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Rescue therapy for acute rejection using 15-deoxyspergualin (DSG) in combination with superoxide dismutase (SOD) on cardiac allografts in rats*

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Abstract. This study was performed to investigate the effect of combination therapy using 15-deoxyspergualin (DSG) plus recombinant human superoxide dismutase (h-SOD) on acute graft rejection in heterotopic rat heart transplantation. DSG was intraperitoneally injected for 10 days at a dose of 5 mg/kg per day, and h-SOD (15,000 or 30,000 μm/kg per day) was continuously administered via external iliac vein for approximately 8 days with a mini-osmotic pump (Alzet model 2001). Administration of the drugs was started on the 4th day after grafting. The grafts treated with h-SOD alone survived slightly longer than the control allografts. The graft survival time was significantly prolonged in the groups treated with DSG alone or DSG plus h-SOD. A higher percentage of induction of immunological unresponsiveness was achieved in the group treated with DSG plus h-SOD at 30,000 μm/kg per day. The ratios of inorganic phosphate (Pi)/phosphocreatine (PCr) and PCr/ATP on the ^{31}P nuclear magnetic resonance spectrogram are useful parameters for assessing the graft injury associated with acute rejection. The ratio of Pi/PCr and that of PCr/ATP were found to increase and decrease, respectively, in proportion to the progress in rejection. In the animals treated with DSG alone, the Pi/PCr ratio was significantly increased from the 4th day, and PCr/ATP ratio decreased from 10th day after grafting. These parameters were not improved during the observation period. However, these parameters were significantly recovered in the animals treated with DSG and h-SOD in combination. Improvement of the parameters seemed to be related to SOD dosage.

These results clearly demonstrated that the oxygen free radical plays a toxic role in cardiac allografts with ongoing rejection and, therefore, the administration of h-SOD in combination with DSG can minimize the graft injury.

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Rejection response involves infiltration of inflammatory cells, i.e., lymphocytes, monocytes, and polymorphonuclear neutrophils (PMNs), at the graft site. There is much evidence that activated monocytes and PMNs can generate the oxygen free radical [10, 18, 26], which is toxic to biomolecules of the grafted organ. In addition, impairment of coronary flow and myocytic necrosis developed during acute rejection in non-immunosuppressed animals [6, 7, 27], although the coronary flow was not observed to decrease in the presence of mild or moderate rejection, which had occurred after a 4-day cessation of immunosuppression [5]. The disturbance of myocardial circulation and myocytic necrosis due to advanced rejection could contribute to myocardial acidosis. The acidosis in muscles may generate the oxygen free radicals through the cyclo-oxygenase pathway of arachidonic acid metabolism [25]. Therefore, a treatment for free radical in combination with direct inhibition of activated immunological cells will be potentially beneficial for the therapy of acute rejection. Kloc et al. [17B] have described a profound decrease in the activity of superoxide dismutase (SOD) in the heterotopically transplanted rat hearts on day 5 day after grafting, while glutathione peroxidase and catalase activities were little affected in the transplanted heart. This indicates that SOD is one of the primary tissue defence enzymes against graft rejection.

Thus, the following study was designed to verify the effect of combination therapy using a new immunosuppressant, 15-deoxyspergualin (DSG), with SOD on acute graft rejection in heterotopic rat heart transplantation. The bioenergetic status of the grafts after transplantation was serially evaluated by ^{31}P magnetic resonance imaging (MRI) spectroscopy.

Materials and methods

Animals

Inbred male ACI rats (RT-1^{av1}) and male WKAH rats (RT-1^{k}), weighing 200–250 g, were used as recipients and

donors, respectively, for the heterotopic heart transplantation.

Drugs

15-Deoxyspergualin (DSG) and recombinant human cuprozinc SOD (h-SOD), both provided in powder forms by Nippon Kayaku Co. (Tokyo), were dissolved in physiological saline to therapeutic concentrations. DSG was daily injected i. p. to the recipients at a dose of 5 mg/kg for 10 days from the 4th day after grafting. h-SOD was continuously administered into the inferior vena cava of the recipients at a daily dose of 15,000 or 30,000 μm/kg with a mini-osmotic pump for approximately 8 days from the 4th day on after grafting.

