to different degrees. Typically, the proportion of  $\gamma\delta$  T cells peaked 3–5 days after infusion, as shown in Fig. 2. On average, the V $\delta$ 2<sup>+</sup>/CD3<sup>+</sup> ratio in the first infusion was 4.83% at the time of apheresis and 13.43% 1 week after infusion.

Serum cytokine contents before and after infusion in the first cycle of treatment were measured by Cell Beads Assay. Levels of serum IFN- $\gamma$ , IL-5, and IL-10 concentrations are shown in Fig. 3a at different time points. IFN- $\gamma$  peaked 1–2 days after infusion and gradually decreased to the levels that had existed before infusion in 7 days and IL-5 3–5 days after infusion and then swiftly decreased. There are, however, no statistically significant differences between the SD/CR patients (dark gray line) and the PD patients (bright gray line). We also measured IL-4, TNF- $\alpha$ , and IL-2, but no remarkable differences were observed between the two groups. In terms of cytotoxicity, SD/CR patients exhibited a slightly higher specific lysis against Daudi, Caki-1, and VMRC-RCW (dark gray bar) than PD patients (bright gray bar), though there was no statistically significant difference between the two groups, as shown in Fig. 3b. In SD/CR patients, tumoricidal activity in the sixth cycle appears to be lower than that in the first cycle, as depicted in Fig. 3c.

### Clinical responses

In order to assess the secondary endpoints, tumor volumes were determined through CT –3 Mo, 0 Mo, +3 Mo, and +7 Mo after the start of treatment. No newly appearing lesions in the lungs were observed in any of the patients during the course of this study. As summarized in Fig. 4a, the tumor volumes of most metastatic lesions increased steeply in the

**Fig. 1** **a** Relative proportions of T-cell subsets in transferred cells derived from patients who completed 6 cycles of the treatment. The proportions of CD3<sup>+</sup> cells (*lozenge*) and those of Vδ2<sup>+</sup> (*rectangle*), CD4<sup>+</sup> (*cross*), and CD8<sup>+</sup> cells (*triangle*) among CD3<sup>+</sup> cells were measured in each cycle of the treatment. **b/c** The absolute numbers of Vδ2<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells among the transferred cells in each cycle of the treatment; SD/CR (**b**) and PD patients **c**

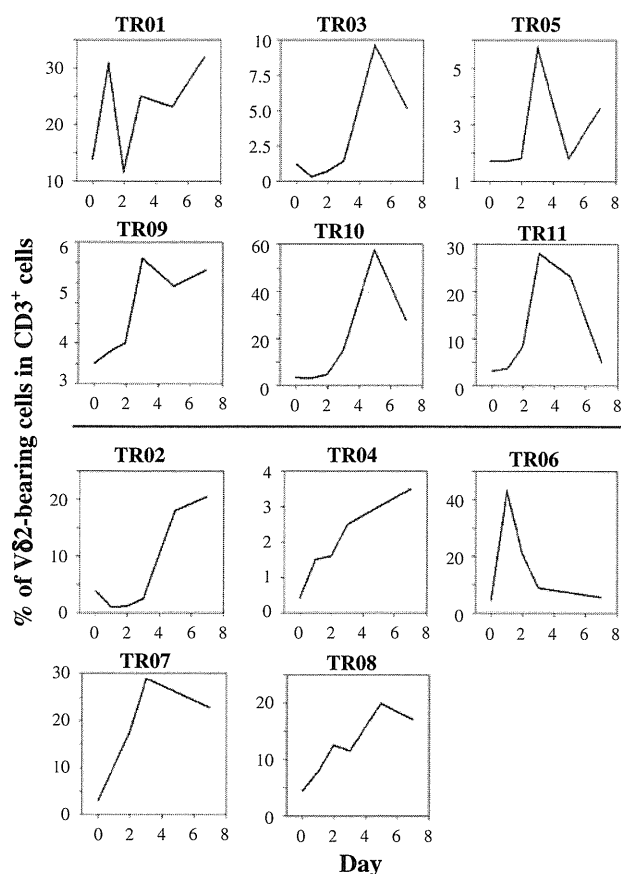


baseline period from -3 months to the beginning of treatment in all patients. After the start of treatment, the tumor volumes of some metastatic lesions began to decrease or continued to increase but at a more moderate rate. As depicted in the upper panels of Fig. 4b, the total tumor volumes in the lungs increased moderately in patients TR07 and TR08, whereas those in TR01, TR03, TR09, TR10, and TR11 did not change significantly. Remarkably, metastatic lesions disappeared within 7 months in TR05. Because the treatment was discontinued in TR02, TR04, and TR06, the total tumor volumes were not measured in this study. A moderate to significant increase in the tumor doubling time was observed in all patients as shown in the lower panels of Fig. 4b. Based on these results, one patient was considered to exhibit CR, five patients SD, and five patients PD (Table 1). Two patients had died as a result of malignant tumors by the end of the study in October 2008 (Table 1). It is encouraging to note that TR05, the patient who exhibited

CR, has remained disease-free to date (more than 36 months after the completion of the treatment). Figure 4d demonstrates representative CT images of the metastatic lesions in TR05 at the beginning of the treatment and 3 months later; it was during this 3-month interval that the two lesions macroscopically disappeared.

