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The effect of cyclosporin on endothelin levels after orthotopic liver transplantation in rats

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Abstract To assess the effects of cyclosporin (CyA) on endothelin-1 (ET-1) in rat liver allograft rejection, we evaluated ET-1 expression in samples obtained from BN($RT1^n$)-to-BN (group 1) rats, DA($RT1^a$)-to-BN (group 2) rats, and DA-to-BN rats treated with 5 mg/kg per day of CyA (group 3). Serum and hepatic ET-1 levels, determined by a radioimmunoassay, remained unchanged in group 1. In group 2, the ET-1 levels peaked on postoperative day (POD) 5 in the liver at 344 ± 31.6 pg/g wet, and on POD 7 in the serum at 38.7 ± 13.1 pg/ml. In group 3, hepatic and renal ET-1 levels showed a progressive increase until POD 10, while serum ET-1 levels remained unchanged. In conclusion, acute rejection caused a temporary increase in the ET-1 level in both the serum and the liver in the early postoperative period what might have been caused by endothelial damage due to ongoing, acute rejection. CyA caused a time-dependent increase in the ET-1 level in both the liver and the kidney without an increase in the serum ET-1 level. The serum ET-1 level might have been affected by the clearance of ET from the liver or kidney.

Key words Endothelin, liver transplantation, rat · Cyclosporin, endothelin, rat liver transplantation · Liver transplantation, endothelin, rat

Introduction

Endothelin (ET) is a potent vasoconstrictor peptide of vascular endothelial cell origin [14]. Both a previous report from this laboratory (manuscript in press) and another study [15] showed that endothelin-1 (ET-1) was actively involved in the development of liver allograft rejection. However, the ET level was also reported to increase in response to cyclosporin (CyA) use, thus mediating both post-transplant renal dysfunction and hypertension in liver transplant recipients [7, 12]. These effects of CyA have not yet been confirmed, however, in experimental liver transplantation. Therefore, we decided to test the physiological role of CyA and ET, as suggested by clinical studies, in controlled, experimental studies in which the effects of both acute rejection and CyA could be evaluated at the same time.

In numerous reports, ET levels in circulating blood have been uniformly employed as tools for investigating the physiological role of ET [2, 5. 15]. However, ET has been observed to exert its physiological effect in a paracrine fashion in vivo [2, 3]. This would seem to suggest that ET levels in circulating blood do not represent the in vivo activities of ET. To investigate the in vivo role of ET in liver transplantation, it may be necessary to serially assess local ET levels in related organs in addition to serum levels. In the present experiment, we did just that. We assessed tissue ET-1 levels in related organs and in serum both quantitatively and serially using a rat orthotopic liver transplantation model.

Materials and methods

Animals

Adult male DA ($RT1^a$) and BN (RT1n) rats, 10–16 weeks of age, were purchased from Ryukyu-Biotec (Okinawa, Japan). All animals were housed in metabolic cages with controlled light/dark cycles, fed a standard laboratory diet, and given free access to water. The "Standards related to the care and management of experimental animals" (Notification No. 6, March 27, 1980, Prime Minister's Office, Tokyo, Japan) for the care and use of animals were carefully followed. The animals used in our studies were handled humanely, in accordance with animal experimental protocols approved by the Animal Care and Use Committee of the University of Ryukyu.

Operative procedure

Orthotopic rat liver transplantation was performed using previously described techniques [4] under sterilized conditions. Each recipient was isolated in a private cage and administered tobramycin, 1 mg/day subcutaneously, for 2 consecutive days post-transplantation.

Experimental groups

Adult male BN ($RT1^n$) rats served as recipients of donor liver grafts obtained either from adult BN ($RT1^{na}$) or DA ($RT1^a$) rats. Our study comprised the following three groups: BN-to-BN rats (group 1), DA-to-BN rats (group 2), and DA-to-BN rats treated with 5 mg/kg per day of CyA vor 10 days (group 3). In each group, nine animals were allowed to survive until death for the survival analysis. An autopsy was performed immediately after death to confirm the patency of the vascular anastomosis, as well as to rule out any biliary obstruction or infection.

Sampling

All specimens were obtained from the sacrificed animals. To obtain liver, kidney, and serum specimens, five rats were sacrificed from each group on postoperative days (POD) 1, 3, 5, 7, and 10 ($n = 5$ on each POD in groups 1–3). All specimens were either snap-frozen or fixed in 10 % formalin. The frozen samples were then stored in liquid nitrogen until use.

