The blocking of CXCR3 and CCR5 suppresses the infiltration of T lymphocytes in rat renal ischemia reperfusion

Koichi Tsutahara1, Masayoshi Okumi1, Yoichi Kakuta1, Toyofumi Abe1, Koji Yazawa1, Shuji Miyagawa2, Katsuyoshi Matsunami3, Hideaki Otsuka3, Junya Kaimori4, Shiro Takahara1 and Norio Nonomura1

1Department of Urology, Osaka University Graduate School of Medicine, Suita, Osaka, Japan, 2Division of Organ Transplantation, Department of Surgery, Osaka University Graduate School of Medicine, Osaka, Japan, 3Department of Pharmacognosy, Hiroshima University Graduate School of Biomedical Science, Hiroshima, Japan and 4Department of Advanced Technology for Transplantation, Osaka University Graduate School of Medicine, Osaka, Japan

Correspondence and offprint requests to Masayoshi Okumi; E-mail: okumi@uro.med.osaka-u.ac.jp

Abstract

Background. Recent studies have identified T cells and natural killer T (NKT) cells as important mediators in renal ischemia–reperfusion (I/R) injury. The recruitment of these cells is induced by chemotaxis factors. We investigated the effects of blocking CXCR3 and CCR5 by an antagonist (TAK) using a rat renal I/R injury model.

Methods. The Sprague–Dawley rats were either subjected to sham operation or left renal occlusion for 45 min followed by reperfusion and contralateral nephrectomy. The control or TAK groups were, respectively, injected phosphate-buffered saline or TAK at 30 min prior to clamp. Serum creatinine, tubular injury, chemokines expression and infiltrating cells were assessed.

Results. TAK treatment significantly suppressed the elevation in serum creatinine (sham 0.40 ± 0.05 mg/dL, control 2.86 ± 0.67 mg/dL, TAK 1.60 ± 0.73 mg/dL) and resulted in a lower tubular injury score compared with the control group (sham 0, control 4.8 ± 0.3, TAK 3.3 ± 1). The mRNA expression of chemokines that bind to CXCR3 and CCR5 in the post-ischemic kidneys was elevated at 1 h after reperfusion in each group. Moreover, the infiltration of CD4+ T cells and CD8+ NKT cells in the control group increased compared with the sham group and TAK injection significantly suppressed the number of CD4+ T cells (sham 13.5 ± 3.5 × 10⁴ cells, control 28.9 ± 15.4 × 10⁴ cells, TAK 11.8 ± 3.5 × 10⁴ cells) and the number of CD8+ NKT cells (sham 11.7 ± 5.4 × 10⁴ cells, control 30.1 ± 8.6 × 10⁴ cells, TAK 11.8 ± 2.9 × 10⁴ cells).

Conclusions. These findings suggest that the blocking of CXCR3 and CCR5 suppress the infiltration of T cells and NKT cells and have a protective effect on kidneys that are injured by I/R.

Keywords: CCR5; CD8+ NKT cell; chemokine; CXCL10; CXCR3; renal ischemia–reperfusion

Introduction

It is well established that kidneys from donation following cardiac death donors have an increased risk of delayed graft function (DGF) compared with live and brain death donors [1]. Although many donor and recipient factors contribute to the risk of DGF, a warm ischemic injury followed by cold ischemic injury during preservation and damage caused by reperfusion are strong influencing factors. The warm ischemic interval is a particularly important contributor to early graft dysfunction [2]. The initial intensity of the ischemia–reperfusion (I/R) injury is also related to both early and late phases of the long-term graft function [3, 4]. Therefore, if damage to kidney grafts caused by I/R injury could be suppressed, this would likely improve the outcome of renal allografts.

Though renal inflammation is usually not detectable by routine histology in human acute kidney injury, some clinical studies of human ischemic renal injury reported that the levels of the proinflammatory cytokines in the plasma can be used as predictors of mortality in patients, and the levels of CXCR3-binding chemokines in the urine can be used to predict acute tubular injury after kidney transplantation [5, 6].

Recent studies have identified T cells as important mediators in experimental renal I/R injury [7–12] as well as I/R injury of the liver [13] and the lung [14]. In addition, other recent reports have shown that natural killer T (NKT) cells contribute to the pathology of I/R injury [12, 15, 16]. NKT cells are relatively minor subsets of T cells, comprising ∼2.5% of T cells in the spleen and up to 30% in the liver [17]. NKT cells are thought to bridge the innate and adaptive immune system by their ability to rapidly release large amounts of cytokines.