**Adverse events**

In order to assess the safety of this protocol as the first primary endpoint, we monitored PS scores, laboratory test values, and adverse reactions throughout the study. Although the patient referred to as TR02 had a PS of 0 at pre-enrollment, his PS rose to 1 at the first apheresis through the second infusion of γδ T cells, and he dropped out of the study after completing the second cycle. TR07, who had also had a PS of 0 at pre-enrollment, had developed a PS of 1 by the fifth γδ T-cell transfer and dropped out of the study after



**Fig. 2** Time course of V $\delta$ 2-bearing  $\gamma\delta$  T-cell circulation in the peripheral blood of patients with advanced RCC after infusion of 2M3B1PP-stimulated PBMC together with IL-2 and Zol. Before and after the infusion, PBMCs were examined for the expression of V $\delta$ 2 and CD3

completing the fifth cycle. The PS scores of the other patients were 0 at pre-enrollment and remained unchanged throughout the study. All patients developed adverse reactions of grade 1 or 2, including fatigue and fever, and ten patients developed adverse reactions of grade 3 or 4, as summarized in Table 2, including lymphopenia, hyponatremia, hypopotassemia, an increase in serum alanine aminotransferase, an increase in serum aspartate aminotransferase, an increase in serum creatinine, and a decrease in hemoglobin. All the deviated laboratory values reverted to normal during the course of the treatment. Patients, TR06 and TR10, whose serum creatinine increased should have been treated with rehydration therapy, but the symptom disappeared without treatment in a week. Almost all AEs occurred in the first cycle, and the frequency of these AEs decreased over the course of treatment.

## Discussion

Kidney cancer is one of the leading cancer types among estimated new cancer cases and deaths [19]. Historically,

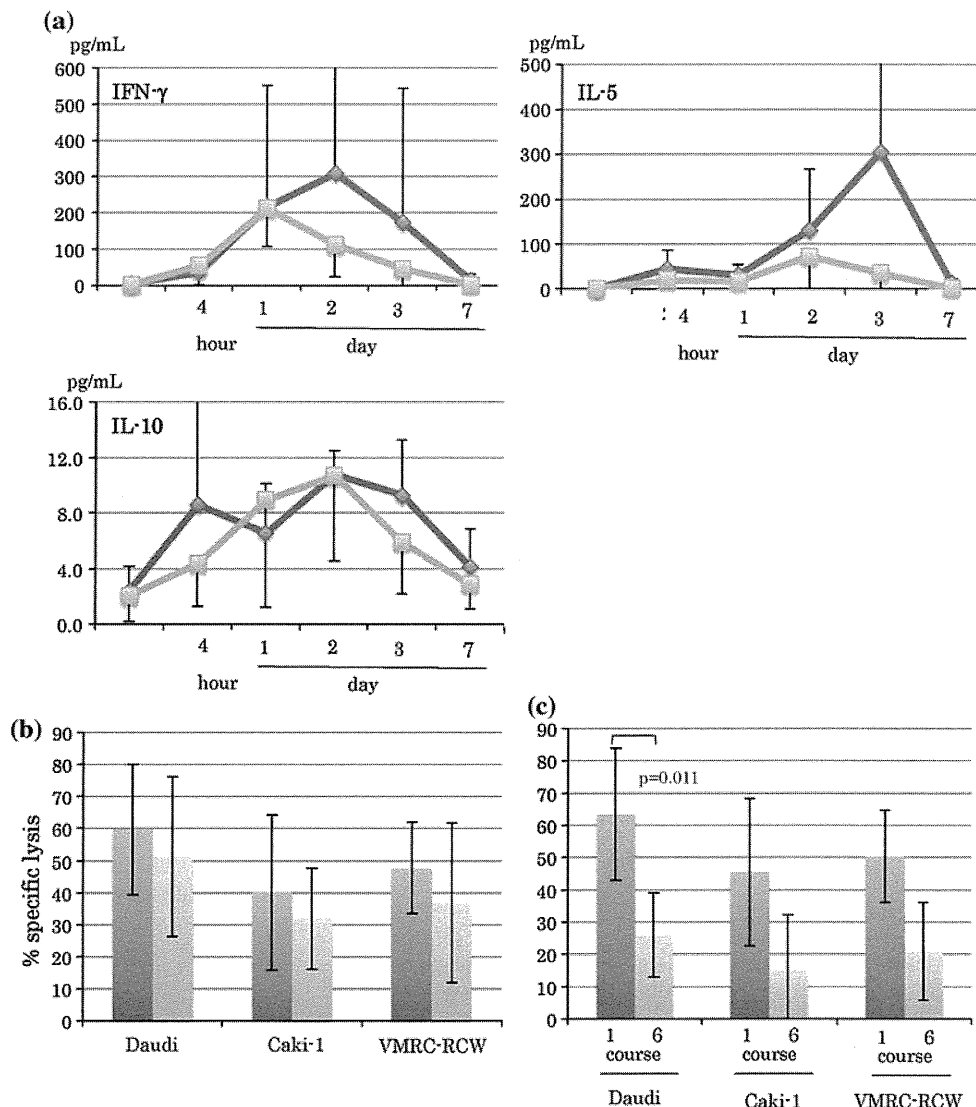
metastatic RCC was relatively unresponsive to traditional chemotherapy, and biological response modifiers such as IL-2 and IFN- $\alpha$  were the favorable treatments for patients with advanced RCC [20]. The recent development of targeted agents has changed the management of metastatic RCC [21]. These agents have distinct modes of action and include multitargeted receptor tyrosine kinase inhibitors, mTOR inhibitors, and vascular endothelial growth factor signaling blockers [22–24]. Although the prognosis for patients with metastatic RCC has improved significantly because of advances in these treatments, it should be noted that the primary achievement of the target therapies is the induction of SD, not CR [21]. IL-2 remains the only agent known to produce durable complete responses [25], but its use is accompanied by severe side effects, including hypotension, capillary leak syndrome, renal insufficiency, and the like, and is limited to good responders to the lymphokines [20]. Notwithstanding the availability of a variety of therapeutics, no satisfactory regimen for advanced RCC has yet been defined.