Histologic grading

The histologic grading of the severity of acute rejection was determined according to the criteria established by Snover et al. [10]. Using these criteria, the development of acute liver rejection was then classified according to the histology of the allograft. "Nondiagnostic" indicated mixed portal infiltration and less than 50 % bile duct damage, while "mild" included endothelialitis, "moderate" referred to mixed portal infiltration and greater than 50 % bile duct damage, either with or without endotheliitis, and "severe" meant a paucity of bile ducts, or central ballooning with confluence dropout of hepatocytes.

Blood chemistry

Liver function tests – SGOT and SGPT – and renal function tests – creatinine and blood urea nitrogen (BUN) – were then performed with an automated technique (Hitachi 7150 Auto-analyzer, Hitachi, Tokyo, Japan).

Radioimmunoassay for ET-1

The tissue samples were weighed and then heated in distilled water at 100 °C for 5 min. After they had cooled and acetic acid had been added, the tissue was homogenized and centrifuged at 15000 rpm at 4 °C for 40 min. The supernatant, serum, and urine samples were then analyzed using a highly sensitive radioimmunoassay (RIA) developed by Ando et al. [1]. Each 0.1-ml sample or standard was incubated with a 0.1-ml assay buffer and 0.1 ml of anti-hET serum (obtained from Sumitomo Bio-science). Incubation was carried out at 4 °C for 24 h, followed later by the addition of 0.05 ml ^{125}I-hET (Amersham International, England). The sample was further incubated at 4 °C for 48 h. After the addition of a secondary antibody, the samples were centrifuged at 3000 rpm at 40 °C for 30 min. The radioactivity in the precipitate was then counted by a spectrometer.

Routine histopathology of the liver

All liver specimens were fixed in 10 % formalin, embedded in paraffin, and stained with hematoxylin-eosin for routine histologic examination.

Statistical analysis

All data are expressed as the mean ± standard error (SE) of the mean value. Group comparisons were performed with the t-test. The values from groups 2 and 3 were always compared to the values from group 1. The differences were considered to be statistically significant at a P level below 0.05.

Results

Survival data and graft histology

Postoperative death related to technical failure occurred in less than 5 % of all recipients, and these recipients were excluded from the study analysis. Rats in the syngeneic control group (group 1) survived indefinitely, but those in the allogeneic combination group (group 2) and CyA-treated group (group 3) died within 12.8 ± 2.7 days and 35.3 ± 15.2 days posttransplantation, respectively, due to histologically proven rejection. In the allogeneic group (group 2), the liver histologically showed a rapid development of acute rejection, with mononuclear cell infiltration to the portal tract area as early as POD 3; all animals reached a "severe" rejection stage in liver histology [11] by POD 10. Endothelialitis was observed as early as POD 3 in our model, and was apparent after POD 5. In contrast, in the CyA-treated

group (group 3), all animals remained free from rejection during the first 10 days, and graft histology remained "nondiagnostic" for more than 20 days.

Serum creatinine and blood urea nitrogen

Serum creatinine and BUN were measured in the serum samples. In all groups, the creatinine levels remained normal and did not exceed 1.0 mg/ml. The BUN level also remained under 50 mg/dl, with no differences observed between the three groups.

RIA

ET-1 levels in the serum, liver, and kidney were determined using a RIA and were expressed as picograms per gram wet (pg/g wet) in the tissue specimens, and picograms per milliliter (pg/ml) in the serum samples.

In the serum, ET-1 levels showed a mild increase until POD 7 in group 2, going from 14.3 ± 1.5 pg/ml on POD 1 to 14.9 ± 2.3 pg/ml on POD 3, to 22.0 ± 3.1 pg/ml on POD 5, and to 38.7 ± 13 pg/ml on POD 7. They then decreased on POD 10 to 29.3 ± 2.5 pg/ml. The corresponding ET-1 levels in group 1 were 11.3 ± 0.5 pg/ml on POD 1, 20.2 ± 3.3 pg/ml on POD 3, 15.6 ± 2.2 pg/ml on POD 5, 22.0 ± 4.4 pg/ml on POD 7, and 18.8 ± 2.2 pg/ml on POD 10. The ET-1 level in group 2 was statistically higher on POD 10 than that in group 1 ($P < 0.05$). In group 3, the ET-1 levels were: 13.5 ± 1.4 pg/ml on POD 1, 13.8 ± 0.8 pg/ml on POD 3, 13.5 ± 4.1 pg/ml on POD 5, 15.1 ± 3.1 pg/ml on POD 7, and 11.2 ± 3.4 pg/ml on POD 10 (Fig. 1 A).

