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Nitric oxide-related neural components in the rat small intestine after transplantation

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Abstract The changes in nitric oxide (NO)-related neural components in the transplanted small intestine are unknown. In this study, the NO neural component was examined using electrophysiological and NADPH-diaphorase histochemical techniques in a rat small bowel transplantation model. Syngeneic total small bowel transplantation was performed in 26 male Lewis rats using microsurgical techniques. The rats were divided into four groups: an untreated control group and animals at 1 (G1), 2 (G2), and 4 (G4) weeks after transplantation. Jejunal strips were mounted in a superfusion apparatus for examination. In the presence of atropine and guanethidine, the effect of the NO synthesis inhibitor L-N^G-nitro-arginine (L-NNA, 100 μM) relaxation mediated by the nonadrenergic, noncholinergic (NANC) neural system was assessed following electrical stimulation at 2 Hz. The inhibitory effect of L-NNA on relaxation was taken as an indicator of NO production. The percentage of inhibition in the control group, and in G1, G2, and G4 was 43.30 % ± 6.08 % (mean ± SE), 42.10 % ± 6.69 %, 43.62 ± 10.00 %, and 52.46 % ± 6.00 %, respectively. Inhibition in G4 was significantly higher than in the other groups ($P < 0.01$). The percentage of NADPH-diaphorase-positive fibers in the control group, G1, G2, and G4 was 25.06 % ± 4.70 % (mean ± SE), 26.27 % ± 2.17 %, 24.73 % ± 2.87 %, and 30.76 % ± 3.19 %, respectively. Again, G4 showed a significantly higher level than the other groups ($P < 0.01$). We conclude that increased NO production may play a significant role in maintaining the intrinsic nervous system of the small intestine after transplantation.

Key words Small intestine transplantation, nitric oxide · Nitric oxide, small intestine transplantation · Nonadrenergic, noncholinergic, small intestine transplantation

Introduction

Since most advances and interest in the field of bowel transplantation have centered on the immunobiology of the transplanted bowel, the changes in enteric neurons that occur after transplantation are still not completely understood. In a previous investigation, we evaluated the effect of transplantation on intestinal smooth muscle contractility and the associated neural control mechanisms. We found changes in both the contraction and relaxation responses to electrical transmural stimulation (ETS), with contraction being significantly enhanced. In addition, we found that the increase in intestinal contractile motility after transplantation was associated with a marked increase in the nonadrenergic, noncholinergic (NANC) neural component, with the

dominant intrinsic neural component changing from cholinergic to NANC after 4 weeks [6, 8]. The NANC component is thought to be stimulated by peptides, amino acids, and catecholamines, but while NANC inhibitory nerves play an important regulatory role, the effects of these neurotransmitters are less clear.

Recently, nitric oxide (NO) has been characterized as a messenger molecule [11]. It has been said to act as an inhibitory NANC neurotransmitter and as a potent mediator of NANC muscle relaxation in the gastrointestinal tract [3, 13]. However, no studies have been performed on the changes in the NO-related neural component in the transplanted intestine. The purpose of this study was to examine the role of NO in the intrinsic neural system of the grafted small bowel.

Materials and methods

Experimental design

Twenty-six Lewis rats were divided into four groups: an untreated control group ($n = 8$, group C) and animals at 1 ($n = 6$, group G1), 2 ($n = 6$, group G2), and 4 ($n = 6$, group G4) weeks after transplantation. To evaluate the physiological properties of smooth muscle and nerves in the four groups, 9 samples from group G1, 7 from group G2, and 9 from group G4 were compared with 12 samples from group C. For histochemical evaluation, we used 8 samples from group G1, 8 from group G2, 12 from group G4, and 8 from group C. The jejunal grafts removed from each animal were compared with the normal jejunum in group C. Bowel strips were cut into 15-mm and 10-mm lengths, and the 10-mm segments were processed for light microscopy with sectioning at an appropriate thickness.

Animals

Male Lewis rats (from the LEW/Crj strain) weighing 200–300 g were obtained from Charles River Japan. These rats were used as both small intestine donors and recipients since syngeneic transplantation avoids the risk of rejection and the need for immunosuppression. Animals were fasted for 12–18 h before operation.

