Transpl Int (1997) 10: 121–124

ORIGINAL ARTICLE

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Involvement of endothelin in graft-versus-host disease after rat small bowel transplantation

Received: 11 June 1996
Received after revision: 3 September 1996
Accepted: 28 October 1996

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Abstract Endothelin (ET-1) expression was evaluated by radioimmunoassay in both plasma and various tissue specimens serially obtained from LBN-F1 recipients of LEW heterotopic small bowel allografts. The recipients showed graft-versus-host disease (GVHD), which histologically became apparent on postoperative day (POD) 13. The ET-1 levels peaked on POD 9 in the kidney, lung, and host intestine at 51.0 ± 21.1, 90.9 ± 59.6, and 25.4 ± 11.8 pg/g wet, respectively, and peaked on POD 11 in the plasma at 7.7 ± 3.2 pg/ml; thereafter, they decreased to basal levels in both the plasma and tissue specimens on POD 13. An immunohistochemical study of these organs showed a corresponding increase in ET-1 staining in both the endothelial and epithelial cells on PODs 5 and 9, and a reduction in staining on POD 13. In conclusion, ET-1 was found to be increasingly released from the target cells of GVHD before any histological changes became apparent, thus suggesting the pathophysiological involvement of ET-1 in intestinal GVHD.

Key words Endothelin, small bowel transplantation, rat · Small bowel transplantation, rat, endothelin · Graft-versus-host disease, endothelin, small bowel transplantation

Introduction

The small intestine is unique among vascularized organ allografts because it can induce both graft-versus-host disease (GVHD) and vigorous graft rejection in the recipient. GVHD after small bowel transplantation is initiated by a large number of T cells contained in the graft intestine and mesenteric lymph nodes [2], but such mechanisms of tissue injury are still not clearly understood. At present, inflammatory cytokines are thought to be the mediators of acute GVHD. The clinical manifestations of GVHD are mostly caused by the dysregulated production of cytokines by T cells and other effector cells, with each cytokine playing a distinctive role in the pathophysiology of GVHD [4].

Endothelin, which is known to be the most potent endothelium-derived vasoconstrictor, is upregulated by inflammatory cytokines [7]. Recent data have also shown that ET-1 plays a key role in various pathologic states, such as septic shock [12], kidney dysfunction of various types [8], and pulmonary fibrosis [5, 14]. Since all of these symptoms are also observed in the acute GVHD after rat small bowel transplantation, ET-1 is assumed to be involved in the pathophysiology of GVHD. To assess the involvement of ET-1, we investigated ET-1 expression in serially collected plasma and tissue specimens using both the radioimmunoassay (RIA) procedure and immunohistochemistry.

Materials and methods

Animals

Adult male Lewis × Brown Norway F1 (LBN-F1) and Lewis (LEW) rats weighing 200–250 g were purchased from Ryukyu Biotec (Okinawa, Japan). All animals were cared for uniformly in ac-

cordance with the guidelines set forth by the Ryukyu University Committee on Animal Care and Use.

Experimental groups and sampling

Adult male LBN-F1 rats served as recipients in all of the experimental groups. Our study included the following two groups: LBN-F1-to-LBN-F1 isografts (group 1) and LEW-to-LBN-F1 allografts (group 2). Each group consisted of 26 recipients. In each group, five recipients were sacrificed by exsanguination under general anesthesia on postoperative days (PODs) 1, 5, 9, and 13 ($n = 5$ on each POD). The remaining six recipients in each group were allowed to survive until death for a survival analysis. The host intestine, liver, kidney, and lung were obtained and either fixed in 10% buffered formalin for 24 h or stored in liquid nitrogen until use. The spleen index [11] was calculated as an indicator of the severity of GVHD. Light microscopic examinations were performed on serially obtained tissue samples from the liver, kidney, lung, and host intestine in group 2.

Operative procedures

Heterotopic small bowel transplantation was performed as previously described [10]. Briefly, the entire small intestine from the ligament of Treitz to the ileocecal valve as well as the mesenteric lymph nodes was harvested from the donor and then transplanted into the recipient as an accessory heterotopic graft, with venous and arterial anastomoses to the vena cava and infrarenal aorta, respectively. The proximal and distal ends of the graft intestine were exteriorized to the abdominal wall. The recipient's native intestine was left intact. After the operation, all recipients were isolated in a private, metabolic cage and weighed daily.