In the liver, ET-1 levels in group 1 were: 204 ± 19.5 pg/g wet on POD 1, 249 ± 15.5 pg/g wet on POD 3, 186 ± 20.2 pg/g wet on POD 5, 228 ± 6.5 pg/g wet on POD 7, and 181 ± 16.4 pg/g wet on POD 10. ET-1 levels in group 2 peaked at POD 5 and decreased slightly thereafter until POD 10. The values were: 208 ± 51.7 pg/g wet on POD 1, 200 ± 38.9 pg/g wet on POD 3, 344 ± 31.6 pg/g wet on POD 5, 293 ± 29.2 pg/g wet on POD 7, and 261 ± 77.0 pg/g wet on POD 10. In contrast, group 3 showed a time-dependent increase in ET-1 levels until POD 10. Values in this group were 216 ± 24.0 pg/g wet on POD 1, 313 ± 76.5 pg/g wet on POD 3, 326 ± 26.0 pg/g wet on POD 5, 428 ± 98.9 pg/g wet on POD 7, and 501 ± 160 pg/g wet on POD 10 (Fig. 1 B).

In the kidney, group 2 showed the same pattern of postoperative changes in ET-1 levels as seen in the liver, with a single peak on POD 5 and decreasing levels between POD 7 and POD 10. The values were: 296 ± 97.8 pg/g wet on POD 1, 365 ± 56.4 pg/g wet on POD 3, 720 ± 211 pg/g wet on POD 5, 365 ± 92.2 pg/g wet on POD 7, and 300 ± 23.5 pg/g wet on POD 10. In

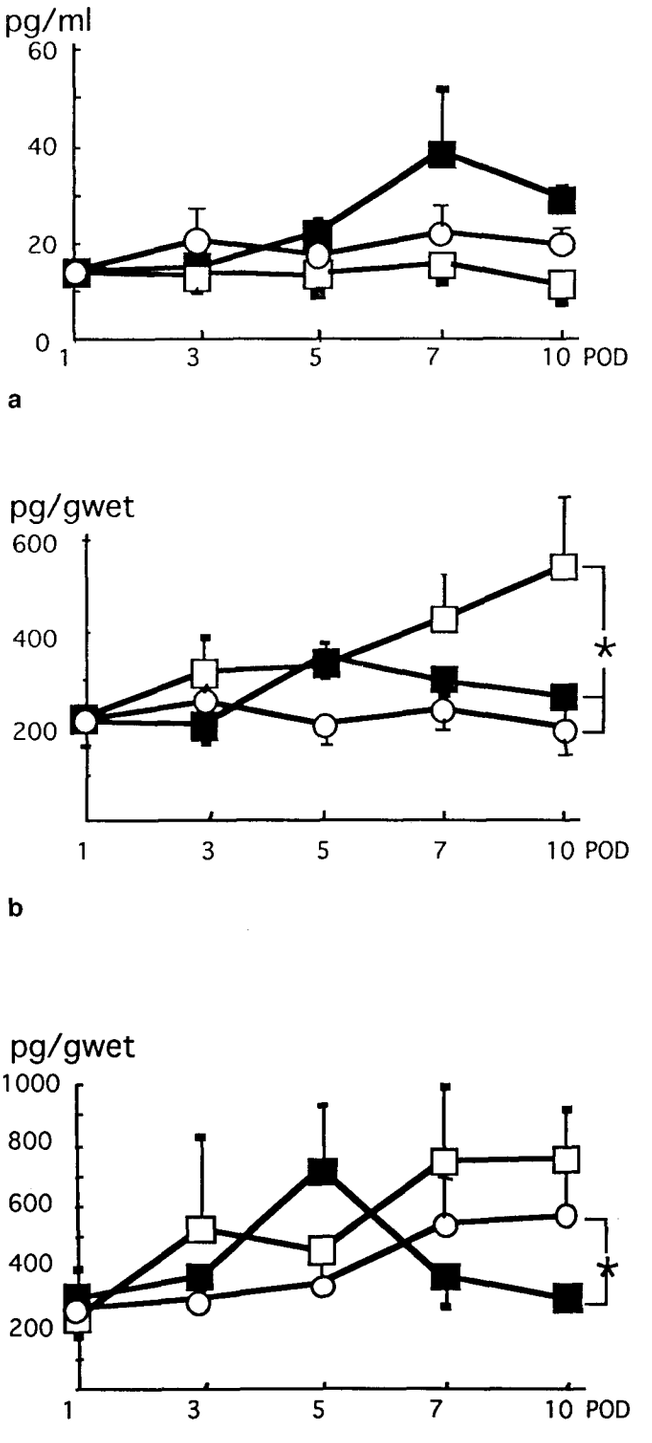

Fig. 1 a–c ET-1 levels in the: **a** serum, **b** liver, and **c** kidney in the syngeneic group 1 (–○–), allogeneic group 2 (–■–), and CyA-treated group 3 (–□–). In the tissue specimens, ET-1 levels were expressed as the mean ± SE pg/g wet. * $P < 0.05$ compared to group 1

group 3, ET-1 levels progressively increased until POD 10 from 233 ± 60.1 pg/g wet on POD 1 to 524 ± 306 pg/g wet on POD 3, 456 ± 110 pg/g wet on POD 5, 747 ± 249 pg/g wet on POD 7, and 751 ± 174 pg/g wet on POD 10. Group 1 also showed a statistically lower level of ET-1 than groups 2 and 3 on POD 10 ($P < 0.001$; Fig. 1 C).

