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Yukihiro Tatekawa
Hiromichi Kanehiro
Yoshiko Dohi
Takatsugu Yamada
Hideki Kanokogi
Yoshiyuki Nakajima
Saiho Ko
Michiyoshi Hisanaga
Yukio Aomatsu
Hiroshige Nakano

Intragraft expression of p38 and activated p38 MAPK (mitogen-activated protein kinase) in rat small bowel transplantation

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Y. Tatekawa (✉) · H. Kanehiro · Y. Dohi
T. Yamada · H. Kanokogi · Y. Nakajima
S. Ko · M. Hisanaga · Y. Aomatsu
H. Nakano
First Department of Surgery,
Nara Medical University,
Kashiharasi Nara, 634–8522, Japan,
Fax: + 81-744-24-6866

Y. Dohi
Department of Public Health,
Nara Medical University, 840 Shijo-cho,
Kashiharasi, Nara, 634–8522, Japan

Abstract Recent studies suggest that cytotoxic T-lymphocytes expressing p38 mitogen-activated protein kinase (p38MAP kinase) contribute to allograft rejection in clinical heart transplantation. Interleukin-2 (IL-2), a potent T cell mitogen, activates the p38MAP kinase pathway, resulting in phosphorylation of target transcription factors. In this study we investigated the expression of activated p38MAP kinase in intragraft cell infiltrates following rat heterotopic small bowel transplantation and examined the effects of the immunosuppressant FK506 on intragraft expression of activated p38MAP kinase and allograft rejection. Allografts receiving FK506 (0.5 mg/kg per day i.m.) for 7 days as primary anti-rejection therapy had a significant reduction in histopathological evidence of allograft rejection on Day 7, compared to allograft controls. In addition, Western blotting analysis of intragraft cell infiltrates showed a reduction in the expression of activated p38MAP kinase in allografts treated with FK506. We conclude that intragraft cell infiltrate expression of activated p38MAP kinase is an important marker of acute rejection in this animal model of small bowel transplantation, and that FK506 is an effective immunosuppressant, in this situation, that may act in part by preventing the activation of p38MAP kinase.

Keywords Mitogen-activated protein kinase (MAPK) · p38 · FK506 · Rat small bowel transplantation

Introduction

The histological pattern of acute rejection in rat small bowel transplantation, which includes the intragraft infiltration of specific T lymphocyte subpopulations and macrophages, has been previously described [7, 19]. The cytokines interleukin 2 (IL-2) and interferon-gamma (IFN-γ) are recognized as crucial in promoting acute allograft organ rejection. IL-2 is produced by two coordinate signals; the interaction of antigen with the T cell receptor-CD3 complex and a secondary stimulus from accessory co-stimulatory molecules [20, 26]. It is, furthermore, a key factor in driving the proliferation of activated T lymphocytes [21]. IFN-γ is produced by activated CD4 Th1 cells, and IFN-γ mRNA is significantly increased in the rat small bowel and may mediate crypt epithelial cell injury and apoptosis [11]. The immunosuppressant FK506, (Tacrolimus), blocks both IL-2 transcription, thereby inhibiting CD4 Th1 lymphocyte response to foreign antigens and inhibits cytokine secretion of IFN-γ [13, 22].

Recent studies suggest that donor-derived cytotoxic T lymphocytes expressing p38 mitogen-activated protein kinase (p38MAP kinase) contribute to allograft rejection in clinical heart transplantation [5]. p38MAP kinase is a member of the MAP kinase family that plays an important role in signaling cascades involved in cell proliferation, differentiation and apoptosis [16]. p38MAP kinase is activated by cellular stress stimuli (heat, osmotic stress, UV irradiation), pro-inflammato-

ry cytokines (interleukin-1, tumor necrosis factor-α) and IL-2 [4, 6, 9, 14, 17, 18]. Activated (phosphorylated) p38MAP kinase translocates from the cytoplasm to the nucleus, where it catalyzes phosphorylation and subsequent activation of target transcription factors [10, 25, 27]. The expresson of p38MAP kinase in different leukocyte subsets has been studied [23], and four types of p38MAP kinase (α, β, γ and δ) have been described [8]. p38α and p38δ are the major isoforms in lymphoid tissue. p38α is the dominant form of p38MAP kinase in monocytes and macrophages and is also expressed by neutrophils, CD8 + and activated CD4 + T lymphocytes [8, 23].

In this study we sought to assess the expression of activated p38MAP kinase in intragraft infiltrating cells during rat small bowel transplantation and to examine the effects of the immunosuppressant FK506 on intragraft expression of activated p38MAP kinase and allograft rejection.