It is well known that RCC sometimes evokes immune responses that lead to complete tumor remissions [20]. We recently conducted a pilot study investigating the safety and feasibility of the adoptive transfer of  $\gamma\delta$  T cells concomitantly with IL-2 [26].  $\gamma\delta$  T cells exert tumoricidal activity via two distinct mechanisms, natural killer-like activity and  $\gamma\delta$  TCR-dependent cytotoxicity [27].  $\gamma\delta$  TCR is involved in the antitumor activity that results when tumor cells are treated with N-BPs [5]. Whereas it is most likely that IPP per se or its derivatives, which are accumulated in the tumor cells via the inhibition of farnesyl pyrophosphate synthase by N-BPs, are presented to  $\gamma\delta$  T cells, the translocation or induction of antigenic proteinaceous entities to the cell surface is also plausible [28]. Generally,  $\gamma\delta$  TCR-dependent cytotoxicity is more potent than natural killer-like activity. We therefore examined the safety and clinical outcomes of the infusion of  $\gamma\delta$  T cells plus IL-2 and Zol in this study.

Regarding primary endpoints, we successfully observed an increase in the proportion of V $\gamma$ 2 V $\delta$ 2-bearing  $\gamma\delta$  T cells in peripheral blood after the infusion of  $\gamma\delta$  T cells. There was a lag of several days between the infusion and the observed increase in the number of peripheral blood  $\gamma\delta$  T cells. This is probably because  $\gamma\delta$  T cells were trapped immediately after infusion in the reticuloendothelial system and the lungs. It is worth noting that the proportions of  $\gamma\delta$  T cells among the CD3<sup>+</sup> cells cultured with 2M3B1PP and IL-2 decreased as the number of treatment cycles increased, from 77.54% in the first cycle to 33.54% in the sixth cycle. Consequently, the tumoricidal activity of the cultured cells decreased, from 36.4 and 42.9% in the first cycle against Caki-1 and VMRC-RCW, respectively, to 15.2 and 21.0% in the sixth cycle. The lower proportion of  $\gamma\delta$  T cells in the cultured cells of the



**Fig. 3** **a** Serum cytokine concentrations at different time points of the first cycle of the treatment. *Dark gray line* indicates cytokine concentrations in sera obtained from SD/CR patients and *bright line* PD patients. The cytokine concentrations are shown as the average  $\pm$ SDs. Cytotoxicity exhibited by cultured cells against Daudi, Caki-1, and VMRC-RCW was measured by the standard  $^{51}\text{Cr}$  release assay. **b** Comparison of cytotoxic activity exhibited by cultured cells derived from SD/CR (*dark gray column*) and that from PD patients (*bright gray column*). **c** Comparison of cytotoxicity exhibited by cultured cells at the first and the sixth cycle of treatment; the first cycle (*dark gray column*) and the sixth cycle (*bright gray column*). The average and SDs of specific lysis were determined in SD/CR patients who had completed the six cycles of the treatment