The recruitment of these T cells into the post-ischemic kidney-mediating tissue injury is induced by chemotaxis factors including chemokines. Chemokines regulate all
steps that are required for the recruitment of leukocytes to the inflammation sites. CXC chemokines, such as CXCL9 [a monokine induced by interferon (IFN)-γ], CXCL10 (IFN-γ-induced protein-10) and CXCL11 (IFN-inducible T-cell α-chemoattractant), bind to CXCR3 and CC chemokines, such as CCL3 (macrophage inflammatory protein-1α), CCL4 (macrophage inflammatory protein 1β) and CCL5 (regulated on activation, normal T cells expressed and secreted), bind to CCR5 [18–20].

Because both CXCR3 and CCR5 are expressed on CD4 T cells and CXCR3 is expressed on NKT cells [21], the blocking of CXCR3 and CCR5 would be expected to suppress these T-cell infiltrations into the inflammation sites. Therefore, we hypothesized that the blocking of CXCR3 and CCR5 could be an effective strategy for protecting organs against I/R injury by suppressing T-cell infiltrations.

We investigated the expression of chemokines in the early phase of I/R injury and the effects of blocking CXCR3 and CCR5 on infiltrating cells into I/R-injured organs, using a synthetic, non-peptide CCR5 and a CXCR3 antagonist (TAK-779) [22] in a rat I/R injury model.

Materials and methods

Animals

The male Sprague-Dawley rats weighing 250–300 g were purchased from Japan SLC Inc. (Shizuoka, Japan) and were maintained under specific pathogen-free conditions in the animal facility of Osaka University. The animals were fed a standard diet and received water ad libitum. All procedures were performed in accordance with the principles of the Guidelines of Animal Experimentation at Osaka University.

CXCR3 and CCR5 antagonist (TAK-779)

A non-peptide, synthetic CXCR3 and CCR5 antagonist (TAK-779), (N,N-dimethyl-N-[4-[[2-(4-methylphenyl)-6,7-dihydro-5H-benzo[cyclohepten-8-yl]carbonyl]amino][benzyl]-tetrahydro-2H-pyran-4-aminium chloride) initially developed for the treatment of HIV infections, was used [23] (Figure 1). TAK appears to selectively inhibit the migration of T cells expressing CXCR5 and CXCR3 to joint [24].

Animal experimental protocols

The rats were divided into three groups: (i) a sham-operated group (sham group), (ii) a phosphate-buffered saline (PBS) treatment group (control group) and (iii) a TAK treatment group (TAK group) at each time point. All rats were anesthetized with isoflurane. The rats were given intraperitoneal injections of PBS for the control group or TAK (10 mg/kg/mL) for the TAK group at 30 min prior to inducing I/R injury [25]. Renal I/R procedures were performed as described previously [26]. Briefly, left renal pedicles were exposed and clamped for 45 min. By releasing the clamp, reperfusion began and was visually confirmed by the restoration of normal color. After confirming reperfusion, a right nephrectomy was performed and the incision was closed. Rats in the sham-operated group were subjected to the same operative procedure as the injury group, but the kidneys were not clamped. Kidneys were sampled after being flushed with cold PBS at each time point (1, 3, 6 and 24 h after reperfusion). Serum was collected and creatinine levels were determined at 24 h.

Histological evaluation

For histological examination, kidneys were extracted from rats and embedded in 4% paraformaldehyde. Paraffin sections were stained with periodic acid Schiff (PAS) and assessed by light microscopy. Tubular injury was scored on PAS by estimating the percentage of tubules in the corticalmedullary junction that showed epithelial necrosis, loss of brush border or had necrotic debris or cast as follows: 0, none; 1, <10%; 2, 10–25%; 3, 26–45%; 4, 46–75%; 5, 75%–100% [27]. Ten viewing fields randomly selected from the corticomedullary junction which is the most vulnerable part of the kidney for I/R [28] on each slide section were examined at 200 magnification.