Materials and methods

Animals

Inbred male Brown Norway rats (BN)(RT-1^n), weighing 200–250 g (donors in allogeneic transplants) and Lewis rats (LEW)(RT-1^l), weighing 200–250 g (donors in syngeneic transplants) and 300–350 g (used as recipients in allogeneic transplants), were purchased from Charles River, Osaka, Japan. All animals were housed in accordance with institutional animal care policies and had access to water and standard laboratory chow ad libitum. All of the animals were cared for in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 86–23, revised 1985).

Small bowel transplantation

Heterotopic small bowel transplantation was performed under general anesthesia. A 10-cm segment of small intestine was harvested from the distal ileum of the donor. The portal vein of the donor was covered with a cuff of 14 gauge angiocather tube (inside diameter 1.6 mm, outside diameter 2.1 mm).

The left renal vessels of the recipient were dissected, and the native kidney was removed. The left renal artery of the recipient was covered with a cuff of 22-gauge angiocather tube (inside diameter 0.60 mm, outside diameter 0.85 mm). Using the technique described by Wallander, et al. [24], an aortic cuff and portal venous drainage of the graft were anastomosed by an end-to-end cuff technique, respectively. The proximal and distal ends of the graft were exteriorized as stomata to the left flank. The recipient's own intestine was left intact.

Experimental groups

Small bowel transplantations ($n = 13$) were performed using the following rat strain combinations: Group 1. Lewis × Lewis (LEW × LEW) ($n = 3$, syngeneic), Group 2. Brown Norway × Lewis (BN × LEW) ($n = 5$, allogeneic, no treatment), Group 3. BN × LEW ($n = 5$, allogeneic with FK506, 0.5 mg/kg per day i.m., for 7 days, from days 0–6 after transplantation). FK506 (Tacrolimus, Fujisawa Pharmaceutical, Osaka, Japan) was dissolved in a carrier solvent (HCO-60, D-mannitol;) and diluted in normal saline.

Histology

The entire transplanted small intestine graft was harvested by killing the recipient rat 7 days after transplantation. Non-transplanted small bowel tissue was also obtained from BN animals ($n = 3$, native small intestine). Samples of the graft and native intestine tissue were fixed in 10% formalin, embedded in paraffin and sectioned at 4 μm.

The sections, stained with hematoxylin and eosin, were graded for acute rejection using the following semiquantitative scheme: 0 = no evidence of rejection, 1 = mild acute rejection (inflammation of the lamina propria, minimal individual cell necrosis, mild villous blunting), 2 = moderate acute rejection (inflammation of the lamina propria and submucosa, easily identifiable cell necrosis, varying degrees of villous blunting), and 3 = severe acute rejection (inflammation of the lamina propria, submucosa, and muscularis propria, diffuse individual epithelial cell necrosis, complete villous blunting).

Statistical differences in the acute rejection scores were determined using the Mann-Whitney *U*-test. Values are given as mean ± SD. Differences with a P-value < 0.05 were considered significant.

Immunohistochemical staining of p38MAP kinase and activated p38MAP kinase proteins

Immunohistochemistry was performed using an immunoperoxidase technique. The primary antibodies used for immunohistochemistry were mouse anti-mouse p38-α-MAPkinase monoclonal antibody (Zymed laboratories, USA, (cross-reactivity: rat, 1:100)) and rabbit anti-human phosphospecific (Thr180/Tyr182) p38 antibody (New England Biolabs, USA, (cross-reactivity: rat, 1:50)). Paraffin sections of the intestine were deparaffinized, hydrated, and subjected to microwave antigen retrieval. The sections were incubated with the appropriate concentrations of primary antibodies at 37 °C for 60 min and then incubated at 4 °C for 12 h with p38 antibody, and 24 h with phosphospecific p38 antibody. The sections were then immersed in methanol with 3 % hydrogen peroxide for 30 min at room temperature to block endogenous peroxidase, and then incubated with the immunohistochemical staining reagent Histofine Simple Stain PO (M) (Nichirei, Tokyo, Japan) and p38 antibody for 60 min at 37 °C, or Histofine Simple Stain PO (R) and phosphospecific p38 antibody for 120 min at 37 °C, respectively. Development of peroxidase was achieved using a peroxidase chromogen substrate solution, Histofine simple Stain DAB solution (3–3′diaminobenzine tetrahydrochloride substrate for horseradish peroxidase solution, Nichirei) at room temperature. Finally, the sections were counterstained with Gill's hematoxylin and examined by light microscopy. The assessment of p38 expression in immunohistochemical staining was done in a blinded fashion.

