Effects of a phenolic compound, resveratrol, on the renal function and costimulatory adhesion molecule CD86 expression in rat kidneys with ischemia/reperfusion injury

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Summary. Recent studies have suggested that an ischemia/reperfusion (I/R) injury enhances the expression of costimulatory adhesion molecules on the vascular endothelium. In the present study, we investigated the protective effects of resveratrol, a phenolic product, on the renal function and expression of CD86 in rat kidneys with I/R injury. Wistar rats were divided into four groups; 1) an I/R group with right nephrectomy and 1-hour clamping of the left renal pedicle; 2) a vehicle group, I/R plus 10% ethanol (0.1 ml/kg/day) administered by intra-peritoneal injection from day -1 through to 7; 3) a resveratrol group, I/R plus 4 mg/kg/day of resveratrol; and 4) a sham group. Blood samples were obtained via the tail vein at 1 day before, and 1, 3, and 7 days after the operation (day 0) for the measurement of serum creatinine (Scr) levels. The expression of CD86 protein was analyzed by immunofluorescence staining, and the level of CD86 messenger RNA (mRNA) was evaluated quantitatively by a real-time reverse transcription-polymerase chain reaction (RT-PCR) in the renal cortex at day 3. Scr levels of the resveratrol group were significantly lower than those of the

I/R and vehicle groups on days 1 and 3 after the operation. From the immunohistochemical study, the expression of CD86 in the glomerular endothelium and peritubular vessels was found to be attenuated in the resveratrol group compared with the I/R or vehicle group. In the resveratrol group, the CD86 mRNA level was significantly lower than that in the I/R or vehicle group, and it was significantly decreased by about one fifth of that in the sham group. Our results suggest that resveratrol markedly reduces renal dysfunction and attenuates the mRNA and protein expression of CD86 following I/R injury.

Introduction

An ischemia/reperfusion (I/R) injury, a main etiologic factor for the acute failure of native and transplanted organs, has microvascular endothelial damage as one of its main causes (Carden *et al.*, 2000). The oxidative stress due to I/R injury in vascular endothelial cells produces oxygen-derived free radicals, with subsequent lipid peroxidation leading to the production and release of inflammatory mediators. The oxidative stress also enhances the biosynthesis of adhesion molecules that mediate leukocyte-endothelial cell adhesion (Parvums *et al.*, 1999).

The costimulatory pathways, such as B7-CD28-CD152 and CD40-CD154, are essential to the proliferation of T cells (Sayegh *et al.*, 1998). The costimulatory signals are provided by cell surface adhesion molecules after binding to their ligands on antigen-presenting cells (APCs). CD28 and CD152 are potent mediators for the costimulatory signal on T cells (Linsley *et al.*, 1991), and CD80 (B7-1) and CD86 (B7-2) have been respectively identified as the

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ligands for CD28 and CD152 (Azuma *et al.*, 1993; Freeman *et al.*, 1993), on APCs. A costimulatory blockade has been proposed to include allograft tolerance. While a blockade of CD80-CD152 accelerates rejection, a CD86-CD28 blockade significantly prolongs graft survival (Haanstra *et al.*, 2003). Therefore, the enhanced expression of CD86 may play an important role in the occurrence of acute rejection.

We recently showed that ICAM-1, CD80, and CD86 proteins are localized on hepatic sinusoidal endothelial cells in the liver and glomerular endothelial cells as well as the peritubular vascular endothelium in the kidney, and that these proteins and their messenger RNAs (mRNAs) are up-regulated after I/R (Kojima *et al.*, 2001; Satoh *et al.*, 2002) and further enhanced by renal dysfunction (Satoh *et al.*, 2002). Our results suggest that the hepatic sinusoidal and glomerular endothelial cells play a pivotal role as APCs by expressing CD80 and CD86 in the induction of a tissue injury associated with I/R injury (Kojima *et al.*, 2001; Satoh *et al.*, 2002).

