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ORIGINAL ARTICLE

Epigallocatechin-3-gallate protects kidneys from ischemia reperfusion injury by HO-1 upregulation and inhibition of macrophage infiltration

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Summary

Epigallocatechin-3-gallate (EGCG) shows diverse chemical and biological activities. We investigated the effects of EGCG in a rat renal ischemia reperfusion (I/R) injury model. Sprague–Dawley rats received intraperitoneal injection of 50 mg/kg EGCG 48 h, 24 h, and 30 min prior to I/R injury. The animals were subjected to left renal occlusion for 45 min. EGCG treatment suppressed the peak in serum creatinine. EGCG-treated kidneys showed significantly less tubular damage and a decreased number of apoptotic cells. The I/R-induced elevation in the renal MDA level was significantly decreased in the EGCG group. Reverse-transcriptase polymerase chain reaction showed that EGCG significantly decreased the expression of MHC class II, TLR2, TLR4, MCP-1, IL-18, TGF-β1, procollagen Ia1, TIMP-1, and Kim-1. ED-1 staining showed reduced macrophage infiltration and α-SMA staining revealed less interstitial expression. Heme oxygenase-1 (HO-1) expression in I/R kidneys was upregulated in the EGCG group based on the results of both RT-PCR and Western blotting analysis. Blockade of HO-1 gene induction by SnPP increased renal tubular damage and macrophage infiltration. These findings suggest that EGCG protects the kidneys against I/R injury by reducing macrophage infiltration and decreasing renal fibrosis. These beneficial effects may be mediated, in part, by augmentation of the HO-1 gene.

Introduction

Renal ischemia-reperfusion (I/R) injury is unavoidable in renal transplantation and may lead to acute post-transplant tubular necrosis and delayed graft function. The initial intensity of the I/R injury is related to both early and late long-term graft function [1,2]. Ischemic complications may occur not only with cadaveric kidney transplantation but also with living kidney transplantation from elderly donors whose kidneys have fewer cardiovascular reserves because of age-related arteriosclerosis. Therefore, methods of reducing the kidney graft damage caused by I/R injury could potentially improve the outcome of renal transplantation.

I/R injury has a complex pathophysiology with cellular mediators of immunity, such as dendritic cells, neutrophils, macrophages, natural killer cells and T cells. Macrophage become a quantitatively dominant infiltrating cell soon after I/R injury, and play a crucial role in early period by the release of proinflammatory cytokines and chemokines. Macrophage depletion attenuated renal injury in a rat renal I/R injury model [3]. Some reports demonstrated that macrophages contribute to the development of renal fibrosis induced by I/R injury via the

TGF-β1 signaling pathway [4]. The strategies that reduce early macrophage infiltration of activation may be useful in the treatment of I/R injury.

Heme oxygenase-1 (HO-1) has attracted considerable attention in organ transplantation because of its cytoprotective function [5]. Local HO-1 overexpression in the graft itself ameliorates IR injury [6], suggesting that HO-1 may play a key role in maintaining antioxidant/oxidant homeostasis in I/R injury after transplantation.

Epigallocatechin-3-gallate (EGCG), the most abundant catechin in green tea, shows antiinflammatory, antioxidant [7,8], anticancer [9,10], and immunomodulatory activities [11]. EGCG promotes the preservation of tissues, such as blood vessels [12], nerves [13], islet cells [14], and skin [15]. Here, we report that EGCG treatment markedly attenuated tubular damage and reduced I/R-induced apoptosis in a severe rat renal I/R model by reducing macrophage infiltration leading to a decrease in renal fibrosis. These protective effects may be associated with the augmentation of HO-1.

Subjects and methods

Animals

Male Sprague–Dawley rats weighing 200–250 g were purchased from Japan SLC Inc. (Shizuoka, Japan), and maintained under standard conditions until the experiments were performed. Animals were fed a standard diet and water *ad libitum*. All procedures were performed in accordance with the principles of the Guidelines of Animal Experimentation at Osaka University.

Animal experimental protocols

The rats were randomly divided into three groups: (i) a sham-operated group ($n = 20$), (ii) a saline-treated group (control group; $n = 34$), and (iii) an EGCG treatment group (EGCG group; $n = 34$). Rats in the control and EGCG groups were given intraperitoneal injections of 1 ml of saline or 50 mg/kg of EGCG, respectively, at 48 h, 24 h, and 30 min prior to I/R injury. All rats were anesthetized with sodium thiopental (30 mg/kg intraperitoneally). The animals were subjected to left renal occlusion for 45 min by clamping the renal pedicles with artery clips. Reperfusion began with removal of the artery clips and was confirmed visually by a blush. After confirmation of reperfusion, right nephrectomy was performed. The rats were sacrificed to collect each eight samples at 4, 24, and 72 h, six samples at 7 days, and four samples at 14 days after commencement of reperfusion. To assess whether EGCG treatment increased HO-1 expression, two additional EGCG groups were treated with 10 μmol/kg tin protoporphyrin (SnPP: Frontier Science, Logan, UT, USA), a competitive inhibitor of HO-1 (EGCG with SnPP group, $n = 5$) or 10 μmol/kg copper protoporphyrin (CuPP: Frontier Science), vehicle control (EGCG with CuPP group, $n = 5$) at 1 h prior to I/R injury. Both groups were sacrificed at 72 h. Rats in the sham-operated group, which were given no treatment, were subjected to the same surgical procedure, including right nephrectomy but without inducing I/R, and were sacrificed at 4, 24, and 72 h, 7 days, and 14 days ($n = 4$) after reperfusion.