Western immunoblotting for phosphospecific p38 MAP kinase

The entire transplanted intestinal tissue was quickly removed, frozen in liquid nitrogen, and stored at –80 °C for later analysis. Frozen intestinal tissue was ground into powder. The separation and

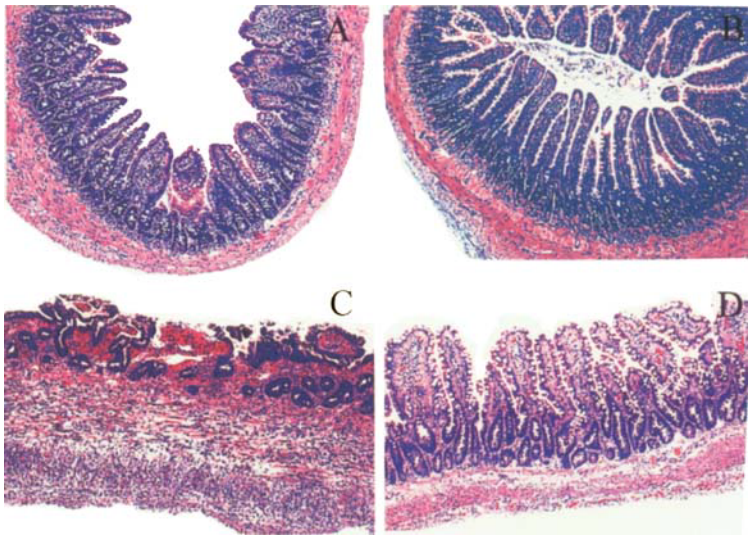

Fig. 1 A–D Histology of small bowel grafts, 7 days after transplantation.There was no histological evidence of acute rejection in native intestine **A** and isografts **B**, while moderate to severe rejection was seen in untreated allografts **C**. Scant evidence of acute rejection was observed in allografts treated with FK506 for 7 days **D**

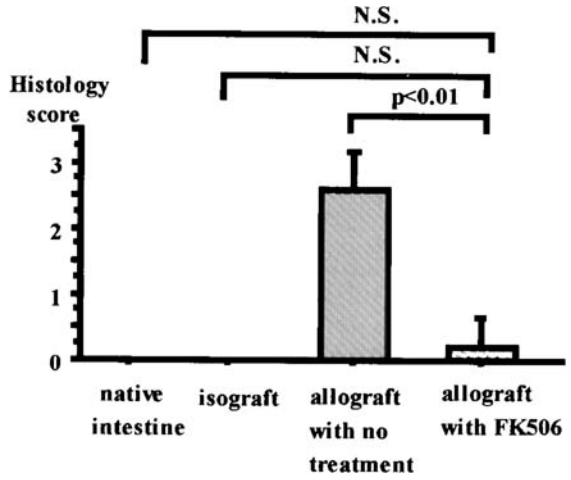

Fig. 2 Histology score for acute rejection using the following semi-quantitative scheme: *0* no evidence of rejection, *1* mild acute rejection, *2* moderate acute rejection, and *3* severe acute rejection). Primary treatment with FK506 for 7 days significantly suppresses histological evidence of acute rejection when compared to untreated allografts ($P < 0.01$) (Mann-Whitney U-test, NS; no significant)

purification of nuclear protein extracts were performed using the method of Blobel-Potter (density gradient centrifugation, [2]. Nuclear protein extracts were homogenized in homogenization buffer (20 mM Tris-HCl, pH 7.4; 50 mM β-glycerophosphate, 5 mM EGTA, 1 mM Na3VO4, 0.1 mM Na2MoO4, 0.5 mM DTT, and protein inhibitor cocktail for mammalian cell extracts (Sigma, St. Louis, USA)). Protein concentration was determined by the bicinchoninic acid technique (Pierce Analytical Research, Rockford, Ill., USA) using bovine serum albumin (BSA) standards. The protein extracts were then lyophilized using a Freeze dryer FD-5 N (Rikakikai, Tokyo, Japan).