RIA for the ET-1 assay

The tissue samples were weighed and heated in distilled water at 100 °C for 5 min. After cooling, acetic acid was added and the tissue specimens were homogenized and centrifuged at 15000 rpm for 40 min. The supernatant and plasma samples were analyzed for ET-1 using a highly sensitive RIA. Each 0.1-ml sample or standard was incubated with 0.1 ml assay buffer and 0.1 ml anti-ET-1 monoclonal antibody (1:25000 dilution, TR-ET no. 48.5, Affinity Bioreagents, Colo., USA). Incubation was carried out at 4 °C for 24 h, followed by the addition of 0.05 ml ^{125}I-hET (Amersham International, England), after which incubation was continued for 2 more days at 4 °C. After adding a secondary antibody, the samples were centrifuged at 3000 rpm for 30 min; then the supernatant was aspirated and the radioactivity of the pellet was measured in a spectrometer. The ET-1 levels were expressed as pg/ml in the plasma, and as pg/g wet in the tissue samples.

Immunohistochemistry

The immunohistochemical staining of ET-1 was performed on 10% neutral buffered formalin-fixed tissue specimens by the labeled streptavidin-biotin method (Universal Dako LSAB Kit for use on rat specimens, Dako, Japan). The procedure included the sequential application of primary antibody (1:500 dilution, anti-ET-1 monoclonal antibody TR-ET no. 48.5, Affinity Bioreagents, Colo., USA) for 1 h, followed by a biotinylated link secondary antibody and then the streptavidin-biotin-peroxidase complex. All slides were processed further with diaminobenzidine) solution and then counterstained with methylgreen.

Negative controls were obtained by omitting the anti-ET-1 primary antibody. In each tissue type, the endothelial cells and epithelial cells were evaluated separately, and then according to the percentage of positive cells. The staining intensity was thus deemed to be one of the following: "none" (= no staining cells), "weak" (= less than 50% staining cells), "moderate" (= more than 50% but not all staining cells), or "strong" (= 100% staining cells). In addition, routine H&E staining was performed to examine the histological changes and to confirm the localization of ET-1 immunoreactivity.

Statistical analysis

The quantitative data were expressed as the mean ± standard error of the mean (SEM). All group comparisons were performed with Student's t-test when the data were applicable. The differences were considered to be statistically significant at a P level below 0.05.

Results

Survival and spleen index

Postoperative death related to technical failure occurred in less than 5% of all recipients and was, thus, excluded from the study analysis. Isogeneic animals in group 1 survived indefinitely, while the semiallogeneic animals in group 2 died within 16.4 ± 3.4 days, due to GVHD. The first signs of GVHD, erythema of the ears and eyelids, began to appear on POD 8.4 ± 0.6. The spleen index in group 2 was as high as 4.29 ± 0.78 on POD 13, which was compatible with the findings of GVHD.

Histological evaluation

In the liver, minimal cellular infiltration was observed in the portal tract and the central vein on POD 13, but neither cellular infiltration nor hepatic cell regeneration was seen in the parenchyma throughout the course.

In the kidney, no glomerular changes were observed by either H&E or PAS staining, but tubular damage was evident as GVHD progressed. That is, there was mild lymphocyte invasion in the tubules on POD 5, and tubular cell loss and necrosis on POD 13.

In the lung, an alteration in the alveolar septa, as characterized by the loss of epithelial cells and the increased thickness of the septa, was the principal lesion. This became evident on POD 13.

In the host intestine, the mucosal epithelium showed a normal histology up until POD 9, when the number of goblet cells started to decrease. Finally, the total loss of goblet cells and moderate villous atrophy were observed by POD 13.