Malondialdehyde measurement

Malondialdehyde (MDA), as an index of lipid peroxidation, was measured in renal tissue using BIOXYTECH MDA-586 (OxisResearch, Portland, OR, USA). The MDA-586 method is based on the reaction of a chromogenic reagent, *N*-methyl-2-phenylindole (NMPI), with MDA at 45 °C. Briefly, kidney tissue was weighed and homogenized. Butylated hydroxytoluene was added to a final concentration of 5 mM prior to homogenization of tissue to preventing new lipid peroxidation. NMPI and hydrochloric acid were added to 0.2 ml homogenate tissue and the mixture was heated at 45 °C for 60 min. The MDA concentration was calculated from the intensity of the color of the final product at 586 nmol.

Histological evaluation

The kidneys were fixed by immersion in 4% (w/v) paraformaldehyde for 16 h and then embedded in paraffin. The tissues embedded in paraffin were cut into sections of 6-μm thick. The sections were mounted on silane (2% 3-aminoprophltriethoxysilane)-coated slides and then deparaffinized with xylene. Each section was stained with Periodic acid-Schiff (PAS) and Masson's trichrome according to standard protocols. Immunohistochemical staining 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 activities were blocked by the avidin-biotin technique using the Dako-Cytomation Biotin Blocking System (Dako). Endogenous peroxidase activities were blocked with 3% hydrogen peroxide for 5 min. The primary antibodies were diluted in Dako REAL Antibody Diluent (Dako) at specific concentrations, and incubated for 24 h at 4 °C with α-smooth muscle actin (α-SMA; Lab Vision, Fremont, CA, USA), ED-1 (AbD Serotec, Oxford, UK), OX6 (Abcam, Cambridge, MA, USA), Toll-like receptor (TLR)2 (Abbiotec, San Diego, CA, USA), TLR4 (Abcam) and kidney injury molecule-1 (Kim-1; R&D Systems, Minneapolis, MN, USA), and for 45 min at room temperature with cleaved

caspase-3 (Cell Signaling Technology, Beverly, MA, USA). This was followed by incubation with secondary antibodies. Color was developed with 3,3′-diaminobenzidine tetrahydrochloride (Dako).

SYBR Green real-time reverse-transcriptase polymerase chain reaction

Total RNA was extracted from cell monolayers using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's directions. Total RNA was reverse transcribed using a first-strand cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ, USA) following the manufacturer's instructions. Aliquots (2 μg) of the reverse transcription product were quantified by SYBR Green two-step real-time reverse-transcriptase polymerase chain reaction (RT-PCR) with the exception of HO-1, and three-step real-time RT-PCR for HO-1 on a Thermal Cycler Dice Real-Time System (Takara Bio, Shiga, Japan). The PCR mixture was prepared using SYBR Premix Ex Taq 2 (Takara Bio). Each sample was analyzed in duplicate using the conditions recommended by the manufacturer. The PCR primer sets used for HO-1, Monocyte chemoattractant protein-1 (MCP-1), RT-1a, TRL2, TRL4, IL-18, Transforming growth factor-β1 (TGF-β1), procollagen Ia1, tissue inhibitor of metalloproteinase 1 (TIMP-1), Kim-1, and β-actin cDNA amplification were as follows: HO-1, forward: 5′-TCTATCCGTGCTCGCATGAAC-3′, reverse: 5′-CAGCTCCTCAAACAGCTCAA-3′; MCP-1, forward: 5′-CTATGCAGGTCTCTGTCACGCTTC-3′, reverse: 5′-CAGCCGACTCATTGGGATCA-3′; RT-1a, forward: 5′-TCCCTACATCGACAGCCTGAAG-3′, reverse: 5′-TACGTAGCCATGTGACATTGAGCA-3′; TRL2, forward: 5′-AGCAGGATTCCTATTGGGTGGAG-3′, reverse: 5′-ATGATCCATTTGCCCGGAAC-3′; TRL4, forward: 5′-CCGCTCTGGCATCATCTTCA-3′, reverse: 5′-CCCACTCGAGGTAGGTGTTTCTG-3′; IL-18, forward: 5′-GACTGGCTGTGACCCTATCTGTGA-3′, reverse: 5′-TTGTGTCCTGGCACACGTTTC-3′; TGF-β, forward: 5′-TGC GCC TGC AGA GAT TCA AG-3′, reverse: 5′-AGG TAA CGC CAG GAA TTG TTG CTA-3′; procollagen Ia1, forward: 5′-CATCTCCATGGCCTCTGCAA-3′, reverse: 5′-CACATGTGTGGCCGATGTTTC-3′; TIMP-1, forward: 5′-CGAGACCACCTTATACCAGCGTTA-3′, reverse: 5′-TGATGTGCAAATTTCCGTTCC-3′; Kim-1, forward: 5′-CGGTGCCTGTGAGTAAATAGAT-3′, reverse: 5′-CTGGCCATGACACAAATAAGAC-3′; and β-actin, forward: 5′-GGAGATTACTGCCCTGGCTCCTA-3′, reverse: 5′-GACTCATCGTACTCCTGCTTGCTG-3′. Real-time RT-PCR data were plotted as the ΔRn fluorescence signal versus the cycle number. The cycle threshold was defined as the cycle number at which the ΔRn crossed this threshold using the manufacturer's software.