Discussion

Although CyA is known to increase the endothelial production of ET in vitro [6], the in vivo effects of CyA have not yet been clarified in the literature. Since acute rejection itself is involved in the production and release of ET in vivo [12, 13], the in vivo effects of CyA should be evaluated in an acute rejection model. In this experiment, ET-1 expression was evaluated after allogeneic and syngeneic liver transplantation in rats, some of which had been given CyA.

ET-1 levels in the liver peaked on POD 5 and decreased thereafter in the nonimmunosuppressed group 2 animals. Since endothelialitis also became apparent on POD 5 in the grafted livers, the ET released from the damaged endothelial cells would seem to account for the temporarily increased ET in the liver on POD 5 and the decreased ET content of the graft liver after POD 7. These findings were identical to those of Watschinger et al. [13], who showed a decrease in ET staining intensity in the vascular rejection of a kidney graft using kidney transplant biopsies. They quantitatively evaluated the staining intensity and then concluded that the intrarenal content of ET had decreased in the vascular rejection model. The CyA dose administered to group 3 rats had, in previous experiments in our laboratory, been determined to be the minimal dose necessary for the complete suppression of acute rejection in this allogeneic combination. Indeed, the CyA completely suppressed the occurrence of acute rejection; in addition, no endothelialitis was observed from POD 1 to POD 10. In contrast to groups 1 and 2, group 3 animals showed a time-dependent increase in ET levels until POD 10. Although the data were not included in the results, ET-1 in the CyA-treated syngeneic combination (BN-to-BN) showed increased levels in the liver and kidney (453 ± 91 pg/g wet and 775 ± 125 pg/g wet, respectively, on POD 7) and decreased levels in the serum (22.5 ± 3.3 pg/g wet on POD 7). Thus, the hepatic ET-1 levels in group 3 were attributable to the increased production of ET-1 caused by the CyA and not to the rejection process.

The kidney showed a pattern of ET-1 kinetics similar to that observed in the liver. The nonimmunosuppressed animals demonstrated a single peak in the ET-1 level on POD 5. Since the kidney is thought to be one of the organs that can clear circulating ET [10], the ET-1 released from the liver might have accumulated in the kidney on POD 5. However, the decreased ET-1 levels in the kidney after POD 7 might have indicated something else, namely, that the increased amount of ET-1 was released from the kidney itself, thus resulting in the decreased level of ET-1 in the kidney. Although no apparent endothelialitis was detected in the histology, a systemic immunological process, similar to a humoral reaction to the endothelial cells [8], was suggested as a possible mechanism. ET-1 levels in group 3 animals showed a time-dependent increase, as observed in the liver. The CyA-induced increase in ET-1 expression was also less apparent in the kidney than in the liver.

Serum ET-1 levels in group 2 animals peaked on POD 7. However, in group 1 and 3 animals, these levels remained unchanged throughout the experiment, despite a progressive increase in tissue ET-1 levels in the liver and kidneys of group 3 animals. Based on our previous report, in which we investigated the production and clearance of ET in vivo, the major clearance routes of ET from the liver and kidney were the bile and urine, respectively [9]. These facts suggest that the increased serum ET-1 levels in group 2 animals reflected the decreased clearance capacity of the graft lifer due to the acute rejection. Hence, CyA is thought to preserve the clearance capacity of the graft liver by suppressing acute rejection of the liver.

In clinical organ transplantation, ET has been described as a mediator of CyA-induced renal dysfunction [2]. In these reports, the circulating ET in the blood was thought to play a major role in a systemic fashion. In our experiment, serum ET-1 levels did not respond to CyA, whereas tissue ET-1 levels continuously increased in response to the administration of CyA in both the liver and kidney. The increase in tissue ET-1, but not in serum ET-1, might thus explain the proposed mechanisms of CyA-induced renal dysfunction in clinical transplantation.

In sum, ET-1 was found to be increasingly released in the liver and kidney in response to acute rejection in the early post-transplant period, resulting in decreased tissue levels of ET-1 in these organs. At the same time, CyA caused a time-dependent increase in ET-1 production in the liver and kidney, resulting in a time-dependent increase in tissue ET-1 levels. Based on these findings, one may conclude that ET-1 levels reflect the clearance capacity of the liver and kidney.

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