Implantation of the mini-osmotic pump

A mini-osmotic pump (Alzet 2001, 200 μl of reservoir) with a polyethylene infusion tube (0.58 mm inside diameter and 90 mm long) was used for continuous administration of h-SOD. The reservoir and infusion tube of the pump were filled with h-SOD at experimental concentrations. The left iliac portion of the recipient was incised to expose the external iliac vein, and the infusion tube was inserted through this vein into the inferior vena cava. The pump was then subcutaneously implanted on the abdominal wall.

Heterotopic heart transplantation

The heterotopic, auxiliary cardiac grafts were placed in the neck of recipient rats by a modification of the method of Miller et al. [20], as previously reported [28, 31, 32]. Briefly, the pulmonary artery of the donor heart was anastomosed to the right external jugular vein of the recipient in a continuous end-to-side manner with 9-0 monofilament nylon suture, and the donor brachiocephalic artery was anastomosed to the recipient left common carotid artery in an end-to-end manner with continous 10-0 nylon suture. Finally, the aorta was flushed with saline and ligated distally with 3-0 silk suture. The day of grafting was regarded as day 0, and cardiac pulsation was assessed by daily palpation. The day of cardiac arrest was defined as the last day of graft survival. A graft that survived longer than 100 days was regarded as an indefinite survivor (recipients acquired immunological unresponsiveness).

^{31}P MRI spectroscopy

The ^{31}P MRI technique was applied to investigate in vivo energy metabolism of the graft in the period after transplantation. Details of the ^{31}P MRI method have been reported previously [20, 30, 31]. In brief, anesthetized recipient rats were fixed on a probe, and a four-turn, 13-mm-diameter surface coil was placed over the cervical allograft in direct contact with the skin. The recipient was then placed in a 6.34-Tesla, 89-mm vertical bore superconducting magnet (Oxford-270/89) operating with a JNM-SMR 270 spectrometer. The coil was tuned to 270 mHz for ^{1}H and 109 mHz for ^{31}P. The magnetic field was scanned using the ^{1}H water signal from the tissue. ^{31}P MRT spectra were obtained with a 12-μs pulse width and 500 scans were made at 1.9-s intervals.

Experimental groups

The study was divided into five experiments: group 1, control receiving no drug; group 2, treated with h-SOD alone at a dose of 30,000 μm/kg per day; group 3, treated with DSG alone; group 4, treated with DSG plus h-SOD (15,000 μm/kg per day); group 5, treated with DSG plus _h-SOD (30,000 μm/kg per day).

Concentration of h-SOD

Separately from the above-mentioned experimental groups, five normal ACI rats were continuously injected with h-SOD at a dose of 30,000 μm/kg/day with a mini-osmotic pump. These animals were bled from the jugular vein on the day before h-SOD administration, and on days 2, 4 and 7 after administration. Plasma concentrations of h-SOD were determined by enzyme immunoassay using monoclonal antibody against h-SOD in accordance with the method of Adachi et al. [1].

Statistical analysis

The data obtained from each of the groups were expressed as mean ± SD. Intergroup differences in graft survival time were analyzed by generalized Wilcoxon's test and intergroup differences in ratios calculated from each phosphate areas on ^{31}P MRI spectra by the unpaired *t*-test.

Results

Graft survival (Table 1)

In the control allograft group (group 1), the graft survived for 9.2 ± 2.2 days (n = 10). The grafts in group 2 (n = 10) survived slightly but significantly $P < 0.01$) longer (12.0 ± 0.7 days) than in group 1. Compared with groups 1 and 2, graft survival time was significantly ($P < 0.01$) prolonged in group 3 (n = 10), group 4 (n = 10) and group 5 (n = 10). Indefinite graft survival was noted in two recipients in group 3, one in group 4 and four in group 5.