Surgical procedure

Orthotopic entire small bowel transplantation was performed using a microsurgical technique similar to that described previously [12]. Briefly, anesthesia was induced with pentobarbital (40 mg/kg intraperitoneally). The entire small bowel of the donor rat was harvested from the ligament of Treitz to the terminal ileum, along with a vascular pedicle consisting of the superior mesenteric artery and the portal vein. The intestinal lumen was flushed with 0.5 % neomycin, and the superior mesenteric artery was flushed with iced saline containing 10 U/ml heparin. In the recipient rat, the infrarenal aorta and vena cava were clamped, and an end-to-side anastomosis was performed between the recipient aorta and the donor superior mesenteric artery along with the aortic cuff, as well as between the recipient vena cava and the donor portal vein. The entire small bowel was resected from the recipient, and the graft was anastomosed end-to-end with the duodenum and the remnant terminal ileum.

Recording of mechanical activity

The strips were suspended in a superfusion apparatus [7] equipped with two platinum electrodes and were superfused with Krebs solution of the following composition: NaCl, 118 mM; KCl, 4.8 mM; $CaCl_2$, 2.5 mM; $NaHCO_3$, 25 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; and glucose 11 mM. The medium was maintained at 37 °C, adjusted to pH 7.4, and constantly aerated with a mixture of 95 % O_2 and 5 % CO_2. In all experiments, the solution also contained atropine and guanethidine (1.0 μM and 5.0 μM, respectively). Motor activity was recorded isometrically on a recticoder (RJG-4128; Nihon Kohden, Tokyo). The resting tension (1.0 g) was kept constant by readjustment during the equilibration period. Experiments were started after a 60 to 90-min equilibration period, and antagonists were given 15 min before the application of electrical transmural stimulation (ETS). L-NNA and L-arginine were respectively added 15 and 20 min prior to ETS. The superfusate flow rate was adjusted to 1 ml/min by using a peristaltic pump (SJ-1220; Atto, Tokyo). Electrical pulses were delivered from an electrical stimulator (S-7272B; Nihon Kohden, Tokyo) that provided stimulus trains lasting 10 s at 4-min intervals. A voltage of 20 V and a duration of 1.0 ms were used in all experiments, while the impulse frequency was altered from 1.0 Hz to 10.0 Hz.

Drugs

The following drugs were used: atrophine sulfate, guanethidine sulfate, L-N^G-nitro-arginine (L-NNA), and L-arginine.

NADPH-diaphorase histochemistry

The strips were immersed in 300 ml of ice-cold fixative containing 4 % paraformaldehyde and 0.1 % glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4). After 3–4 h, the specimens were placed on glass slides as whole-mount preparations and were stained in a free-floating state. NADPH-diaphorase activity was visualized by incubating the tissue in 0.1 M PB (pH 8.0) containing 0.01 mM β-NADPH, 0.02 mM nitroblue tetrazolium, and 0.3 % TritonX-100 for 2–4 h at 37 °C. The reaction was terminated by washing the sections in 0.1 M PB [1] and the sections were observed under a light microscope.

Quantitative measurements

Measurements were performed using an IBAS 20 computer image analysis system (Zeiss, Germany), which is capable of identifying and counting positive fibers with a fixed optical density. The sections were also examined by a single observer using a × 20 objective. Image segmentation is the process of separating objects of interest from the background features in an image. Even though the NO fibers were easily distinguishable from the background and selection of the black levels between which the pixels of the required images fell could consequently be done with confidence, black level scaling and editing were used to correct for slight variations in background intensity, as it was sometimes impossible to compensate for this by simple shade correction. The density of the NO nerve fibers and the frame area were assessed using the IBAS 20 image analyzer, and the total number of nerve fibers was expressed as the plexus area to frame area ratio.

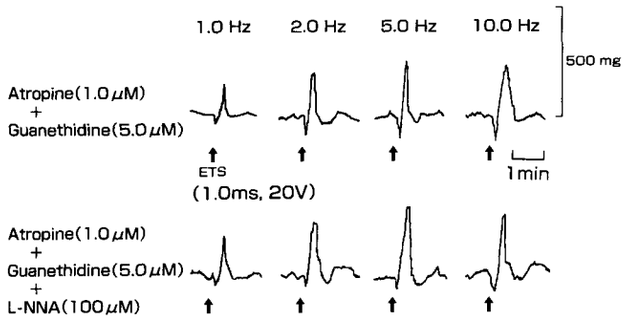

Fig. 1 Effect of L-N^G-nitro-arginine (*L-NNA*, 100 μM) on the motility of longitudinal muscle strips obtained from control jejunum in the presence of atropine (1.0 μM) and guanethidine (5.0 μM). Electrical transmural stimulation *(ETS)* provoked a biphasic response in a frequency-dependent manner (ETS; 1.0 ms, 20 V, 1.0–10.0 Hz)