Interest in the study of phenolic compounds contained in red wine has grown since epidemiological research indicated an inverse correlation between red wine consumption and the incidence of cardiovascular disease (Nanji *et al.*, 1986). Resveratrol, a member of the viniferin family of polymers, was identified as a biologically active compound in red wine in 1992 (Siemann *et al.*, 1992). Numerous studies have assessed the abilities of resveratrol, especially as a free radical scavenger, to prevent multiple pathophysiological processes. Resveratrol has the ability to inhibit the peroxidation of lipid membranes (Fauconneau *et al.*, 1997), to decrease the concentration of low- and very-low-density lipoproteins (Frankel *et al.*, 1993), and to inhibit platelet aggregation (Kimura *et al.*, 1985). Resveratrol also has anti-inflammation (Kimura *et al.*, 1985; Marier *et al.*, 2002) and cancer chemoprotective properties and induces apoptosis in leukemia and human breast carcinoma (Jang *et al.*, 1997; Mgbonyebi *et al.*, 1998; Lu R *et al.*, 1999). Based on these findings, we decided to investigate whether resveratrol has a protective role in renal function and to attenuate the expression of costimulatory adhesion molecules, especially CD86, after I/R injury in rat kidneys.

In this study, we examined the effects of the phenolic compound, resveratrol, on the renal function and expression of CD86 protein and mRNA in rat kidneys with I/R injury.

Materials and Methods

Animal model

Wistar male rats with a mean weight of 249 g (range, 223–

269 g) were purchased from Japan SLC Co. (Sendai) and maintained with free access to conventional food and water in an air-conditioned room (23°C). The rats were divided into four groups: 1) an I/R group with right nephrectomy and 1-hour clamping of the left renal pedicle; 2) a vehicle group, I/R plus 10% (v/v) ethanol (0.1 ml/kg body weight) administered by intra-peritoneal injection once a day from 1 day before through to 7 days after the operation; 3) a resveratrol group, I/R plus 4 mg/kg of resveratrol (Sigma, Saint Louis, MO, USA) in 10% (v/v) ethanol (0.1 ml/kg body weight) administered by intra-peritoneal injection once a day from 1 day before through to 7 days after the operation; and 4) a sham group with right nephrectomy and exposure of the left kidney and renal pedicle. The animals were anesthetized with an intra-peritoneal injection of pentobarbital (50 mg/kg body weight), and then both kidneys were exposed via incisions in the right and left flanks. In the I/R, vehicle, and resveratrol groups, the left renal pedicle was occluded with a microvascular clip for 1 hour after right nephrectomy and then the left kidney was reperfused. The incisions were closed after these procedures. To investigate renal function, blood samples were obtained *via* a tail vein at 1 day before, and 1, 3, and 7 days after the operation (6 rats per group). To investigate the expression of CD86 protein and mRNA, the left kidney and spleen were obtained via an abdominal midline incision under anesthesia on day 3 after the operation in other rats (3 rats per group). The protocol for the present study was approved by the Animal Research Committee, Akita University School of Medicine.

Serum creatinine determination

A 0.5 ml blood sample obtained from the tail vein of each rat was centrifuged at 3000 rpm for 5 min, and the concentration of serum creatinine (Scr) was determined using an Olympus AU800 autoanalyzer (Olympus, Tokyo).

Immunofluorescence staining

To avoid the intravascular blood cell reaction and to fix the renal tissues, the descending aorta was occluded proximally to the left renal artery and first perfused with 5 ml of phosphate-buffered saline (PBS, 10 mM sodium phosphate, pH 7.4, containing 0.15 M NaCl and 40 IU/ml heparin) *via* a silicon tube (0.3 mm diameter) catheterized from the distal aorta, and then with 5 ml of Zamboni fixative [4% (w/v) paraformaldehyde containing 0.2% (w/v) picric acid in a phosphate-buffer (PB, 100 mM sodium phosphate, pH 7.4)]. The left kidney was removed, further fixed in Zamboni solution for 12 h, and cryoprotected in 15% (w/v) sucrose in PB for 12 h and then in 30% (w/v) sucrose in PB for 24 h.