^{31}P MRI spectroscopy

As previously reported [20, 30, 31], the inorganic phosphate (Pi)/phosphocreatine (PCr) and PCr/ATP ratios on the ^{31}P MRI spectrogram were calculated from the peak areas. Only the peak of beta-phosphate of ATP was read as the ATP level since this peak contains only the signal from ATP. The mean values of Pi/PCr and PCr/ATP ratios were determined by 28 measurements using ^{31}P MRI spectroscopy in syngeneic grafts in the postoperative peri-

Table 1. Graft survival days in each experimental groups

Groups	DSG[a] (mg/kg)	h-SOD[b] (μm/kg)	Survival days	Significance	
				vs group 1	vs group 2
1 (n = 10)	0	0	9.2 ± 2.2		
2 (n = 10)	0	30,000	12.0 ± 0.7	$P < 0.01$	
3 (n = 10)	5	0	8, 10, 21, 21, 22, 23, 25, 26, > 100, > 100	$P < 0.01$	$P < 0.01$
4 (n = 10)	5	15,000	9, 16, 20, 20, 25, 25, 29, 30, 38, > 100	$P < 0.01$	$P < 0.01$
5 (n = 10)	5	30,000	7, 18, 20, 26, 26, 30, > 100, > 100, > 100, > 100	$P < 0.01$	$P < 0.01$

[a] DSG was intraperitoneally administered for 10 days from the 4th day after grafting.

[b] h-SOD was continuously administered for approximately 8 days, using the mini-osmotic pump (Alzey 2001), from the 4th day on after grafting

od from days 3–30 they were previously reported [30] to be 0.38 ± 0.11 and 1.88 ± 0.42, respectively. In all of the present groups, the Pi/PCr and PCr/ATP ratios on day 3 were not significantly different from those in the syngeneic grafts.

As shown in Fig. 1, an increase in the Pi/PCr ratio and a decrease in the PCr/ATP ratio were observed as time elapsed after grafting in both groups 1 and 2. The increase in the Pi/PCr ratio was observed earlier than the decrease in the PCr/ATP ratio. There were no significant daily differences in these ratios between groups.

In groups 3, the Pi/PCr ratio rapidly increased from the 4th day and PCr/ATP ratio gradually decreased from the 10th day after grafting. Although there were no significant daily differences in the ratios between groups 3 and 4, these ratios tended to recover on day 17 in group 4 (Fig. 2). Furthermore, compared with group 3, Pi/PCr and PCr/ATP ratios in group 5 were significantly recovered from the 12th day and 14th day on, respectively (Fig. 2).

The Pi/PCr and PCr/ATP ratios from the grafts that survived for less than 19 days in groups 3–5 were excluded from the present analysis.

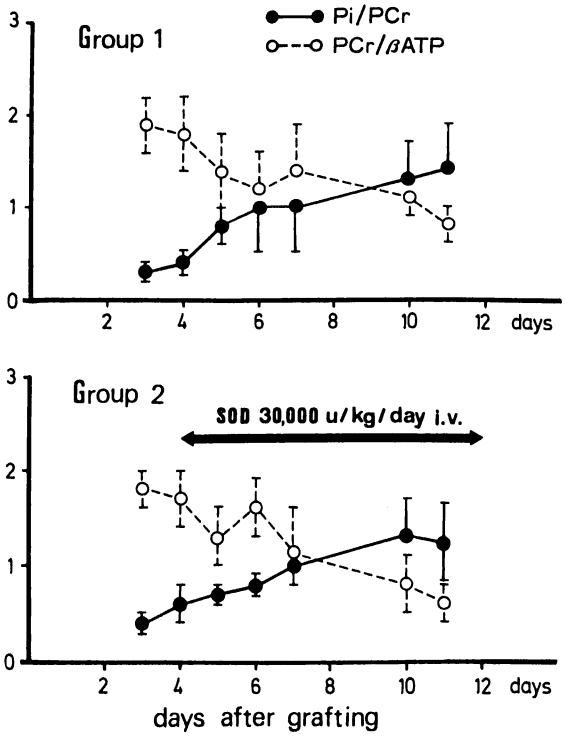

Fig. 1. Pi/PCr and PCr/ATP ratios in the control group (group 1) and in treated with h-SOD alone (group 2). h-SOD was continuously injected at a dose of 30,000 μm/kg per day via the external iliac vein with a mini-osmotic pump (Alzet model 2001) from the 4th day after grafting. In groups 1 and 2, Pi/PCr ratio increased and PCr/ATP ratio decreased in proportion to the progress in rejection. The increase in Pi/PCr ratio preceded the decrease in PCr/ATP ratio. There were no significant daily differences in these ratios between the two groups

Concentrations of h-SOD in plasma

Figure 3 demonstrates the changes in the plasma concentration of h-SOD in rats continuously administered the enzyme with the mini-osmotic pump. It was clear that a constant plasma level of h-SOD was maintained by this administration method.