Real-time reverse transcription–polymerase chain reaction

Quantitative real-time polymerase chain reaction (PCR) was performed to assess the expression of CXCR3- and CCR5-binding chemokine mRNA in the post-ischemic kidney. The PCR primer sets used for CXCL9, CXCL10, CXCL11, CCL3, CCL4, CCL5 and GAPDH cDNA amplification are listed in Table 1. Isolated total RNA from post-ischemic kidneys was reverse transcribed to cDNA using the PrimeScript RT reagent Kit (TAKARA Bio Inc.) following the manufacturer’s guidelines. Real-time reverse transcription (RT)-PCR was performed on a Thermal Cycler Dice Real Time System TP800 (TAKARA Bio Inc.) with SYBR premix Ex Taq II (TAKARA Bio Inc.). Each sample was analyzed in duplicate using the conditions recommended by the manufacturer. Chemokine levels were normalized to the GAPDH mRNA level using the comparative Ct method. We then compared the chemokine levels with those for the sham.

Immunohistochemical evaluation

Immunohistochemistry staining of T cells was performed using the LSAB+ System-HRP (Dako, Hamburg, Germany), according to the manufacturer’s instructions. Antigen retrieval was performed for 10 min in preheated 10 mmol/L sodium citrate (pH 7) using an autoclave. Endogenous biotin and peroxidase activities were determined according to the manufacturer’s instructions. The first antibody, CD3 (BD Pharmingen, Franklin Lakes, NJ, USA), was diluted in Dako Real Antibody Diluent (Dako) to a specific concentration and incubated overnight at 4°C. This was followed by incubation with the secondary antibody. Color was developed with 3,3′,5-diaminobenzidine tetrahydrochloride (Dako). Ten randomly selected fields from the corticomedullary junction at a 200 magnification were viewed and the mean number of T cells was estimated from these data.

Immunofluorescence staining involved the following procedure: frozen kidney samples embedded in OCT were cut into 4 μm sections in a cryostat, fixed in acetone for 15 min and incubated with the first antibodies, i.e. biotin mouse anti-rat CD16a (BD Pharmingen), rabbit anti-rat CXCR3 (Abbiotec, San Diego, CA) and rabbit anti-rat CCR5 (Abcam, Cambridge, MA), overnight at 4°C. They were then incubated with secondary antibodies, Alexa Fluor 488 goat anti-mouse and Alexa Fluor 568 goat anti-rabbit (Invitrogen) for 60 min, at room temperature. Images were captured with a confocal laser-scanning microscopy system, LAM-510 META (Carl Zeiss).

Isolation of kidney mononuclear cells from post-ischemic kidneys

Mononuclear cells were isolated from kidneys at 3 h after the reperfusion procedure. At the time of sacrifice, the rats were first exsanguinated and the kidneys, flushed with cold PBS, were collected. The kidneys were minced and incubated with Type I A collagenase (1 mg/mL; Wako, Osaka) in cold PBS buffer for 40 min at 37°C. The suspensions were filtered through a 70 μm BD Falcon cell strainer and centrifuged at 300 g for 10 min at 4°C. The cell pellet was resuspended in ACK Lysis Buffer, and the suspension incubated on ice for 5 min. The suspension
was then centrifuged and washed with PBS twice and the number of kidney mononuclear cells (KMNCs) counted.

Flow cytometry analysis
KMNCs were incubated with anti-rat CD32 Fc receptor (BD Pharmingen) for 15 min to block non-specific Ab binding. The surfaces of the KMNCs were stained with mAb anti-CD3 FITC, CD8 PE, CD4 APC and CD161a Biotin (BD Pharmingen) for 30 min at 4°C and washed twice with PBS buffer. They were then incubated with secondary antibodies, streptavidin PE-Cy7 (Invitrogen) for 30 min, at 4°C. KMNCs were analyzed using a FCAS CantoII instrument (BD Biosciences). KMNCs were identified using forward scatter and side scatter to exclude debris. The data were analyzed using the FlowJo software program (Tree Star, Inc). The absolute number of infiltrating T cells was calculated by multiplying the total number of KMNCs by the percentage of positive cells, as determined by flow cytometry [12].

Statistical analysis
Statistical analyses were performed by JMP. Data are expressed as means ± SD. Statistical comparisons between groups were performed with the unpaired t-test, the Turkey test or the Steel–Dwass test. Differences were considered to be statistically significant at P < 0.05.