Tissue specimens frozen on dry ice were cryosectioned at a 5 micrometer thickness, and the sections were mounted on 3-aminopropyltriethoxysilane-coated glass slides, air-dried for 1 h at room temperature, washed in PBS, and postfixed in 4% paraformaldehyde in PB. After washing in PBS, the sections were incubated in PBS containing 1% (w/v) bovine serum albumin and 0.1% (w/v) Triton X-100 (Sigma) (B-PBS-T) for 30 min, reacted with a primary antibody solution (1:100 dilution in B-PBS-T) overnight at 4°C, and incubated in B-PBS-T containing a secondary antibody (1:200 dilution in PBS-T) for 1 h at room temperature. The tissue sections were stained with an Alexa Fluor 488-labeled tertiary antibody (1:200 dilution in B-PBS-T) in a dark room for 1 h at room temperature. Antibodies used in this study were as follows: rabbit polyclonal anti-rat CD86 antibody (Santa Cruz Biotechnology, Santa Cruz, UA, USA), mouse anti-rabbit IgG antibody (Jackson Immuno Research Laboratories, West Grove, PA, USA),

and Alexa Fluor 488-labeled donkey anti-mouse IgG antibody (Molecular Probes, Eugene, OR, USA). Nuclei were stained with 100 nM SYTOX-Orange (Molecular Probes) in PBS. Fluorescence signals were analyzed using a confocal laser-scanning microscope LSM-510 and LSM system soft version 3.2 (Carl Zeiss, Jena, Germany).

RNA isolation and CD86 mRNA analysis by RT-PCR

Total RNA was isolated using TRIZOL Reagent (Life Technology Inc., Grand Island, NY, USA) from the frozen kidney specimens (80–120 mg), and then poly(A)⁺ RNA was isolated using a MACS mRNA Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The first-strand complementary DNA (cDNA) was generated by using reverse transcription using a ReverAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) and poly(A)⁺ RNA preparations as templates. The

Fig. 1. Changes in serum creatinine (Scr) levels. Scr levels until 7 days after the operation in the Ischemia/Reperfusion (I/R) group, right nephrectomy and 1-hour clamping of the left renal pedicle; the vehicle group, I/R plus 10% ethanol by intra-peritoneal injection; the resveratrol group, I/R plus 4 mg/kg resveratrol in 10% ethanol by intra-peritoneal injection; and the sham group, right nephrectomy and exposure of the left kidney and renal pedicle. Scr levels in the I/R, vehicle, and resveratrol groups peaked at day 1 after the operation and decreased gradually, but these levels were significantly higher than those in the sham group until day 7 after the operation. The Scr levels in the resveratrol group were significantly lower than those in the I/R and vehicle groups at days 1 and 3.

first-strand cDNA was amplified by real-time PCR using a LightCycler (Roche Diagnostics, Mannheim, Germany) with the specific upstream and downstream primers for CD86 mRNA analysis under the following reaction conditions: denaturation at 95°C for 2 min, then 30 cycles of denaturation at 95°C for 0 sec, annealing at 55°C for 5 sec, and extension at 72°C for 15 sec. The sequences of the upstream and downstream primers for CD86 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as follows: 5'-GCTCGTAGTATTTTGGCAGGACC-3' and 5'-CGGGTATCCTTGCTTAGATGAGC-3' for rat CD86; and 5'-TCCCACCACCCTGTTGCTGTA-3' and 5'-ACCACAGTCCATGCCATCAC-3' for rat GAPDH. The product sizes for CD86 and GAPDH were 337 base pairs and 455 base pairs, respectively. The amplified products were subjected to a melting curve analysis and agarose gel electrophoresis using a 1.5% gel followed by staining with ethidium bromide and/or SYBR Green I (FMC Bio Products, Rockland, ME, USA).

Statistical analysis

Results are expressed as the means $\pm$ standard deviation (SD). The Fisher least significant difference method of Student's *t*-test was used to assess the difference in Scr levels as well as CD86 mRNA levels normalized with the GAPDH mRNA level in each poly(A)⁺ RNA preparation between each group. When necessary, the *t*-test was modified to all for unequal variances. A *p*-value of less than 0.05 was considered to be statistically significant.