Discussion

Oxygen free radicals such as superoxide anion and its derivertives, hydrogen peroxide and hydroxyl radicals, mediate tissue injury during inflammation [9, 19, 24] and during reoxygeneration following myocardial ischemia [4, 8]. Abundant radicals may be generated at the graft site with ongoing rejection by activated macrophages and PMNs, as well as in the course of arachidonic acid metabolism. There is a defense system of intracellular enzymes, radical scavengers, to protect the tissue against oxygen toxicity [14]. However, it is postulated by Kloc et al. [17] that SOD activity may be decreased in rejecting allograft due to destruction of SOD in situ and/or to a decrease in SOD synthesis. Therefore, the graft with acute rejection must be attacked by both activated lymphocytes and the oxygen free radical.

The remarkable immunosuppressive effect of DSG has been confirmed in various organ transplantations using the mouse, rat, and dog [2, 11, 20, 21, 31, 35]. Furthermore, an indefinite graft survival was induced by short-term administration of DSG in allogeneic kidney [35], liver [12, 33], and heart [20, 31] transplantation in rats.

The immunological mechanism of action of DSG has not been well clarified. The production of IL-1 and IL-2 from macrophages and T cells, respectively, was not inhibited in DSG-treated rats [22]. The initiation of DSG treatment at the onset of acute rejection in rat heart trans-

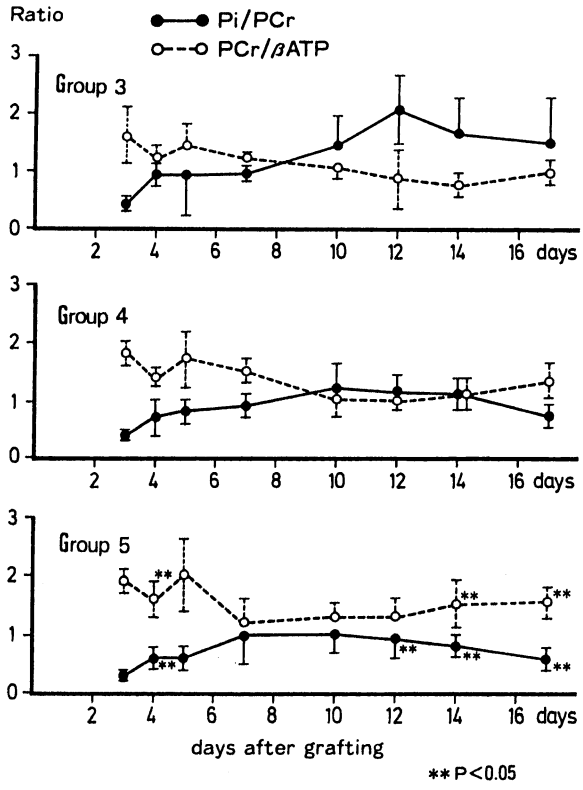

Fig. 2. Pi/PCr and PCr/ATP ratios in the allografts treated with DSG alone (group 3), DSG plus h-SOD at 15,000 μm/kg per day (group 4), and DSG plus h-SOD at 30,000 μm/kg per day (group 5). DSG was intraperitoneally injected for 10 days at a dose of 5 mg/kg per day from the 4th day. Continuous administration of h-SOD performed via the recipient external iliac vein with a mini-osmotic pump (Alzet 2001) from the 4th day after grafting. Statistical significance in group 3: Pi/PCr: $P < 0.01$, day 3 vs day 4, 5, 7, 10, 12, 14 and 17; PCr/ATP: NS, day 3 vs day 4, 5 and 7; $P < 0.05$, day 3 vs day 10 and 12; $P < 0.01$, day 3 vs day 14 and 17. Although there were no significant daily differences in the ratios between groups 3 and 4, these ratios tended to recover on the 17th day in group 4. Compared with group 3, the Pi/PCr and PCr/ATP ratios in group 5 were significantly recovered from the 12th day on and the 14th day, respectively. The significance of the daily ratios in groups 3–5 were: ** $P < 0.05$; others, NS