Each concentrated protein sample (40 µg) was added to 20 µl of a sodium dodecyl sulfate (SDS)-sample buffer. The tubes were heated to 95–100 °C for 5 min. After reheating, the samples were loaded onto a 10 % SDS-polyacrylamide gel and electrophoresed. Protein extracts from phosphorylated C6 glioma cells (New England Biolabs), were run in parallel as a positive control. After electrophoresis and gel transfer to a polyvinylidene difluoride (PVDF) membrane, the membrane was incubated for 1 h at room temperature with blocking buffer (TBST; 10 % w/v nonfat dry milk) and then incubated overnight at 4 °C with the phosphospecific p38 antibody (1:1000) in dilution buffer (TBST; 5 % w/v BSA). After a subsequent incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000) in blocking buffer for 1 h at room temperature, the membrane was developed using the Phototope-HRP Western Blot Detection Kit (LumiGLO Chemiluminescent Substrate kit, New England Biolabs) at room temperature. The membrane was exposed to X-ray film.

Results

FK506 treatment suppresses acute rejection in small bowel allograft transplantation. Histopathologic examination of both native small intestine, and small intestine isografts (group 1, $n = 3$, syngeneic) on day 7 after transplantation (Fig. 1A and B) showed no evidence of rejection (histology scores: 0). However, moderate to severe rejection (histology scores 2, 2, 3, 3, 3; mean 2.6 ± 0.5) was observed in allografts that received no treatment (Group 2, $n = 5$, Fig. 1C). Histopathologic examination of allografts that had received FK506 (Group 3, $n = 5$) showed few signs of rejection (Fig. 1D) (histology scores: 0, 0, 0, 0, 1; 0.2 ± 0.4). The histology score difference between the latter two groups was statistically significant ($P < 0.01$, see Fig. 2).

Activated p38MAP kinase expression is increased in intragraft cell infiltrates during acute rejection of small bowel allografts.

Immunostaining, using a monoclonal antibody specific to p38MAP kinase or a monoclonal phospho-specific antibody to activated (phosphorylated) p38MAP

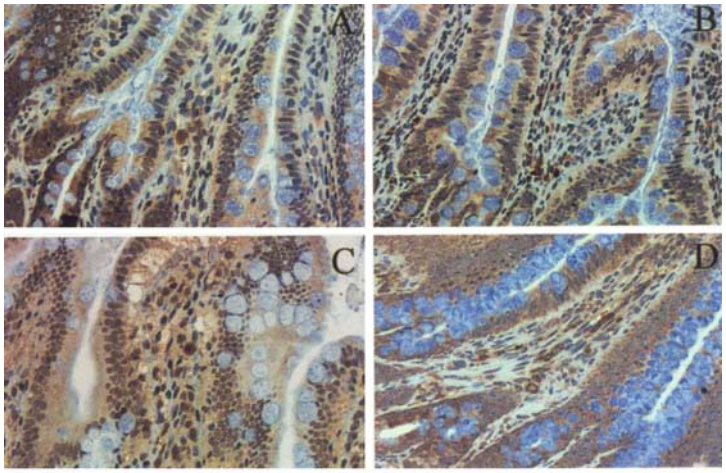

Fig. 3 A–D Immunostaining for p38MAP kinase in small bowel allograft sections (at Day 7). Positive staining for p38MAP kinase was observed in the perinuclear regions of infiltrating mononuclear cells found in the lamina propria and submucosal layers. p38MAP kinase staining was equivalent for all three groups and native intestine (**A** native intestine, **B** isograft, **C** untreated allograft, **D** allograft treated with FK506)

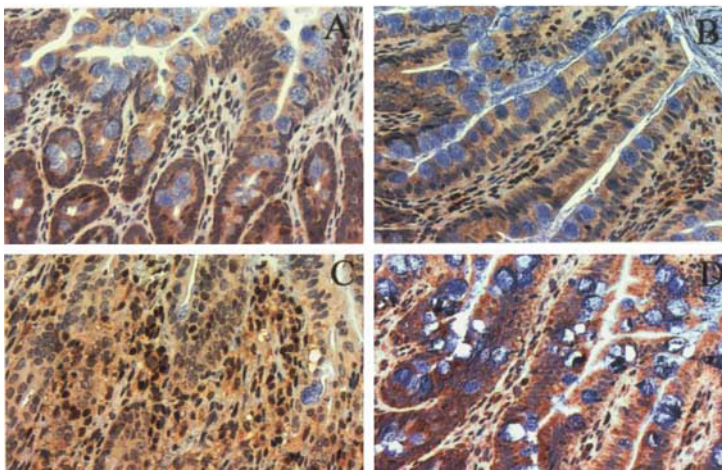

Fig. 4 A–D Immunostaining for activated (phosphorylated) p38MAP kinase is increased in small bowel allografts undergoing acute rejection (at Day 7).Positive staining for activated p38MAP kinase was observed in the nuclei of infiltrating mononuclear cells found in the lamina propria and submucosal layers. Increased levels of phosphorylated p38MAP kinase were detected in untreated allografts **C**, relative to native intestine **A**, isografts **B** and allografts treated with FK506 **D**