Results

Serum creatinine levels

Scr levels in each group for up to 7 days after the operation are shown in Figure 1. In the sham group, Scr levels did not change remarkably. Scr levels were significantly higher in the I/R, vehicle, and resveratrol groups than in the sham group until day 7 after the operation. Scr levels were significantly lower in the resveratrol group than the I/R and vehicle groups at days 1 and 3 after operation (day 1 after operation: 2.82 ± 0.98 , 1.92 ± 0.70 , 0.75 ± 0.32 , and 0.41 ± 0.43 mg/dl; and day 3: 1.71 ± 0.96 , 0.95 ± 0.34 , 0.50 ± 0.05 , and 0.41 ± 0.03 mg/dl in the I/R, vehicle, resveratrol, and sham groups, respectively). Scr levels were lower in the vehicle group than I/R group at days 1 and 3, but not significantly. These results suggested that resveratrol reduced renal dysfunction after I/R injury.

Localization and intensity of CD86 protein

Since it has been reported that the CD86 protein is expressed in B cells in spleen tissue as well as in other APCs (Maeda *et al.*, 1997), rat spleen tissue was used as a positive control to verify anti-CD86 antibodies for immunofluorescence staining. As expected, CD86-positive cells, probably APCs, were consistently detected in the marginal sinus of these tissues (data not presented). The localization of CD86 in the renal tissue was evaluated by indirect immunofluorescence staining using specific polyclonal antibodies against CD86 on day 3 after the operation. The protein was expressed in the glomerular and peritubular vascular endothelium after I/R as previously reported (Sato *et al.*, 2002). In the glomerular endothelium, CD86 protein was detected in the sham group, but the intensity was lower than that in the I/R and vehicle groups (Fig. 2, a, b, d). In the resveratrol group, the expression was weaker compared to that in the I/R and vehicle groups (Fig. 2, a, b, c). From these results, resveratrol attenuated the expression of CD86 proteins in the glomerular endothelium after I/R. In fact, the localization of CD86 in the glomerular endothelial cells had been confirmed by counterstaining with von Willebrand factor, a marker protein of the endothelial cells, in our previous study (Sato *et al.*, 2002). The fluorescence signals of the CD86 protein were also detected in the peritubular microvessels, and the intensity was higher in the I/R and vehicle groups compared with the resveratrol group (data not shown).

RT-PCR analysis of CD86 mRNA

To evaluate the quantitative mRNA levels by real-time RT-PCR, total RNA and then poly(A)⁺ RNA were isolated from the renal cortex on day 3 after nephrectomy-warm I/R. The first-strand cDNA was generated against the poly(A)⁺ RNA preparation and used for PCR amplification for CD86 mRNA as well as GAPDH mRNA as an internal control by using a LightCycler (Roche). The PCR products for CD86 and GAPDH were characterized by melting-curve analysis and analyzed by agarose gel electrophoresis (Fig. 3 and Table 1). The CD86 mRNA level was normalized using the GAPDH mRNA level in each poly(A)⁺ preparation, and the quantitative data on alterations of CD86 mRNA levels in each group are summarized in Table 1. CD86 mRNA levels were significantly higher in the I/R and vehicle groups than the sham group, 4 times higher for the former and 12 times for the latter. The CD86 mRNA level was lowest in the resveratrol group, which was about one fourth of that in the sham group. These results also indicated that resveratrol markedly attenuated the expression of CD86 mRNA after I/R injury.

Fig. 2. Immunofluorescence staining of CD86 proteins (green) in the renal cortex of the ischemia/reperfusion (I/R) group (a), the vehicle group (b), the resveratrol group (c), and the sham group (d) on day 3 after the operation, respectively. CD86 proteins were mainly located on the glomerular endothelium. In the sham and resveratrol groups, the expression of CD86 protein in the glomerular endothelium was weak as compared with those in the I/R or vehicle groups. Scale bar: 50 μ m

Fig. 3. Agarose gel electrophoresis of RT-PCR products. CD86 mRNA (a), as well as GAPDH mRNA (b) as an internal control, in the renal cortex from each group at day 3 after surgery was analyzed by real-time RT-PCR. The PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide. Lane M: DNA size marker, lanes 1 and 2: Ischemia/reperfusion (I/R) group, lanes 3 and 4: vehicle group, lanes 5 and 6: resveratrol group, lanes 7 and 8: sham group. The arrows indicate the size of PCR products: 337 base pairs for CD86 (a), and 455 base pairs for GAPDH (b). The expression of CD86 mRNA was higher in the I/R group (lane 1, 2) and the vehicle group (lane 3, 4) than in the resveratrol group (lane 5, 6) and the Sham group (lane 7, 8). The quantitative data are shown in Table 1.