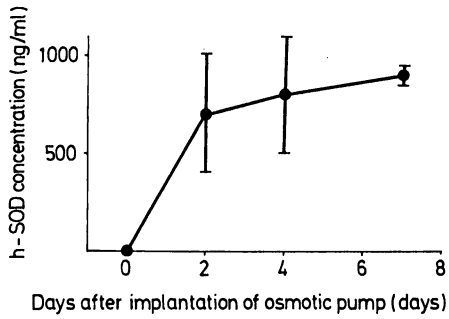

Fig. 3. Plasma concentration of h-SOD in rats continuously administered the enzyme with mini-osmotic pump. A constant plasma level of h-SOD was maintained in the h-SOD-infused rats

plantation induced higher degree of immunological unresponsiveness [31]. This indicates that the immunological mechanism of DSG action includes specific inhibition of expandes lymphocyte clones. Therefore, the drug may be used for the treatment of acute graft rejection. Indeed, DSG pulse therapy was highly effective on acute kidney rejection in canine transplantation [17A], as well as in clinical recipients [3, 29].

DSG has a significant rescue effect on acute rejection as mentioned above, although it seems to have no effect on the oxygen free radical. Therefore, combination therapy using DSG and SOD was considered to be highly effective for recovery from rejection. In the present study, the grafts treated with h-SOD alone survived slightly longer than the control allografts. In the groups treated with DSG alone or DSG plus h-SOD, the graft survival was significantly longer than the control allografts. Furthermore, a higher percentage of induction of immunological unresponsiveness was dose-dependently achieved with h-SOD in the DSG-treated groups.

The generation of oxygen free radical during acute rejection is different, in terms of duration, from that during reperfusion following myocardial ischemia; the former may last for several days, and the latter during the early phase of reperfusion [4]. Therefore, constant and long-lasting blood concentration of SOD should be necessary to protect the graft from free radicals during acute rejection. SOD is known to be rapidly cleared from the circulation. The circulatory half-life of this enzyme is less than 10 min [34]. Long-lasting derivatives of SOD have been prepared by coupling with polymers [15, 23, 24] or by entrapping with liposomes [16, 34]. In this study, we continuously administered native h-SOD, using the mini-osmotic pump, to maintain the constant circulating level of the enzyme. This method, in combination with DSG therapy, provided excellent protection from graft injury during acute rejection.

Previously, we reported [30] that Pi/PCr and PCr/ATP ratios on ^{31}P MRI spectrogram are useful parameters for assessing the metabolic dysfunction associated with acute graft rejection in heterotopically allografted hearts in rats. Fraser et al. [13] demonstrated that metabolic abnormality precedes functional or histological changes in allografts with ongoing rejection, and that an increase in the Pi/PCr ratio is seen earlier than a decrease in the PCr/ATP ratio. We also observed [20, 30, 31] that the onset of the increase in the Pi/PCr ratio indicates the early phase of graft rejection, and the PCr/ATP ratio decreases in proportion to the progress in rejection. The present study also demonstrated that the increase in the Pi/PCr ratio preceded the decrease of the PCr/ATP ratio. Although significant graft survival was obtained by the treatment with DSG alone, the highly increased Pi/PCr ratio from the 4th day and decreased PCr/ATP ratio from the 10th day, indicating severe graft injury, remained during the observation period. However, these parameters were significantly recovered in the animals treated with DSG and h-SOD in combination. Amelioration of the parameters seemed to be related to h-SOD dosage.

These results clearly demonstrated that the oxygen free radical plays a toxic role on cardiac allografts undergoing acute rejection and, therefore, the administration of h-SOD in combination with DSG minimized the tissue destruction. This is a novel and important finding that com-

bination therapy using DSG and h-SOD is highly valuable for a smooth recovery from acute graft rejection.

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