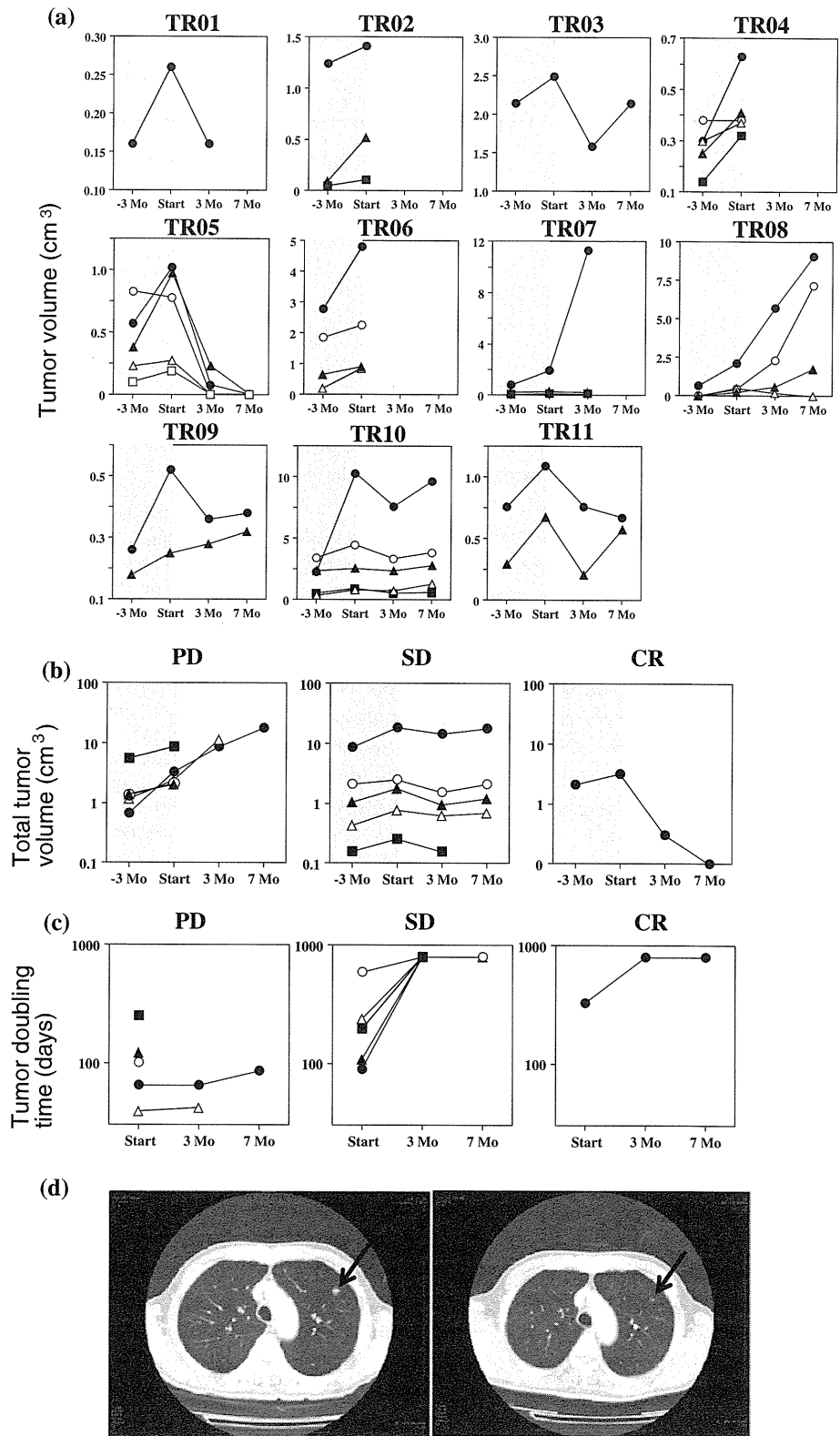


sixth cycles compared to those of the first cycles may account for the reduced cytotoxicity. The proportion of  $\gamma\delta$  T cells among the peripheral blood  $\text{CD}3^+$  cells after infusion also decreased significantly. We did not observe such a decrease in the pilot study, in which expanded  $\gamma\delta$  T cells and IL-2 were infused without Zol [26]. Thus, the induced unresponsiveness or hyporesponsiveness of  $\gamma\delta$  T cells to 2M3B1PP is attributable to the prior infusion of Zol. This finding may lead to a future improvement in the current protocol. An improved regimen could consist of the collection of peripheral blood by apheresis, the storage of purified PMBC aliquots until use, and the expansion of cryopreserved  $\gamma\delta$  T cells. Because our initial objective was to increase the number of  $\gamma\delta$  T cells in peripheral blood by adoptive transfer of ex vivo expanded  $\gamma\delta$  T cells, this new regimen would further our aims. In addition, we should determine the optimum dose of Zol in the future regimen because the current dose of Zol, 4 mg, was originally chosen for the treatment of patients with hypercalcemia, but not with malignant tumors.

Regarding another primary endpoint, we observed some AEs of grades 3 and 4. In our previous pilot study comprising  $\gamma\delta$  T-cell transfer plus IL-2 infusion without Zol, AEs of these magnitudes did not occur during treatment [26]. Because Zol infusion itself is also well tolerated in clinical settings [29–37], the combination of Zol, IL-2, and  $\gamma\delta$  T cells may have caused the AEs. Regardless, the symptoms were manageable and disappeared swiftly during the course of treatment, indicating that the regimen used in this study was well tolerated. In the pioneering study on N-BPs, AEs were induced after the initial infusion of N-BPs and essentially no AEs were observed in the subsequent infusions [38–40]. It is thus possible to reduce the dose of Zol in the initial infusion in order to reduce the severity of AEs. Furthermore, the interval between Zol administration and  $\gamma\delta$  T-cell infusion should be optimized to minimize AEs in future regimens.

It is worth noting that tumor doubling time was successfully increased in all patients. In TR07, for instance, there

**Fig. 4** **a** Time course of individual tumor volumes in the lungs of advanced RCC patients after infusion of 2M3B1PP-stimulated PBMC with IL-2 plus Zol. The tumor size of each lesion was measured by means of CT imaging -3 Mo, 0 Mo, 3 Mo, and 7 Mo after the start of the treatment. **b** Time course of total tumor volumes and **c** tumor doubling time. Total lung tumor volumes of each patient were calculated and plotted against time separately for three patient subgroups: PD (*closed triangle*: TR02, *open circle*: TR04, *closed square*: TR06, *open triangle*: TR07, and *closed circle*: TR08), SD (*closed square*: TR01, *open circle*: TR03, *open triangle*: TR09, *closed circle*: TR10, and *closed triangle*: TR11), and CR (*closed circle*: TR05). **d** CT images of metastatic lung tumors in an advanced RCC patient. Five metastatic lung lesions were observed through CT imaging in TR05; one metastatic site depicted here was measured at  $1.26 \times 1.08$  cm (*not shown*),  $1.33 \times 0.97$  cm (*left*), and  $0.00 \times 0.00$  cm (*right*) at -3 Mo, 0 Mo, and 3 Mo after the start of treatment, respectively



were three metastatic tumors in the lungs, one of which grew consistently, while the other two remained stable. Summing the volumes of the individual tumors revealed

that the tumor doubling time was prolonged even in this PD case. In TR05, there were five metastatic tumors, three of which disappeared within 3 months and two within

**Table 2** Adverse events

Grade >3	Number of patients/frequency (%)					
	1 course (n = 11)	2 course (n = 9)	3 course (n = 8)	4 course (n = 7)	5 course (n = 6)	6 course (n = 5)
Lymphopenia	10 (91%)	3 (30%)	1 (13%)	1 (14%)	0 (0%)	0 (0%)
Neutropenia	0 (0%)	0 (0%)	0 (0%)	1 (14%)	1 (17%)	0 (0%)
Hyponatremia	2 (18%)	0 (0%)	1 (13%)	0 (0%)	0 (0%)	0 (0%)
Hypopotassemia	0 (0%)	1 (11%)	0 (0%)	0 (0%)	0 (0%)	1 (20%)
AST	1 (9%)	0 (0%)	0 (0%)	1 (14%)	0 (0%)	0 (0%)
ALT	1 (9%)	1 (11%)	2 (25%)	1 (14%)	0 (0%)	0 (0%)
Creatinine	1 (9%)	0 (0%)	1 (13%)	0 (0%)	0 (0%)	0 (0%)
Hemoglobin	0 (0%)	0 (0%)	1 (13%)	0 (0%)	0 (0%)	0 (0%)

7 months. To date, this patient has not developed any evidence of recurrent tumors during the nearly three-year period since the completion of the treatment.

In addition, several new lesions that had not been present in the CT images recorded 3 months before the start of the treatment were detected at the start of immunotherapy, but no new lesions were observed in the lungs after the start of immunotherapy. This finding suggests that the present regimen may prevent micrometastasis of renal cell carcinoma. It has recently been reported that the addition of Zol to the standard regimen for the treatment of breast cancer improved disease-free survival times [41]. It is therefore necessary to identify the mechanism by which Zol elicits its therapeutic effects [42].