**Results**

**Assessment of renal function and acute tubular damage after I/R injury**
Renal functions after I/R injury were evaluated by serum creatinine levels at 24 h after reperfusion. Serum creatinine levels increased significantly in both the control and the TAK groups, compared with the sham group. However, the serum creatinine level’s aggravation in the TAK group was significantly suppressed compared with the control group (sham group 0.40 ± 0.05 mg/dL, control group 2.86 ± 0.67 mg/dL, TAK group 1.60 ± 0.73 mg/dL, n = 6) (Figure 2a).

A structural injury in the corticomedullary junction of a rat kidney occurred as the result of I/R (Figure 2b–d). Consistent with the renal function, kidneys of the TAK group had less tubular damage than those of the control group, as evidenced by the acute tubular injury score (Figure 2e).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence Sense</th>
<th>Sequence Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL9</td>
<td>Forward: 5′-AGCCAAGGCACATCCACTACA-3′</td>
<td>Reverse: 5′-TCTAGGCGAGTTTGATCCGTTCC-3′</td>
</tr>
<tr>
<td>CXCL10</td>
<td>Forward: 5′-TTATTGAACGGGAGCCAAAG-3′</td>
<td>Reverse: 5′-GCTGTCCATCGGTCTCGCA-3′</td>
</tr>
<tr>
<td>CXCL11</td>
<td>Forward: 5′-GCTATGTATCATCGGCGGACAA-3′</td>
<td>Reverse: 5′-CGCGTTACTCGGTAATTACAGAA-3′</td>
</tr>
<tr>
<td>CCL3</td>
<td>Forward: 5′-TCAGCAACATGAGGTTCTCCAC-3′</td>
<td>Reverse: 5′-GCAAAGAAGCTGCTGGTCTCAA-3′</td>
</tr>
<tr>
<td>CCL4</td>
<td>Forward: 5′-CTTCTGCGATTCAGGTTGCTCA-3′</td>
<td>Reverse: 5′-GCATAAGGGCTGCTGCTCAA-3′</td>
</tr>
<tr>
<td>CCL5</td>
<td>Forward: 5′-ACACAGACAGAGCAGCTCCCA-3′</td>
<td>Reverse: 5′-AGCTGGTACTGAGCAAGCAA-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5′-GCTGTGGATGATGTGTCGT-3′</td>
<td>Reverse: 5′-TGATTTGAAATTTGTCATTA-3′</td>
</tr>
</tbody>
</table>

Fig. 2. Renal function and acute tubular damage after I/R injury. Serum creatinine levels at 24 h after reperfusion (a). PAS staining of kidney sections from sham (b), control (c) and TAK (d) groups at 24 h after reperfusion; original magnification ×200. Pathological acute tubular injury score in kidney sections were summarized in (e). Data are means ± SD. The statistical test used was the Turkey test for serum creatinine levels and the Steel–Dwass test for acute tubular necrosis score (n = 6 animals per group). *P < 0.05 compared with the corresponding value of the sham; **P < 0.05 compared with the corresponding value of the control.
Chemokine expression in post-ischemic kidneys

Real-time RT–PCR demonstrated the mRNA expression levels of chemokines that bind to CXCR3 and CCR5 in post-ischemic kidneys at different times (1, 3, 6 and 24 h) (Figure 3). CXCL10 and CXCL11 levels were increased from 1 h after the reperfusion and peaked at 3 h in both the control and TAK groups, which was significantly elevated at 3 h compared with the sham group, and then decreased by 24 h after reperfusion. The levels of CXCL11, CCL3 and CCL4 increased following the CXCL10 elevations in the control group. However, the levels of CCL3 and CCL4 in the TAK group were less than those in the control group.

T-cell infiltration into post-ischemic kidneys

Since our present study demonstrated that mRNA expression levels of CXCL10 in post-ischemic kidneys peaked at 3 h, we next evaluated the influence of the elevation of the chemokine levels on cell infiltration at 3 h after reperfusion. The numbers of T cells increased in post-ischemic kidneys of the control group, but the TAK injection reduced significantly the infiltration of T cells.
into the post-ischemic kidney, compared with those of the sham and control groups (sham group: 4.78 ± 0.94/hpf, control group: 12 ± 4.08/hpf and TAK group: 5.68 ± 1.61/hpf) (Figure 4).