Western blotting analysis

Kidney tissues were homogenized in radioimmunoprecipitation assay buffer (10 mmol/l Tris Cl, pH 7.6, 150 mmol/l NaCl, 1% [w/v] sodium dodecyl sulfate, 1% [v/v] aprotinin, 2 mmol/l Na_3VO_4, and freshly prepared leupeptin [1 μg/ml], pepstatin [1 μg/ml], and 1 mmol/l of phenylmethylsulfonylfluoride). Homogenates were centrifuged (11 000 *g*, 10 min, 4 °C), and the total protein in the supernatant was measured using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Total protein lysate (15 μg) containing 1:1 denaturing sample buffer was boiled for 3 min, resolved on 5.0% sodium dodecyl sulfate-polyacrylamide gels, and electrophoretically transferred onto membranes. To detect HO-1, the filters were blocked with 3% bovine serum albumin in PBS for 1 h at room temperature, followed by overnight incubation at 4 °C with a 1:500 dilution of anti-HO-1 monoclonal antibody (Millipore, Billerica, MA, USA) in TBS-T. After washing, the filters were incubated with anti-mouse IgG (1:1000) (Cell Signaling Technology) in TBS-T for 1 h at room temperature and developed to detect specific protein bands using ECL reagent (Amersham Biosciences). The bands on Western blots were quantified using the ImageJ program (National Institutes of Health, Bethesda, MD).

Histopathology scoring

The damage was assessed on PAS stained renal tissue sections by scoring tubular dilatation, cast deposition, loss of brush borders, and necrosis in 10 non-overlapping fields (×200) at the corticomedullary junction. Injury was scored on a five-point scale [16]: 0 = normal, 1 = <10% of the corticomedullary junction, 2 = 10–25%, 3 = 25–50%, 4 = 50–75%, 5 = >75%.

Statistical analysis

Data are expressed as means ± SD. Statistical analysis was performed with unpaired *t*-test or analysis of variance (ANOVA) for multiple comparisons, followed by Scheffe's *F*-test. Differences were considered statistically significant at $P < 0.05$.

Results

EGCG preserved renal function and reduced the extent of acute tubular damage and apoptosis after I/R

Compared with sham-operated rats, serum creatinine was increased significantly at 4, 24, and 72 h, and at 7 days after I/R injury in both control and EGCG groups. The increase in creatinine in the EGCG group was significantly suppressed compared with the control group at 24 h (control

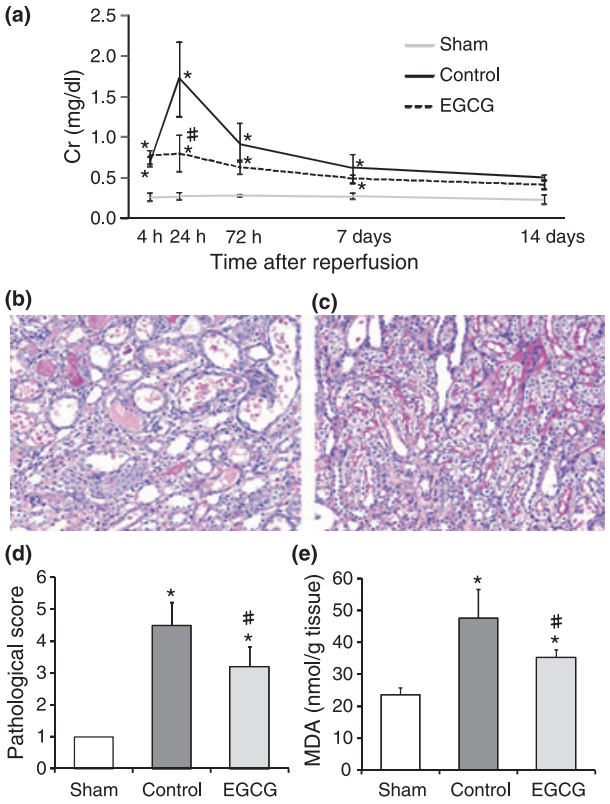

Figure 1 EGCG pretreatment on kidney I/R injury. The transition in serum creatinine after reperfusion (a). PAS staining of kidney sections from control (b) and EGCG (c) groups 72 h after I/R injury (×200). Pathological tubular damage scores in kidney sections are summarized in (d). The MDA level from the kidneys of each group 4 h after reperfusion (e). Data are means ± SD. *$P < 0.05$ compared with the corresponding value of the sham, #$P < 0.05$ compared with the corresponding value of the control.