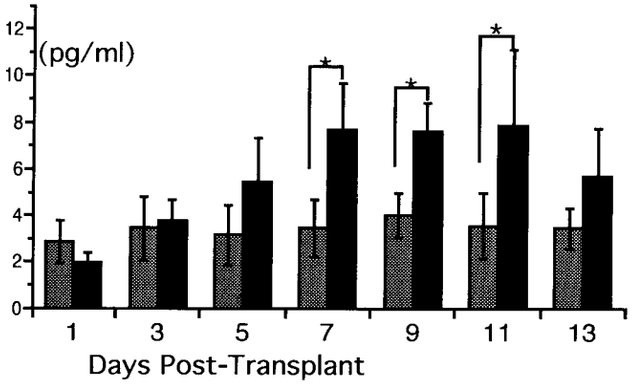

Fig. 1 Serial changes in the plasma ET-1 levels in group 1 (*shaded bars*) and group 2 (*black bars*). The data are expressed as mean ± SEM. $^{*}P < 0.05$ between group 1 and group 2

RIA

In the plasma, the ET-1 levels in group 1 remained low (< 4.5 pg/ml) throughout the observation period. In contrast, plasma ET-1 levels in group 2 increased to 7.7 ± 2.0 pg/ml on POD 7, remained high until POD 11, and then decreased to basal levels on POD 13. The ET-1 level in group 2 was statistically higher than that in group 1 from POD 7 to 11 ($P < 0.05$; Fig. 1).

The ET-1 levels in tissue extracts of the kidney, lung, and host intestine in group 2 increased to 51.0 ± 21.1, 90.9 ± 59.6, and 25.4 ± 11.8 pg/g wet, respectively, on POD 9 and then decreased to basal levels on POD 13. In these organs, the ET-1 levels in group 2 were statistically higher than those in group 1 on POD 9 ($P < 0.05$; Fig. 2B–D). In the liver, the ET-1 levels in group 2 also increased to 8.2 ± 2.7 pg/g wet on POD 9, but there was no significant difference between the groups (Fig. 2A).

Immunohistochemistry

In the liver, a "weak" expression of ET-1 was observed at the portal triad in all of the samples, but the intensity did not change throughout the course in either group.

In the kidney, a "weak" expression of the small vessels and tubuli were observed in all of the samples from group 1. In group 2, the staining intensity increased in the tubular epithelial cells to "moderate" on PODs 5 and 9, and thereafter decreased to "weak" on POD 13, in conjunction with a degenerative change in the tubuli.

In the lung, a "moderate" expression of epithelial cells of bronchus and bronchiole, and a "weak" expression of alveolar cells, were observed in all of the samples from group 1. In group 2, the staining intensity increased in both the bronchial epithelium and the alveolar cells to "strong" and "moderate", respectively, on PODs 5 and 9, but markedly decreased to "none" in the degenerated bronchial epithelium and alveolar cells on POD 13.