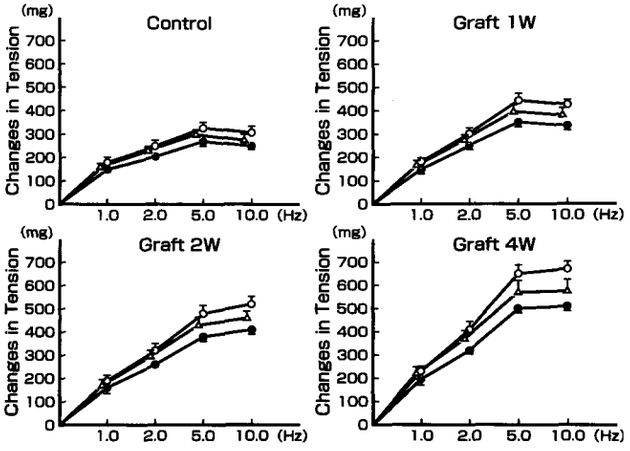

Fig. 2 Frequency response curves showing ETS-induced contraction in the presence of atropine (*Atr*, 1.0 μM), guanethidine (*Gua*, 5.0 μM), L-N^G-nitro-arginine (*L-NNA*, 100 μM), and L-arginine (*L-Arg*, 1.0 mM). ●—● Atr + Gua; △—△ Atr + Gua + L-NNA + L-Arg; ○—○ Atr + Gua + L-NNA

Statistical analysis

The results shown in the text and figures are expressed as the mean ± SE. Statistical analysis was performed using Student's *t*-test in the electrophiological study and using a one-way analysis of variance (ANOVA) in the histochemical study. A *P* value of less than 0.05 was considered significant.

Results

Effect of L-NNA on NANC-mediated relaxation

To study the jejunal activity of NANC components, we used atropine and guanethidine. ETS (1.0–10 Hz) produced a biphasic response, which was comprised of relaxation (first phase) followed by contraction (second phase). Typical jejunal motility patterns of control intestinal segments in the presence of atropine (1.0 μM) and guanethidine (5.0 μM) are illustrated in Fig. 1. L-NNA (100 μM) markedly inhibited the first phase (relaxation) and enhanced the second phase (contraction) of the response to ETS (Figs. 2, 3). This effect of L-NNA was prevented by preadministration of L-arginine (1.0 mM; Figs. 2, 3). The proportional effect of L-NNA was assessed at 2.0 Hz by comparing the responses of control and grafted strips because the maximal relaxation of untreated control intestine was recorded at 2.0 Hz [6]. Inhibition of the response by L-NNA was expressed as a percentage of the relaxation produced by ETS. The percentage of inhibition in groups C (control), G1, G2, and G4 was 43.30 % ± 6.08 %, 42.10 % ± 6.69 %, 43.62 % ± 10.00 %, and 52.46 % ± 6.00 %, respectively. Group G4 had a significantly higher inhibitory response than the other groups ($P < 0.01$).

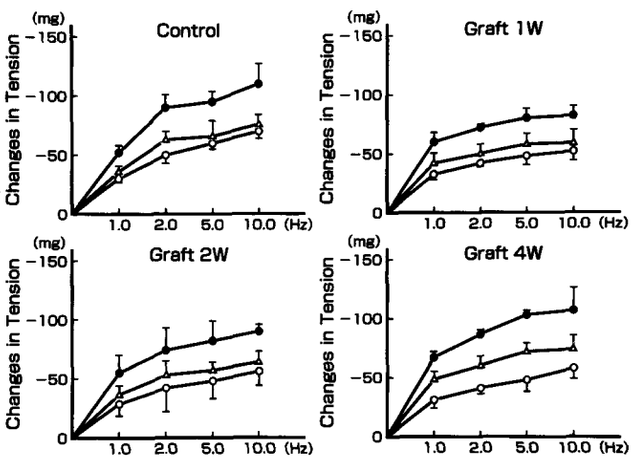

Fig. 3 Frequency response curves showing ETS-induced relaxation in the presence of atropine (*Atr*, 1.0 μM), guanethidine (*Gua*, 5.0 μM), L-N^G-nitro-arginine (*L-NNA*, 100 μM), and L-arginine (*L-Arg*, 1.0 mM). ●—● Atr + Gua; △—△ Atr + Gua + L-NNA + L-Arg; ○—○ Atr + Gua + L-NNA