kinase showed two distinct patterns. An identical pattern of positive-staining for p38MAP kinase was observed in samples from the three groups and native intestine (Fig. 3). However, in samples from untreated allografts (Fig. 4C), there was evidence of increased expression of activated p38MAP kinase relative to native intestine (Fig. 4A), isografts (Fig. 4B) and the allografts treated with FK506 (Fig. 4D). The increased immunostaining was observed in the perinuclear regions and in the nucleus of infiltrating mononuclear cells found in the lamina propria and submucosal layers. The increased immunostaining and the nuclear pattern of staining are consistent with the activation of p38MAP kinase during acute rejection.

Immunoblotting analyses of small bowel cell lysates for expression of activated p38MAP kinase gave similar results, in that immunoreactive bands were substantially stronger in samples from untreated allografts compared to those from native intestine, isografts and allografts treated with FK506 (Fig. 5).

Discussion

In this study we have sought to demonstrate a role for the p38MAP kinase signaling cascade in the cellular infiltrate associated with acute rejection in an animal model of small bowel allograft transplantation. We have shown by immunostaining that in this context, activated p38MAP kinase is uniquely present in the nuclei of intragraft infiltrating cells. This pattern of staining is consistent with a model whereby immunomodulatory cytokine (e. g. IL-2, or type 1 interferons) – induced activation of the p38MAP kinase signaling cascade results in phosphorylation of p38MAP kinase and subsequent translocation of the activated cytoplasmic p38MAP kinase into the nucleus to phosphorylate and thereby activates target transcription factors. This study is also in accordance with a previous study that suggested that donor-specific T cytotoxic lymphocytes have upregulated expression of activated p38MAP kinase in the setting of heart transplantation [5]. The importance of

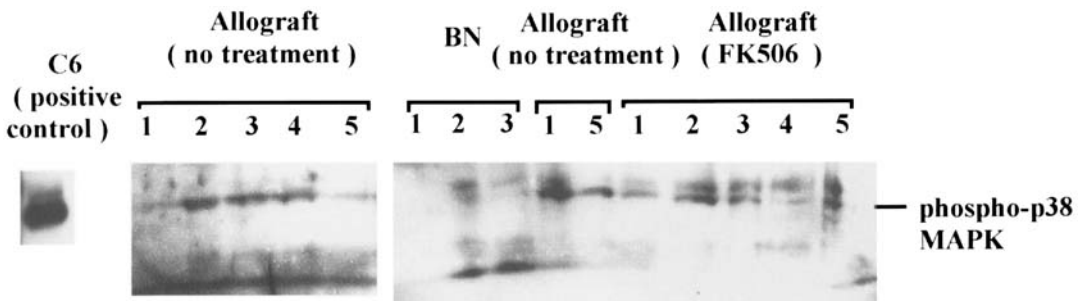

Fig. 5 Phosphorylated p38MAP kinase expression is increased in cell lysates from small intestinal allografts undergoing acute rejection.Immunoreactive bands in cell lysates from untreated allografts stain more intensely than those from native intestine and allografts treated with FK506

p38MAP kinase activation in the immune responses involved in allograft rejection has also been demonstrated in a recent study by Yang et al. [28]. These investigators observed that administration of CNI-1493, a drug that inhibits activation of p38MAP kinase by IL-2 and TNF α [1, 3, 12], resulted in prolonged allograft survival in an animal model of cardiac allograft rejection. We have also shown in this study that primary therapy of rat small bowel allografts with FK506 (Tacrolimus), a specific inhibitor of both IFN-γ secretion, and IL-2 production and IL-2 receptor expression on activated T lymphocytes [11, 13, 22] results in suppression of allograft rejection as evidenced by both histological analyses and a reduction in intragraft cell infiltrate expression of activated p38MAP kinase. Recent studies suggest that Cyclosporin A, which is also effective in preventing allograft rejection, may also function via the p38MAP kinase signaling cascade in that it inactivates an upstream regulator of p38MAP kinase [15].

We conclude that intragraft cell infiltrate expression of activated p38MAP kinase is an important marker of acute rejection in this animal model of small bowel transplantation, and that FK506 is an effective immunosuppressant in this situation. We would suggest that prevention of the activation of p38MAP kinase is an important component of this immune suppression. We would anticipate that more specific inhibitors of the p38MAP kinase signaling cascade may prove to be effective in preventing allograft rejection.

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