Based on the present results and those of other clinical trials, it is most likely that  $\gamma\delta$  T cells exert cytotoxic activity against malignant tumors *in vivo*. The effector roles of  $\gamma\delta$  T cells *in vivo* are still controversial, however. Thus, it is imperative that the immunological properties of  $\gamma\delta$  T cells in tumor-infiltrating lymphocytes be examined and the results applied to the development of efficacious regimens for malignant tumors.

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ORIGINAL

## Significant lowering of plasma ghrelin but not des-acyl ghrelin in response to acute exercise in men

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**Abstract.** Ghrelin, an acylated peptide produced predominantly in the stomach, stimulates feeding and growth hormone (GH) secretion via interaction with the GH secretagogue receptor. Ghrelin molecules are present in two major endogenous forms, an acylated form (ghrelin) and a des-acylated form (des-acyl ghrelin). Recent studies indicated that aerobic exercise did not change plasma total ghrelin levels, however, dynamics of circulating ghrelin and des-acyl ghrelin during aerobic exercise remains unclear. The purpose of this study is to examine the effects of moderate intensity exercise on plasma ghrelin and des-acyl ghrelin concentrations, and to investigate the relationship between ghrelin molecules and other hormonal and metabolic parameters during exercise. Nine healthy males ( $25.2 \pm 0.5$  years) exercised for 60 min at 50% of their maximal oxygen consumptions. We measured the plasma concentrations of ghrelin, des-acyl ghrelin, GH, norepinephrine (NE), epinephrine (E), dopamine (DA), insulin, and glucose. Plasma ghrelin level significantly decreased during exercise, whereas plasma des-acyl ghrelin and total ghrelin levels did not change. Plasma NE, E, DA and GH levels were significantly increased during exercise. Plasma insulin level significantly decreased during exercise, and plasma glucose levels remained steady during exercise. NE, E, DA, and GH were correlated negatively with plasma ghrelin levels. These findings suggest that acute moderate exercise may suppress ghrelin release from the stomach, decrease ghrelin *O*-acyltransferase activity, and/or activate ghrelin utilization in peripheral tissues and that exercise-induced ghrelin suppression may be mediated by activated adrenergic system.

**Key words:** Ghrelin, Des-acyl ghrelin, Growth hormone, Adrenergic system, Exercise

**GHRELIN**, a 28-amino-acid peptide with *n*-octanoylation indispensable for binding to the growth hormone secretagogue receptor (GHS-R), was originally discovered in the human and rat stomach [1]. Ghrelin molecules are present as two major endogenous forms, an acylated form (ghrelin) and a des-acylated form (des-acyl ghrelin). Ghrelin stimulates growth hormone (GH)

release [1, 2], and induces food intake [3], adiposity, and weight gain [4]. Plasma ghrelin levels increase upon fasting [3, 5, 6] and insulin-induced hypoglycemia [6], and decrease in response to oral or intravenous administration of glucose [5, 7]. Plasma ghrelin level also exhibits a diurnal pattern with preprandial increases and postprandial decreases [5]. Fasting plasma ghrelin level elevates in anorexia nervosa [8] and cachexia [9], and reduces in obesity [7, 10].

The systemic circulation during acute exercise is regulated by attenuated vagal efferent activity, known as Bainbridge reflex, and by increased sympathetic nerve activity. The vagal efferent pathway mediates ghrelin release [11, 12]. In addition, gastric vagal afferent serves as the major pathway convey-

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ing ghrelin's signals for starvation and GH secretion to the brain [13]. As exercise stress induces a variety of catabolic processes involving glycolysis, lipolysis, and protein degradation [14], we assume that release and action of ghrelin may change in response to the alternation of the autonomic activity and/or catabolic state during an acute exercise. While long-term exercise resulting weight loss increases plasma ghrelin level [15, 16], short-term running, cycling, or rowing exercise does not alter plasma total ghrelin [17-23]. A few papers, however, reported that plasma total ghrelin level decreased [24-26] or increased [27-29] during short-term exercise. Schmidt *et al.* [22] reported that there were no correlations between total ghrelin levels and either of treadmill exercise at 50%, 70%, or 90% maximum oxygen consumption ( $\dot{V}O_{2max}$ ). Kraemer *et al.* showed that acute resistance exercise reduced circulating total ghrelin levels [30].

The effects of physical exercise on ghrelin and des-acyl ghrelin have yet to be investigated. We here examined the effects of acute exercise on the plasma levels of ghrelin and des-acyl ghrelin in healthy men and studied the relationships between two ghrelin forms and hormonal or metabolic parameters.

## Materials and Methods

### Subjects

We examined nine healthy men (mean age,  $25.2 \pm 0.5$  years; average body mass index (BMI),  $22.6 \pm 0.4$  kg/m<sup>2</sup>, and  $\dot{V}O_{2max}$ ,  $44.8 \pm 1.4$  mL/min/kg) who had not engaged in any regular exercise for at least two years. All subjects were healthy, non-smokers, and non-obese and were not currently taking any medications. Prior to beginning the study, the purpose and risks of the study were explained to them to obtain their written informed consent. The study, approved by the local Ethics Committees of the University of Miyazaki and Fukuoka University, was conducted in accordance with the Helsinki Declaration.

### Measurement of $\dot{V}O_{2max}$

To measure physical fitness, all of the subjects performed a  $\dot{V}O_{2max}$  test on a mechanically braked ergometer (Electric Bicycle Ergometer; Lode's Instrumenten, Groningen, Netherlands). The work rate was initially set at 15 W and thereafter was increased every 1 min by 15 W. The test was continued until the subjects felt exhausted.  $VO_2$  was measured from the mixed

expired gas collected in neoprene bags. The volume of the expired gas was quantified using a twin-drum-type respirometer (Fukuda Irika CR-20, Tokyo, Japan), and both the O<sub>2</sub> and CO<sub>2</sub> fractions were analyzed using a mass spectrometer (ARCO-1000; ARCO System, Chiba, Japan). Electrocardiogram and heart rates were monitored throughout the exercise test using a three-lead electrocardiograph.