Phenotype of infiltrating T cells in post-ischemic kidneys

As the number of T cells in post-ischemic kidneys increased at 3 h, we examined the phenotype of T cells among KMNCs by flow cytometry analysis. Freshly isolated KMNCs were gated by CD3, followed by CD4, CD8 and CD161a gating. Most of CD161a+ T cells expressed CD8, but not CD4, CD4+ cells (CD4+ T cells), CD8+CD161a− cells (CD8+ T cells) and CD8+CD161a+ cells (CD8+NKT cells) were identified (Figure 5). Populations of each phenotype of T cells in the sham group were as follows: CD4+ T cells 26.5 ± 1.5%, CD8+ T cells 38.9 ± 9.8% and CD8+ NKT cells 22.0 ± 6.1%. At 3 h after reperfusion, the percentages of CD4+ T cells and CD8+ NKT cells in the control group increased by 2.9 and 9.9%, respectively, compared with the sham group. However, TAK treatment suppressed increases in the percentages of CD4+ T cells and CD8+ NKT cells in the control group increased by 2.9 and 9.9%, respectively, compared with the sham group. However, TAK treatment suppressed increases in the percentages of CD4+ T cells and CD8+ NKT cells (Figure 5), which lead to a significant decrease in the number of CD8+ NKT cells compared with the control group (P < 0.05; Table 2). Immunofluorescence staining was performed to determine whether the infiltrating CD8+ NKT cells express CXCR3 and CCR5. In the post-ischemic kidneys, the CD8+ NKT cells expressed only CXCR3 (Figure 6a and b).

Discussion

A variety of studies have identified T cells as important mediators in renal I/R injury [7–12]. However, the mechanism of T-cell infiltration during the early phase of renal I/R injury remains unknown. We focused on the chemokines, which are up-regulated during inflammation. To address this issue, we evaluated the chemokine response that is responsible for the recruitment and the characterization of T cells during the early phase of renal I/R injury and studied the effect of tissue protection on I/R injury by blocking CXCR3 and CCR5, which are expressed on T cells.

In our study, CXCL10, a CXCR3-binding chemokine, was the most up-regulated at 3 h after reperfusion and CXCL9 was increased by ~1.3-fold compared with the sham similar to a previous report [29]. On the other hand, the elevation of CXCL11, CCL3 and CCL4 was detected following CXCL10 elevation in both the control and the TAK groups. Concerning CCL3 and CCL4, these chemokines were less up-regulated at 6 h after reperfusion in the TAK group compared with the control group. CXCR3 and CCR5 were not present in damaged tissue, such as...
tubular endothelial cells other than T cells as well as previous reports [30]. It has been reported that CD4+ T cells produce CCL3 and CCL4 upon activation in vitro [31]. At 6 h, the levels of CCL3 and CCL4 in KMNCs were similar between the control and the TAK groups (data not shown). Therefore, it appears that CXCL10 functions as a trigger for the elevation of CXCL11, CCL3 and CCL4 in post-ischemic kidneys, and the up-regulation of CXCL10 then induces the recruitment of T cells in the post-ischemic kidney.

Previous studies using mice I/R injury model reported that invariant NKT cells mediated I/R injury [15, 17]. These cells express a CD1d-restricted T-cell receptor and CD4−CD8− or CD4−CD8−. But, we determined that T cells were trafficked to post-ischemic kidneys at 3 h after reperfusion and the phenotypes of infiltrated T cells in the post-ischemic kidney were CD8+ NKT cells and CD4+ T cells. Especially, the number of CD8+ NKT cells increased significantly at 3 h after reperfusion. The engagement of CD8+ NKT cells was a novel observation in the case of a rat I/R injury. Our results suggest that CD8+ NKT cells as well as CD4+ T cells proliferate at the early phase in the rat I/R injury model and are associated with the characteristics of the rat I/R injury.

Fig. 5. The phenotype of T cells infiltrating into the post-ischemic kidneys. The phenotype of the T cells was identified by flow cytometry. KMNCs were prepared from post-ischemic kidneys and were identified as described in the Materials and methods section. A CD3 mAb was used to identify the total T cell population. After gating on the T cells population, we identified subsets by using CD4 mAb, CD8 mAb and CD161a mAb. The data are organized in three groups. The top column shows CD4 versus CD8 staining on CD3-gated cells, the middle column shows CD8 versus CD161a (NKR) and the bottom column shows CD4 versus CD161a.