group 1.73 ± 0.46 mg/dl, EGCG group 0.81 ± 0.23 mg/dl; $P < 0.05$) (Fig. 1a). There was no significant difference in creatinine levels between the control and EGCG groups at 72 h, 7 days, and 14 days, but the EGCG group had a lower creatinine level than the control group. Consistent with preserved renal function, kidneys at 72 h after I/R injury in the EGCG group had significantly less renal tubular damage than the control group (Fig. 1b–d). We performed immunostaining for cleaved caspase-3 to quantify the number of apoptotic cells in the I/R kidneys. Cleaved caspase-3 was widely expressed among the tubular epithelial cells in the control group, whereas EGCG treatment significantly decreased cleaved caspase-3 expression (Fig. 2).

EGCG decreased the levels of lipid peroxidation products in the kidney tissues after I/R

Lipid peroxidation is a well-established mechanism of cellular injury and is used as an indicator of oxidative stress

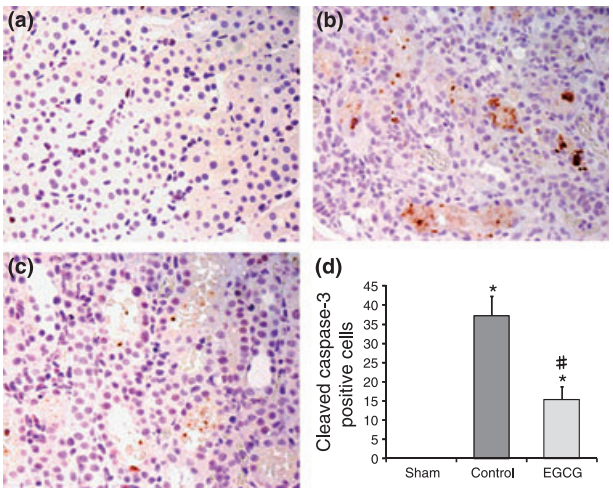

Figure 2 EGCG treatment on apoptotic cell death induced by I/R injury (×400). Light photomicrographs of kidney sections from the sham (a), control (b), and EGCG (c) groups 72 h after reperfusion. The dark brown dots correspond to representative cytoplasmic localization of cleaved caspase-3 in apoptotic cells. The number of cleaved caspase-3-positive cells (×200) is summarized in (d). Data are means ± SD. *$P < 0.05$ compared with the corresponding value of the sham, #$P < 0.05$ compared with the corresponding value of the control.

in cells and tissues. Measurement of MDA is widely used as an indicator of lipid peroxidation. The renal tissue MDA content 4 h after reperfusion was significantly increased by I/R injury (47.7 ± 9.0 nmol/g tissue) compared with the sham-operated group (23.6 ± 2.1 nmol/g tissue; $P < 0.05$). However, EGCG significantly decreased the I/R-induced elevation of the renal MDA level (35.2 ± 2.5 nmol/g tissue; $P < 0.05$, Fig. 1e).

EGCG decreased expression of MHC class II antigen, TLR2 and TLR4 mRNA, and protein in the kidneys after I/R

RT-PCR examination showed that MHC class II, TLR2 and TLR4 mRNA expression at 72 h was elevated in the control group compared with the sham group, and EGCG significantly decreased these increases (Fig. 3a). Figure 3b shows representative immunohistochemistry of MHC class II antigen using OX6, TLR2 and TLR4 in rat kidneys 72 h after I/R injury. In the sham group, immunoreactivity was rarely observed in infiltrating cells by immunohistochemistry of MHC class II. However, in the control group, the number of OX-6 positive cells was increased, and EGCG decreased the number of OX-6 positive cells. The expression and localization of TLR2 and TLR4 protein detected using immunohistochemistry were similar to TLR2 and TLR4 mRNA expression, EGCG decreased the expression of TLR2 and TLR4 protein.