### Exercise test protocol

The subjects were asked to refrain from alcoholic beverages and strenuous physical exercise for at least 3 days prior to each exercise test. After an overnight fast, the subjects performed cycle exercise for 60 min at 50% of  $\dot{V}O_{2max}$ . Exercise consisted of 3 min warm up and cool down. Exercise intensity was calculated based on the results of  $\dot{V}O_{2max}$  test. Blood samples were taken from the antecubital vein before exercise, at 15, 30, 45, 60 min during exercise, and at 90 min in the recovery periods.

### Ghrelin and des-acyl ghrelin assay

Blood samples were immediately transferred to chilled polypropylene tubes containing EDTA·2Na (1 mg/mL) and aprotinin (500 U/mL), and centrifuged at 4°C. Hydrochloric acid (1N) was added to the samples at 10% volume of plasma volume immediately after the separation of plasma and stored the plasma at -30°C until assayed [32]. The levels of ghrelin and des-acyl ghrelin were measured using two commercially available ELISA kits, respectively (Mitsubishi Chemical Medience Corporation, Tokyo, Japan) [33]. The minimal detection limits of ghrelin and des-acyl ghrelin in this assay were 2.5 and 12.5 fmol/mL, respectively. The intra- and inter assay coefficients of variation were 6.5% and 9.8%, respectively, for ghrelin and 3.7% and 8.1%, respectively, for des-acyl ghrelin.

### Other biochemical assay

Plasma GH concentrations were analyzed using a commercially available GH ELISA kit (Biocode SA, Liege, Belgium). Plasma epinephrine (E), norepinephrine (NE) and dopamine (DA) were assayed using high-performance liquid chromatography (SRL Inc, Tokyo, Japan). Plasma glucose was measured using the glucose oxidase methods and plasma insulin using EIA (SRL Inc, Tokyo, Japan).

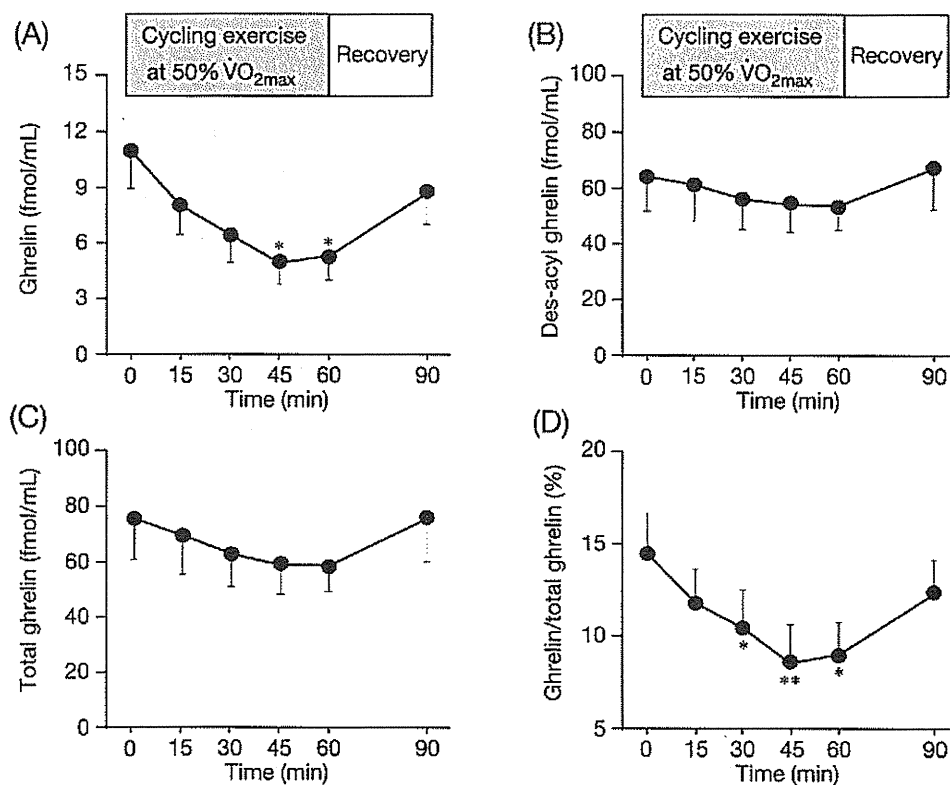


Fig. 1 Plasma levels of ghrelin (A), des-acyl ghrelin (B), total ghrelin (C), and ghrelin/total ghrelin ratio (D) at rest, during exercise, and during post exercise recovery. Data are mean  $\pm$  SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  in comparison with basal levels.

### Statistical analysis

All statistical calculations were performed using JMP statistical software (JMP 8; SAS Institute, Cary, NC, USA). Plasma NE, E, DA, GH, ghrelin, des-acyl ghrelin, glucose, and insulin levels at before and after exercise were analyzed by paired *t*-test. Correlation between the plasma ghrelin level and plasma GH, NE, E, or DA was analyzed by Spearman rank correlation test. All values are shown as means  $\pm$  SE.  $P < 0.05$  was considered to be statistically significant.

### Results

Fasting plasma ghrelin and des-acyl ghrelin levels in the subjects were  $11.0 \pm 2.0$  fmol/mL and  $64.4 \pm 12.8$  fmol/mL, respectively. Plasma ghrelin levels tended to decrease after exercise (15 and 30 min,  $P = 0.061$  and  $P = 0.063$ , respectively), and were suppressed significantly during cycling exercise (45 and 60 min, Fig. 1A). Plasma des-acyl ghrelin and total ghrelin (ghrelin plus des-acyl ghrelin) levels tended to decrease during exercise; however, there was no statistically sig-

nificant (Fig. 1B, C). The ghrelin to total ghrelin ratio significantly reduced during exercise (30–60 min) (Fig. 1D). Plasma GH, NE, E, and DA concentrations increased significantly during exercise and decreased at 90 min (Fig. 2A–D). Plasma glucose concentrations gradually decreased during exercise and became significantly lower at 90 min than pre-exercise level (Fig. 3A). Plasma insulin level significantly decreased during exercise (30–60 min) and recovered at 90 min (Fig. 3B). Spearman rank correlation test showed significant negative linear relationships between plasma ghrelin levels and plasma GH, NE, E, or DA levels (each Spearman's rho =  $-0.943$ ,  $P = 0.0048$ ) (Fig. 4A–D).