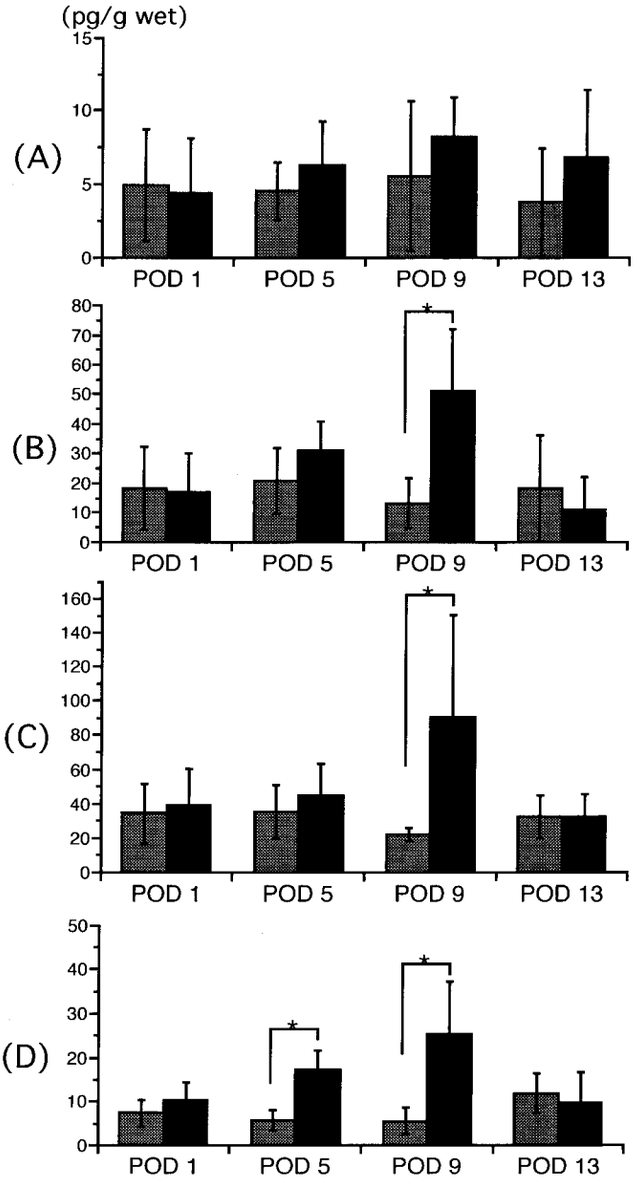

Fig. 2A–D Serial changes in the ET-1 levels in tissue extracts from **A** the liver; **B** kidney; **C** lung; and **D** host intestine of group 1 (*shaded bars*) and group 2 (*black bars*). The data are expressed as mean ± SEM. $^{*}P < 0.05$ between group 1 and group 2

In the host intestine, a "moderate" expression of small vessels in the submucosal layer and a "weak" expression of epithelial cells were observed in all of the samples from group 1. In group 2, the staining intensity increased at both the submucosal layer and the columnar epithelium to "strong" and "moderate", respectively, on PODs 5 and 9. As seen in the kidney and the

lung, a reduction in the staining intensity was observed in the degenerated epithelial cells on POD 13.

Discussion

Acute GVHD has recently been reported to be associated with endothelial cell injury [1], and inflammatory cytokines such as tumor necrotic factor-α and interleukin-1 are regarded as mediators of acute GVHD [9]. Since ET-1 is released from endothelial cells by the stimulation of such cytokines [7], we hypothesized that ET might be an additional mediator of GVHD, and we therefore investigated ET-1 expression in acute GVHD after rat intestinal transplantation.

In the immunohistochemical study, ET-1 staining intensity in group 2 increased on PODs 5 and 9 in the alveolar wall, tubuli of the kidney, and mucosal epithelium of the intestine. This finding is consistent with those of other studies that have pointed out that ET can be released from the epithelial cells of the alveoli [5], tubuli [13], and intestinal mucosa [3], in addition to endothelial cells. During the development of GVHD, intense injuries were observed on these cells by light microscopy, thus suggesting that these tissues are targets of GVHD. ET-1 levels decreased until POD 13, when the tissue injury became histologically apparent. These reductions may be explained by the degenerated epithelial cells that were observed in the histological examination and that resulted in an inability to synthesize ET.

The elevation in ET-1 levels may have some clinical implications. First, ET is known to have a distinct renal effect. In previous rat studies, exogenously infused ET-1 was reported to reduce the renal blood flow [8]. Another author demonstrated the role of endogenous ET-1 in renal impairment during acute liver rejection of rats using ET-1 receptor antagonist [15]. We observed tubular damage in light microscopy in the terminal state of acute GVHD. We also observed a decrease in creatinine clearance and an increase in N-acetyl-beta-glucosaminidase excretion at that time (data not shown). These observations suggest that renal dysfunction in GVHD may be caused by the vasoconstrictive property of ET-1.

At the same time, current studies have also focused on the mitogenic action of ET-1. ET-1 was reported to have a potent mitogenic effect on airway smooth muscle cells [6]. We observed a marked thickening of the alveolar wall and a corresponding reduction in ET-1 staining intensity after the peak increase in ET-1 levels in tissue extracts. Uguccioni et al. reported that ET-1 might play a role in the fibrogenic process of idiopathic pulmonary fibrosis (IPF) [14]. The histological evidence of IPF resembles the pulmonary lesions observed in this study, thus suggesting the fibrogenic process of ET-1 in the course of GVHD. However, further studies are still required to confirm this hypothesis.

In conclusion, we have presented our findings of the increased release of ET in endothelial and epithelial cells of the target organs of GVHD. Based on these findings, we may conclude that the physiological action of ET plays an important role in the pathophysiology of GVHD in rat small bowel transplantation.

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