Table 1. CD86 mRNA levels analyzed by real-time RT-PCR in the renal cortex at day 3 after the operation.

	CD86 (mols $\times 10^{-6}$)
	Mean $\pm$ SD
Ischemia/Reperfusion (n=3)	47.8 $\pm$ 24.2 ^{ab}
Vehicle (n=3)	130.8 $\pm$ 100.5 ^{ab}
Resveratrol (n=3)	2.8 $\pm$ 0.4
Sham (n=3)	11.2 $\pm$ 5.1 ^b

All data indicated were normalized with the GAPDH mRNA level in each sample.

^a $p < 0.05$, compared with the sham group

^b $p < 0.05$, compared with the resveratrol group

Discussion

I/R injury, an inevitable consequence of transplantation, is an important clinical problem leading to endothelial cell dysfunction. Endothelial cell dysfunction of the graft during transplantation is an early event that is critical in I/R injury and probably plays a key role in graft dysfunction after transplantation. Although new immunosuppressive protocols have reduced the number of acute rejections, the incidence of acute renal failure has remained unchanged. Schwarz and Oberbauer (2003) reported that donor factors contribute to approximately 40% of the variability in early allograft function and that adhesion molecules are risk factors at the donor site. Several studies have suggested that the vascular endothelial cells function as resident APCs by presenting not only human leukocyte antigen but also costimulatory adhesion molecules, B7 and CD40/CD40L, the expressions of which were up-regulated both during acute rejection and after I/R injury (Takada *et al.*, 1997; Chandraker *et al.*, 1997; Bartlett *et al.*, 2003). I/R injury may enhance allograft antigenicity in organ transplantation without an antigen-antibody reaction. Consequently, the blockade of T cell costimulatory pathways will be an important strategy for organ failure against organ failure in both acute rejection and I/R injury (Kamoun *et al.*, 2001).

The present study had two purposes. The first was to estimate the protective effect of resveratrol on renal function from I/R. Giovannini *et al.* (2001) reported that resveratrol reduced both the mortality of renal ischemic rats from 50 to 10 % and Scr levels short-term. They also studied the renoprotective effects of resveratrol in rats with 40 min renal ischemia following 24 h after reperfusion (Bertelli *et al.*, 2002). In our previous studies, Scr levels in rats with right-nephrectomy and 1-h left renal I/R were significantly elevated on days 1 and 3 and returned to baseline levels on day 14 after the operation (Sato *et al.*, 2002). Therefore, to investigate the effects of resveratrol on renal function, we administered it to rats at 1 day before to 7 days after reperfusion following right nephrectomy and 1-h clamping of the left renal pedicle, and measured the Scr levels. In the present study, no rats died from a right nephrectomy and 1-h ischemia of the left kidney. The present study is the first report of a consecutive administration of resveratrol and measurement of Scr levels for up to 7 days after I/R injury. The results suggest that the consecutive administration of resveratrol has renoprotective effects in rats with I/R injury by significantly reducing Scr levels. Although the Scr level of rats with the vehicle (10% ethanol) was lower than that of I/R rats, there was no significant difference.

In previous studies, resveratrol was freshly prepared in 50% ethanol (Gupta *et al.*, 2002; Sinha *et al.*, 2002),

and when administered at doses of 20 and 40 mg/kg to rats ranging in weight from 200 to 250 g, the total volume injected intraperitoneally ranged up to the limit of 0.1 ml/kg body weight. Based on these studies, resveratrol was prepared in 50% ethanol and administered at doses of 4, 8, 20, and 40 mg/kg in our preliminary experiment. In the experiment to decide the dose, the mortality was over 50% in rats with 50% ethanol, and 20 and 40 mg/kg of resveratrol in the 50% ethanol groups (data not shown). In regard to the ethanol concentration, ethanol has either an oxidative or anti-oxidative effect, depending on the plasma level (Sacanella *et al.*, 2003). To avoid the influence of alcohol bioactivity, we employed 4 mg/kg of resveratrol prepared in 10% ethanol, the lowest dissolvable concentration of ethanol for resveratrol.