### Discussion

We here reported significant suppression of plasma ghrelin level but not des-acyl ghrelin level during acute moderate exercise. Plasma ghrelin level was negatively correlated with plasma catecholamines and GH levels during exercise. We thus concluded that acute exercise lowered plasma ghrelin concentration: this was likely

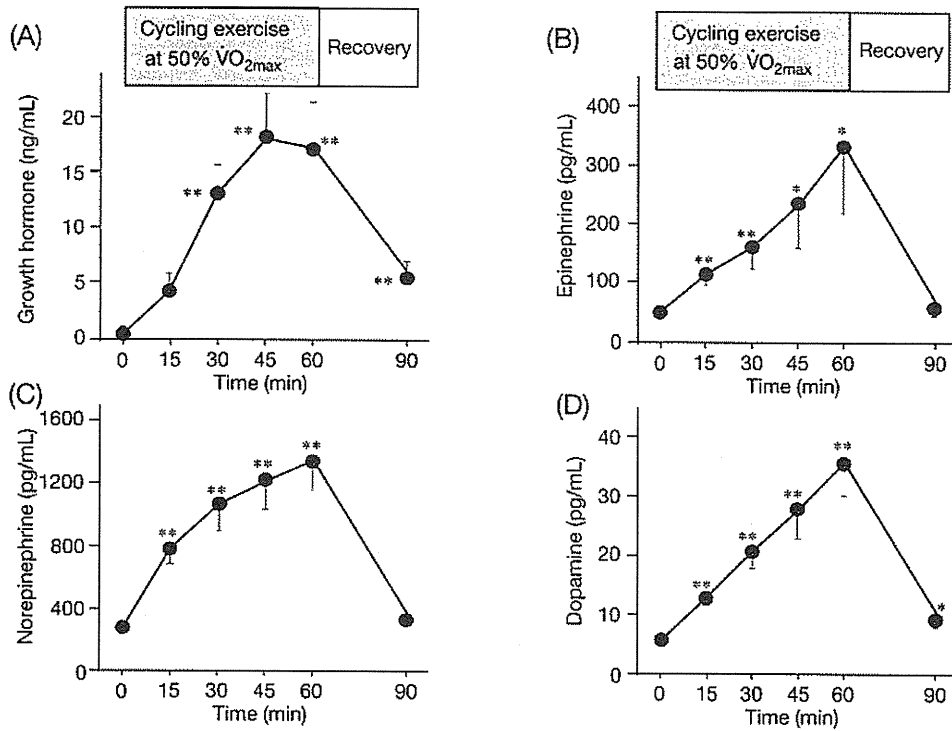


Fig. 2 Plasma levels of GH (A), epinephrine (B), norepinephrine (C) and dopamine (D) at rest, during exercise, and during post exercise recovery. Data are mean  $\pm$  SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  in comparison with basal levels.

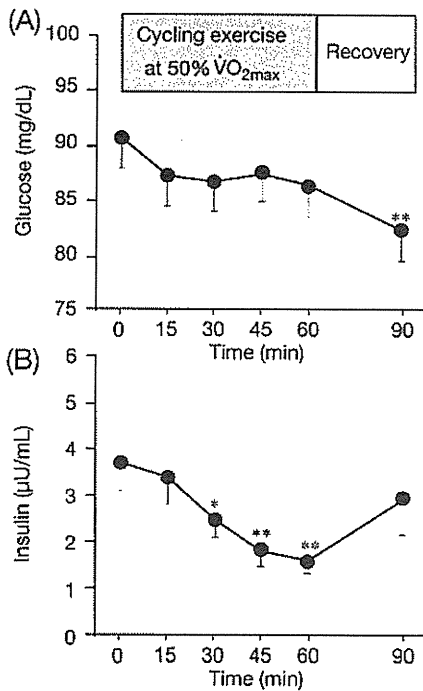


Fig. 3 Plasma levels of glucose (A) and insulin (B) at rest, during exercise, and during post exercise recovery. Data are mean  $\pm$  SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  in comparison with basal levels.

mediated by elevated sympathetic tone, and unrelated to alteration of GH secretion upon exercise.

Plasma ghrelin level decreased during acute moderate exercise in this study. Recently significant suppression or no change of plasma ghrelin level during 60–90 min running or brisk walking were reported, but plasma des-acyl ghrelin and catecholamine concentrations were not examined in these reports [33–35]. Although the physiological significance of ghrelin suppression by exercise is unclear, a number of potential mechanisms may be proposed. In humans, plasma ghrelin concentration increased through the activation of the vagal efferent pathway using sham feeding and by a cholinergic agonist such as pyridostigmine [11, 36]. On the other hand, plasma ghrelin level was decreased by cholinergic antagonists such as pirenzepine and atropine [36, 37]. During acute exercise, vagal efferent activity was attenuated and sympathetic nerve activity increased. Plasma NE, E, and DA levels rose, in fact, during exercise in this study. These findings suggest that the significant suppression of ghrelin level during exercise might be induced by the activation of sympathetic nerve activity and/or relative inactivation of the vagus. Ghrelin, which enhances appetite and increases