Table 2. Population and number of T cells

<table>
<thead>
<tr>
<th>KMNCs (n×10⁶)</th>
<th>Sham</th>
<th>Control</th>
<th>TAK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population of T cells (%)</td>
<td>8.8 ± 2.8</td>
<td>18.8 ± 2.5*</td>
<td>8.8 ± 1.1**</td>
</tr>
<tr>
<td>CD4 (%)</td>
<td>26.5 ± 1.5</td>
<td>29.4 ± 9.0</td>
<td>25.6 ± 3.0</td>
</tr>
<tr>
<td>CD8 (%)</td>
<td>38.9 ± 9.8</td>
<td>32.0 ± 3.2</td>
<td>41.4 ± 7.4</td>
</tr>
<tr>
<td>CD8+ NKT (%)</td>
<td>22.0 ± 6.1</td>
<td>31.9 ± 4.7</td>
<td>26.4 ± 6.1</td>
</tr>
</tbody>
</table>

Number of T cells (n)

<table>
<thead>
<tr>
<th>KMNCs (n×10⁶)</th>
<th>Sham</th>
<th>Control</th>
<th>TAK</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4 (n×10⁴)</td>
<td>13.5 ± 3.5</td>
<td>28.9 ± 15.4</td>
<td>11.8 ± 3.5</td>
</tr>
<tr>
<td>CD8 (n×10⁴)</td>
<td>20.1 ± 8.6</td>
<td>31.1 ± 16.4</td>
<td>19.3 ± 6.8</td>
</tr>
<tr>
<td>CD8+ NKT (n×10⁴)</td>
<td>11.7 ± 5.4</td>
<td>30.1 ± 8.6*</td>
<td>11.8 ± 2.9**</td>
</tr>
</tbody>
</table>

Data are means ± SD. The statistical test used was the Tukey test.

*P < 0.05 compared with the corresponding value of the sham.

**P < 0.05 compared with the corresponding value of the control.
At the late phase (24 h) after I/R injury, it has been shown that the reconstitution of nu/nu mice with CD4+ T cells alone, but not CD8+ T cells alone [8], restored kidney injury after I/R that the phenotypes of KMNCs that increased in post-ischemic kidneys are CD4+ IFN-γ+ T cells but not CD8+ T cells [8, 12]. Although our study also showed that CD4+ T cells increased in renal I/R injury, we failed to detect the induction of these cytokines in detail (data not shown). On the other hand, other reports have shown that CD8+CD161+ subsets produce high levels of cytotoxic granules, although they do not produce cytokines such as IFN-γ [32]. We presume that activated CD8+ NKT cells mediate tissue injury at the early phase in rat I/R and could represent a bridge between innate and adaptive immunity (Figure 7).

TAK treatment improved serum creatinine and tubular damage in post-ischemic kidneys because TAK could suppress CXCR3 on both CD8+ NKT cells and CD4+ T cells and CCR5 on CD4+ T cells. Important details regarding the mechanism of renal protection by blocking T-cell infiltration remain unknown, because the role of T cells in renal I/R injury remains a controversial subject [7–12, 33, 34]. These findings indicate that the accumulation of inflammatory cells at the early phase of post-ischemic kidneys could be a contributing factor to tissue damage and that CXCR3 and CCR5 play an important role in the infiltration of T cells. Thus, chemokines that regulate T cells in post-ischemic kidneys represent potential therapeutic targets for renal I/R injury.

In conclusion, the findings reported herein demonstrate that chemokine production in the kidney is up-regulated from the early phase after reperfusion and the blocking of chemokine receptors on T cells including NKT cells contributes to tissue protection in renal I/R injury. Therefore, the administration of TAK can be considered to be a potential therapeutic method for reducing renal injury and dysfunction caused by I/R injury.

Acknowledgements. The authors wish to thank Dr Milton S. Feather for his editing of the manuscript, Ms Mutsumi Tsuchiya and Ms Mizuki Takeuchi for their excellent technical assistance. This work was supported by a Grant-in-Aid for Young Scientists (B) (23791753 to M.O.). The contents of the article have not been published or submitted for publication in any other journal.

Conflict of interest statement. None declared.
References


Received for publication: 27.12.2011; Accepted in revised form: 10.6.2012