The second purpose in the present study was to evaluate the influence of resveratrol on the expression of costimulatory adhesion molecules by I/R. De Greef *et al.* (2001) found CD80 expression along the endothelial cells of the ascending vasa recta in the kidney soon after I/R injury. We have shown that the expression of CD80, CD86, and ICAM-1 mRNA and proteins increased in the glomerular endothelial cells after I/R injury (peaking at day 3) (Satoh *et al.*, 2002). The biological reactions to the I/R injury of CD80, CD86, and ICAM-1 mRNA and proteins were the similar (Kojima *et al.*, 2001; Satoh *et al.*, 2002). Chandraker *et al.* (1997) reported that CD86 rather than CD80 is critical in the development of organ dysfunction following ischemic injury. Therefore, we investigated the location of the CD86 protein in the renal cortex after I/R injury by immunofluorescence staining, and CD86 mRNA levels on day 3 after I/R by a real-time reverse transcription polymerase chain reaction (RT-PCR).

The localization of CD80, CD86, and ICAM-1 proteins in the glomerular endothelial cells was confirmed by counterstaining with von Willebrand factor in our previous study (Satoh *et al.*, 2002). In it, we reconfirmed that CD86 proteins are mainly expressed on the glomerular endothelial cells and further enhanced by I/R injury. Resveratrol attenuated the CD86 protein expression in rat glomerulus and peritubular vessels with I/R injury. Interestingly, CD86 mRNA levels in rats treated with resveratrol were significantly reduced by about one fourth compared with those in sham-operated rats. Several anti-oxidant drugs have been reported to reduce renal dysfunction and injury associated with I/R of the kidney, perhaps by modulation of both free radical damage and adhesion molecules (Seth *et al.*, 2000; Sivarajah *et al.*, 2003). The reduction in expression of adhesion molecules including CD86 by resveratrol may have helped to prevent renal dysfunction in the present study. On the other hand, the vehicle (10% ethanol) had no effect on the reduction of CD86 mRNA

expression in the present study. Although there was no significant difference, CD86 mRNA levels in rats treated with 10% ethanol were rather high compared with those of control rats. Sacanella and Estruch (2003) mentioned that both ethanol and non-alcoholic components of alcoholic beverages, mainly polyphenols, reduce ICAM-1, vascular cell adhesion molecule-1, and E-selectin expression in the vascular endothelium as well as monocyte adhesion to this endothelium. Moderate alcohol intake has an anti-inflammatory effect on the cardiovascular system and reduces early serum markers of atherosclerosis. However, at higher doses, ethanol may exert an inflammatory effect. The 0.1 ml/kg of 10% ethanol may not influence the expression of adhesion molecules.

In a study of rats with rhabdomyolysis induced by glycerol injection leading to acute renal failure, chronic red wine exposure prior to the injection provided functional and biochemical protection. A less marked degree of functional and biochemical protection was also observed in response to the administration of alcohol-free red wine and ethanol. Rodrigo *et al.* (2004) suggested that red wine protects against functional, biochemical, and morphological damage caused by rhabdomyolysis in the rat, and this protection may be due to the synergistic effects of ethanol and non-alcoholic red wine components. In the present study, resveratrol dissolved in 10% ethanol protected renal function and attenuated CD86 protein expression and mRNA in rat kidneys with I/R injury. Since ethanol had no significant protective effects for renal function and CD86 expression after I/R injury, there were no synergic effects of 10% ethanol and resveratrol in our study.

Several abilities of resveratrol including those as free radical scavenger, and in anti-coagulation and anti-inflammation may contribute toward the renoprotective effect and attenuation of the costimulatory adhesion molecule CD86.

The present study demonstrated that resveratrol dissolved in ethanol markedly reduced renal dysfunction and attenuated the expression of costimulatory adhesion molecule (CD86) mRNA and protein after I/R injury. Resveratrol may be useful for kidney preservation and transplantation because of its synergistic effects of protecting the renal function and diminishing antigenicity via the attenuation of costimulatory adhesion molecule expression.

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