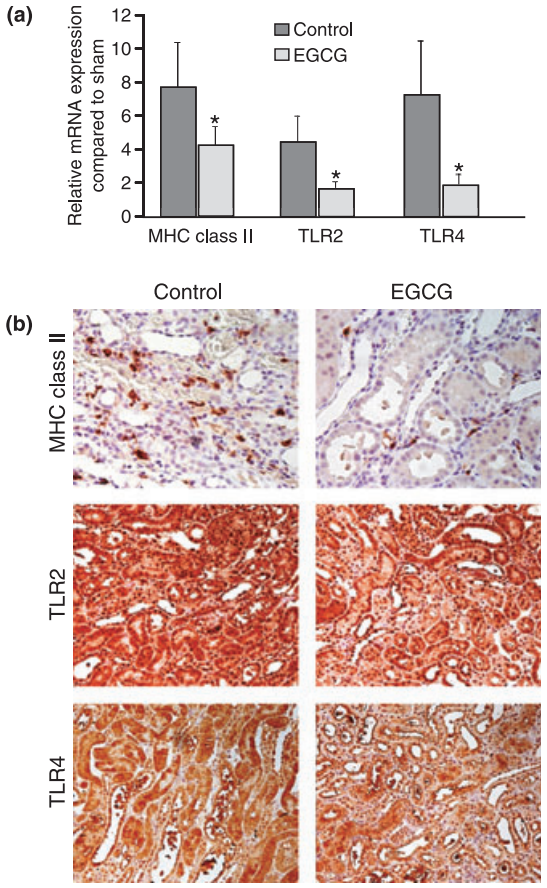

Figure 3 EGCG decreased expression of MHC class II antigen, TLR2 and TLR4 mRNA and protein in the kidneys after I/R. RT-PCR showed that EGCG decreased the expression of MHC class II, TLR2 and TLR4 mRNA (a). Immunohistochemical staining of MHC class II (×400), TLR2 and TLR4 (×200) (b). Data are means ± SD. $*P < 0.01$ compared with the corresponding value of the control.

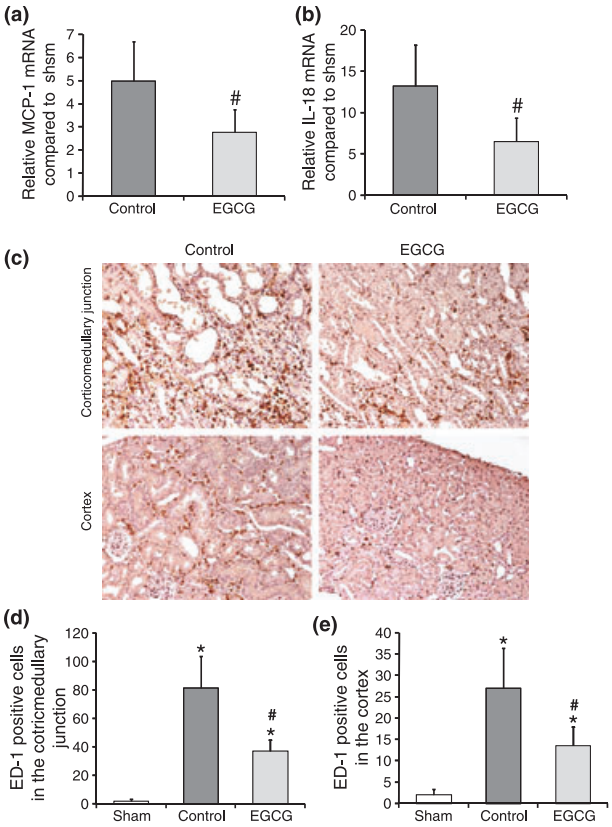

Figure 4 Macrophage infiltration in kidneys after I/R injury. RT-PCR analysis revealed that EGCG treatment markedly decreased MCP-1 mRNA level at 24 h (a) and IL-18 level at 72 h (b) after reperfusion. Immunohistochemical staining of ED-1 of the kidney sections from the control and EGCG groups at the corticomedullary junction and in the cortex (×200) (c). The numbers of ED-1-positive cells in the corticomedullary junction and in the cortex are summarized in (d, e). Data are means ± SD. $*P < 0.05$ compared with the corresponding value of the sham, $\#P < 0.05$ compared with the corresponding value of the control.

EGCG downregulated MCP-1 mRNA expression and reduced macrophage infiltration in the kidneys after I/R

We investigated the levels of MCP-1 mRNA expression, which mediates early monocyte/macrophage infiltration, by RT-PCR. The results of RT-PCR analysis indicated that EGCG significantly decreased MCP-1 mRNA expression in the I/R kidney at 24 h after reperfusion compared with the control group (Fig. 4a). Immunohistochemical analysis indicated that the number of ED-1-positive cells was significantly increased in the kidneys at 72 h after reperfusion in the control group, whereas EGCG treatment suppressed the number of ED-1-positive cells at both the corticomedullary junction and in the cortex of the kidneys (Fig. 4c–e). Moreover, while IL-18 gene expression in whole kidney tissue from 72 h was progressively upregulated in I/R kidneys, EGCG significantly decreased expression of IL-18 (Fig. 4b).