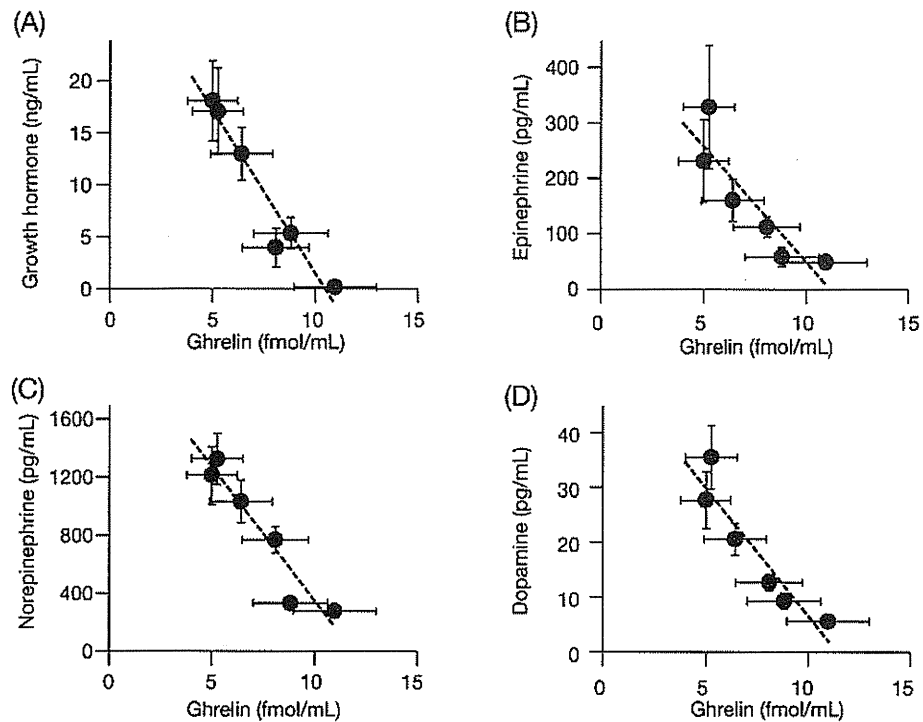


Fig. 4 Correlations between plasma ghrelin levels and plasma GH (A), epinephrine (B), norepinephrine (C) and dopamine (D) levels at rest, during exercise, and during post exercise recovery. Data are mean  $\pm$  SE.

food intake in animals and humans, exhibits a diurnal pattern with preprandial increases and postprandial decreases [3, 5]. Ghrelin has been postulated as a hunger signal and meal initiator [38, 39]. Appetite is suppressed during and after intense exercise, however, this suppression does not last long [40]. We suspect that ghrelin suppression by exercise may reflect the diminution of hunger sensations experienced during and after vigorous exercise. Exercise, especially intense exercise, can lead to a variety of gastrointestinal abnormalities, such as abdominal pain or discomfort, nausea, vomiting, and diarrhea [41, 42]. Exercise induces the development of reduced blood flow in the gastric mucosa in an exercise intensity dependent manner [41, 42]. We hypothesize that this exercise-induced ghrelin suppression may be related, at least in part, to gastric mucosal ischemia. Gastric mucosal ischemia also may decrease the activity of ghrelin *O*-acyltransferase [43], which acylates ghrelin with *n*-octanoic acid in the gastric mucosa. As these mechanisms are speculative theories, further studies will be necessary to clarify the fundamental mechanisms of exercise-induced ghrelin suppression.

Although some studies have examined the relation-

ships between acute exercise and ghrelin, plasma total ghrelin level did not change [19–22], decreased [24–26] or increased [27–29] during acute exercise. The reason for the discrepancy with our results is unclear. We speculate that this discrepancy is due to combined factors including sample collection, storage conditions, and assay systems used. Hosoda *et al.* reported that stabilization and acidification are essential for measuring ghrelin level [31]. In the present study, since ghrelin is rapidly converted to des-acyl ghrelin, blood samples were immediately collected in tubes containing EDTA-aprotinin (a protease inhibitor) and centrifuged as soon as possible. Hydrochloric acid was immediately added to the plasma samples obtained to stabilize *n*-octanoyl modification. Moreover, in this study, we used novel sensitive ELISA kits that can separately detect acylated and des-acyl ghrelin up to 2.5 and 12.5 fmol/mL, respectively [32]. Since assay systems for total ghrelin in previous reports recognized all of ghrelin, des-acyl ghrelin, and fragmented ghrelin, they could not detect the change of only ghrelin. Taken together, these optimum sample treatments and the novel sensitive assay systems may probably allow us to detect the specific alteration of ghrelin during exercise.

*N*-octanoyl modification is essential for the activity of ghrelin. In the present study, no significant changes were found in plasma des-acyl ghrelin concentrations, although we showed the significant suppression of ghrelin and ghrelin/total ghrelin ratio during exercise. These results suggest that ghrelin disappears from the circulation more rapidly than des-acyl ghrelin during acute moderate exercise. In general, the alternative factors of plasma ghrelin level are secretion from the stomach, clearance from the kidneys [44], and utilization in peripheral tissues. Since all the subjects in this study have normal renal function, the suppression of plasma ghrelin level during acute exercise might be due to decreased synthesis and/or increased utilization in peripheral tissues. However, further studies are required to elucidate the mechanisms regulating ghrelin synthesis, acylation and clearance during exercise.

Similar to the previous findings, plasma GH significantly increased during exercise. In addition, negative correlation was found between ghrelin and GH. Exercise is well known to stimulate GH release [45]. GH response to exercise is dependent on the duration/intensity of exercise and the fitness levels of the subjects [46]. Previous studies suggest that exercise stim-

ulates GH secretion without an increase in plasma total ghrelin concentration [17-22, 24-26]. Thus, previous and our findings suggest that ghrelin is less likely to be involved in exercise-induced GH secretion and that decreased ghrelin during exercise may be affected by negative feedback of GH [47].

In conclusion, the present findings indicated that ghrelin but not des-acyl ghrelin levels were suppressed by 60 min moderate cycling exercise. Furthermore, there were negative correlations between ghrelin and catecholamines. These results suggest that exercise-induced ghrelin suppression might be, in part, mediated by the adrenergic system. Further studies will be necessary to clarify the physiological significance of individual ghrelin form in physical exercise.

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