EGCG downregulated TGF-β1 mRNA expression and reduced interstitial fibrosis in the I/R kidney

The RT-PCR analysis revealed that EGCG significantly decreased TGF-β1 mRNA at 24 h after reperfusion, and decreased procollagen Ia1, TIMP-1, and Kim-1 mRNA expression at 72 h in the I/R kidneys compared with the control group (Fig. 5). EGCG decreased the expression of Kim-1 protein in I/R injury kidneys, as determined using immunohistochemistry, compared with the control group (Fig. 6a). Interstitial α-SMA expression in the I/R kidneys, which associated with interstitial damage and fibrosis, was increased in kidneys from control rats 72 h and 7 days after reperfusion, whereas EGCG treatment significantly suppressed interstitial expression of α-SMA (Fig. 6a,b). Moreover, the presence of collagen deposition,

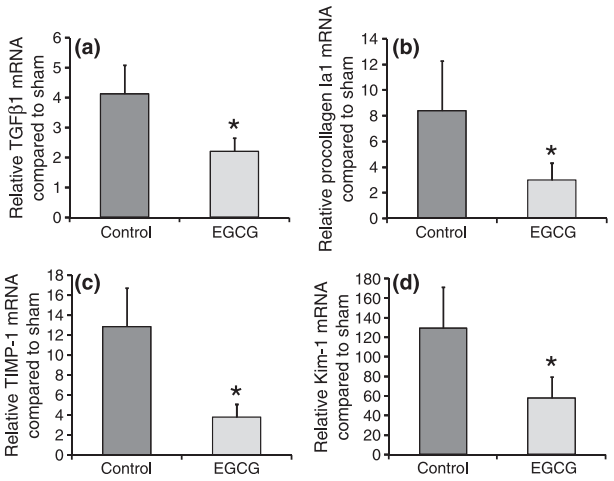

Figure 5 RT-PCR analysis revealed that treatment with EGCG markedly decreased TGF-β1 mRNA (a), procollagen Ia1 mRNA (b), TIMP-1 mRNA (c), and Kim-1 mRNA (d). Data are means ± SD. $*P < 0.01$ compared with the corresponding value of the control.

determined using Masson trichrome staining, was reduced in the EGCG group at 7 and 14 days after reperfusion (Fig. 6a,c).

EGCG augmented HO-1 in rat kidneys after I/R, and blockade of HO-1 gene induction increased renal tubular damage and macrophage infiltration

We investigated HO-1 expression levels in the kidneys after I/R injury. RT-PCR analysis indicated that EGCG markedly increased HO-1 mRNA expression in I/R kidneys at 4 and 24 h after reperfusion (Fig. 7a,b). On Western blotting analysis, HO-1 was almost undetectable in sham-operated kidneys at 24 h after I/R injury, whereas HO-1 level increased at 24 h after I/R injury in both the control and EGCG groups. HO-1 expression levels increased further in the kidneys from the EGCG group compared with the control group (Fig. 7c). When we blocked HO-1 gene induction by SnPP in the EGCG group, kidneys from these animals showed a significant increase in renal tubular damage in the outer medulla 72 h after I/R injury compared with the EGCG with CuPP group, the vehicle control with no effect on HO-1 induction (Fig. 7d). Similarly, ED-1 immunohistochemical staining showed that macrophage infiltration increased in the EGCG with SnPP group at 72 h compared with those in the EGCG with CuPP group (Fig. 7e).

Discussion

Delayed graft function (DGF) is a common complication immediately after cadaveric kidney transplantation. The

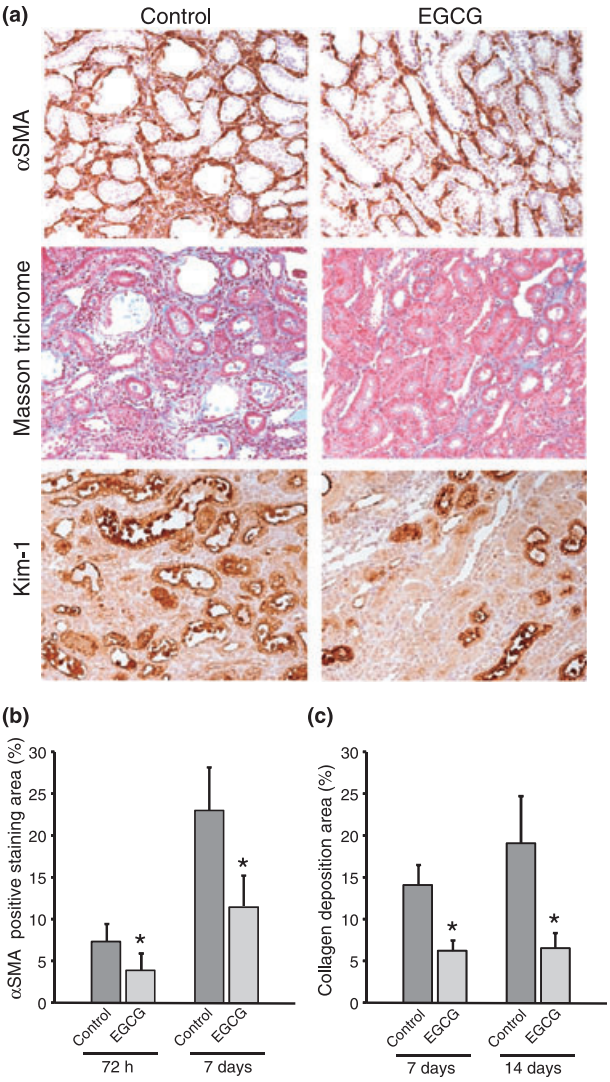

Figure 6 EGCG pretreatment on renal fibrosis after I/R injury. Immunohistochemical findings of α-SMA (7 days after reperfusion; ×200), the deposition of collagen as determine by Masson Trichrome staining (14 days; ×200), and Kim-1 (72 h after reperfusion; ×200) (a). The percentage of α-SMA-positive staining area is summarized in (b). The percentage of collagen deposition area is summarized in (c). Data are means ± SD. $*P < 0.01$ compared with the corresponding value of the control.

main cause of DGF is I/R injury, which has a complex pathophysiology with a number of contributing factors, such as local neutrophil accumulation, lymphocyte/macrophage activation, and release of proinflammatory cytokines, which lead to cell injury and culminate in graft failure. The initial intensity of the I/R injury is related to both early and late long-term graft function [1,2]. I/R injury represents an important problem affecting the outcome of organ transplantation. EGCG, the flavonoid

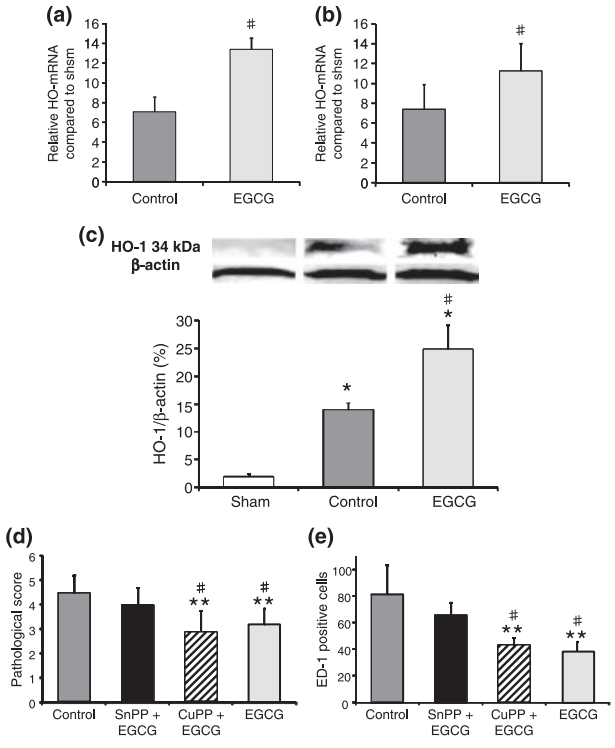

Figure 7 Expression of HO-1 in kidneys after I/R injury. RT-PCR analysis revealed that treatment with EGCG markedly increased HO-1 mRNA expression in I/R kidneys at 4 (a) and 24 h (b). Western blotting analysis showed that EGCG augmented HO-1 protein at 24 h (c). When induction of HO-1 gene expression was blocked by tin protoporphyrin (SnPP), I/R kidneys from these rats at 72 h after reperfusion showed significantly more renal tubular damage on PAS staining (d), and a significant increase in macrophage infiltration on ED-1 staining (e). Data are means ± SD. $*P < 0.05$ compared with the corresponding value of the sham, $\#P < 0.05$ compared with the corresponding value of the control, $**P < 0.05$ compared with the corresponding value of the SnPP + EGCG group.

extracted from green tea, is a naturally occurring polyphenolic compound with antiinflammatory, antioxidant [7,8], anticancer [9,10], and immunomodulatory activities [11]. The results of this study confirmed the protective effects of EGCG on I/R-injured rat kidneys via reduction of macrophage infiltration.

Renal tubular epithelial cells express both TLR2 and TLR4, and expression of both TLRs is increased by I/R injury [17,18]. TLR2 and TLR4 activation in renal tubular cells may be a link between toxic tubular cell injury and the onset of the innate inflammatory response in the pathogenesis of I/R injury. EGCG decreased the TLR2 and TLR4 mRNA expression by RT-PCR, and both TLRs protein expression by immunohistochemistry. Moreover, MHC class II mRNA expression was reduced in the EGCG group, and immunohistochemistry of MHC class II antigen using OX6 presenting macrophages and interstitial dendritic cells showed that EGCG decreased the infiltration of MHC class II positive cells in rat kidneys after I/R injury. These findings indicate that EGCG may inhibit the innate immune response in I/R injured kidneys.

Thus, we investigated the effect of EGCG on macrophage infiltration in I/R injured kidneys. Early infiltration of macrophages plays an important pathogenic role in renal I/R injury, presumably mediated by the release of proinflammatory cytokines and chemokines [3]. Macrophage infiltration is mediated by MCP-1, expression of which in the I/R kidney is correlated with the renal infiltration of monocytes/macrophages into the kidneys [19]. Furuichi *et al.* reported that mice deficient in CCR2, a receptor of MCP-1, were protected from acute tubular necrosis and cell infiltration after renal I/R injury [20]. Thus, MCP-1 may be a therapeutic target for I/R injury. Some previous studies have shown that EGCG inhibits MCP-1 expression and secretion in vascular endothelial cells *in vitro* [21] and in a human monocyte cell line [22]. In this study, EGCG inhibited MCP-1 expression in rat kidneys after I/R compared with the control group and inhibition of macrophage infiltration into I/R kidneys was observed in the EGCG group. Moreover, the expression of IL-18, a proinflammatory cytokine produce by macrophages that contributes to renal damage after I/R injury [23], was decreased in the EGCG group. This effect of EGCG contributed to the preservation of renal function and reduced the extent of acute tubular damage after renal I/R injury.

Macrophages induce tubulointerstitial fibrosis after I/R injury via the TGF-β1 signaling pathway, and TGF-β1 expression are reduced by macrophage depletion [4]. We demonstrated that EGCG has an inhibitory effect on TGF-β1 upregulation after I/R injury. Although the activity of TGF-β1 was not measured in this study, the mRNA expression of procollagen Ia1 and TIMP-1, which parallel the activity of TGF-β1, was also decreased in the EGCG group. These findings suggest that EGCG may reduce both the production and activity of TGF-β1. TGF-β1 has been associated with the formation of fibrosis and causes differentiation of fibroblasts into myofibroblasts, characterized by *de novo* α-SMA synthesis. EGCG treatment suppressed the expression of α-SMA, which was accompanied by deposition of collagen, as determined using immunohistochemical staining and Masson trichrome staining. Kim-1 is a membrane protein maximally upregulated in proliferating and dedifferentiated tubular epithelial cells after renal ischemia, and is associated with their loss of polarity and the development of interstitial fibrosis [24,25]. EGCG decreased the expression of Kim-1 mRNA and protein in I/R injured kidneys. We consider that the decrease in macrophage infiltration by EGCG may lead to

suppression of renal fibrosis via reduction of TGF-β1 expression.

The HO system is the rate-limiting step in the conversion of heme into biliverdin, bilirubin, carbon monoxide, and free iron [26], and play a key role in maintaining antioxidant/oxidant homeostasis during periods of cellular injury [27]. The cytoprotection associated with local HO-1 overexpression may include several mechanisms, such as antioxidant function, maintenance of microcirculation, antiapoptotic function, and antiinflammatory function [28]. There has been a great deal of interest in the induction of HO-1 in renal transplantation because HO-1 is overexpressed in the kidney after I/R injury and represents an important endogenous antioxidative defense mechanism against postischemic tissue damage [6]. Recent studies have shown that *in vitro* EGCG upregulates HO-1 expression in endothelial cells in both a concentration- and a time-dependent manner [29]. In this study, EGCG treatment boosted HO-1 expression after I/R injury, as shown by RT-PCR and Western blotting analysis. We consider that the beneficial effect of EGCG on the development of I/R injury may be mediated, in part, by augmentation of the HO-1 gene. It has been reported that EGCG acts on the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, and upregulate the HO-1 [29]. Nitric oxide induces HO-1 in many cell types [30], so the activation of the PI3K/AKt pathway, one target of which is eNOS activation and NO release, may be a partial pathway in the induction of HO-1 by EGCG. However, in our investigation, there was no significant difference in the expression of eNOS mRNA between the control group and the EGCG group (data not shown). Thus, in the renal I/R injury model, EGCG may have another pathway, in addition to the PI3K/Akt pathway, for inducing HO-1 gene induction. When SnPP, a competitive inhibitor of HO-1 gene induction, was added to the EGCG-treated group, the protective effects of EGCG on I/R injury were reduced, and macrophage infiltration was augmented, compared with the vehicle group. Our investigation showed that MCP-1 mRNA was expressed at higher levels in the EGCG with SnPP group than in the vehicle group (data not shown). This suggests that upregulation of HO-1 expression by EGCG may have partially contributed to the decrease in macrophage infiltration during I/R injury. However, It was also reported that higher-dose SnPP than that used in our study induced HO-1 protein and protected the kidneys from I/R injury independently of its enzyme activity [31]. SnPP may have another mechanism to protect kidneys from I/R injury independent of HO activity. Thus, further investigation, including an investigation of HO enzyme activity, is needed to confirm the possibility that EGCG may act as an inducer of HO-1 leading to a decrease in the damage of kidney I/R injury.

In conclusion, we demonstrated that EGCG protected rat kidneys from I/R injury, and postulated that this was attributed to decreased macrophage infiltration via MCP-1 downregulation, and decreased renal fibrosis via TGF-β1 downregulation. It is possible that this beneficial effect of EGCG may be mediated by HO-1 augmentation. This is the first study to show that EGCG increases HO-1 mRNA and protein under hypoxic conditions *in vivo*. Thus, the systemic administration of EGCG could be used to reduce renal injury and dysfunction caused by I/R injury in kidney transplantation. However, further investigations are needed to establish the feasibility and efficacy of using EGCG in a clinical setting.

Authorship

YK: participated in research design, the performance of the research, data collection, data analysis, and the writing of the manuscript. MO: participated in research design, the performance of the research, data analysis, and the writing of the manuscript. YI: participated in research design and data analysis. KT, TA, KY, NI, ST and NN: participated in research design. KM and SHH: contributed reagents.

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