NADPH-diaphorase activity

Positive fibers extended from the myenteric plexus to the inner circular and deep muscular layers. In whole-mount preparations of the jejunal muscle layer, fine networks of NADPH-diaphorase-positive fibers were clearly visible (Fig. 4). We compared the positivity for NADPH-diaphorase staining of the control and transplanted jejunum, and expressed the results as a percentage. The positivity rate in groups C, G1, G2, and G4 was 25.06 % ± 4.70 %, 26.27 % ± 2.17 %, 24.73 % ±

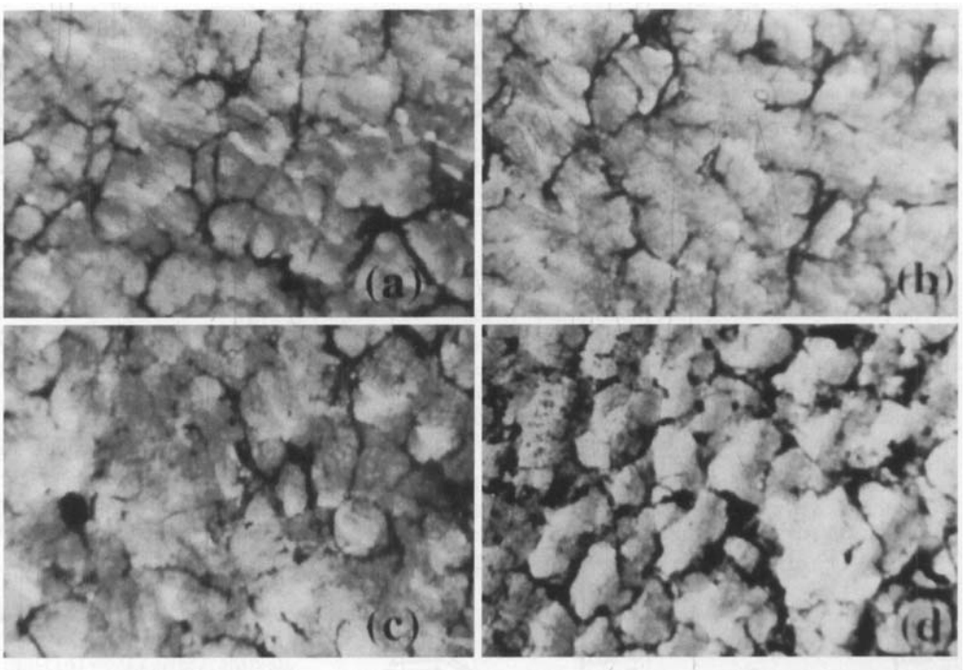

Fig. 4 a–d NADPH-diaphorase staining in whole-mount preparations of control and transplanted jejunum. Positive fibers extend from the myenteric plexus to the muscular layer in: **a** the control group, **b** G 1, **c** G 2, and **d** G 4 (× 20)

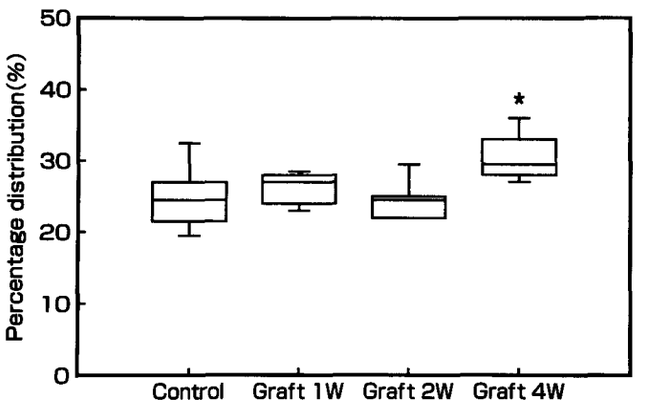

Fig. 5 Percentage distribution of NADPH-diaphorase-positive fibers in the jejunal muscle layers from each group. * $P < 0.01$ vs other groups (ANOVA)

2.87 %, and 30.76 % ± 3.19 %, respectively. The rate for group G 4 was significantly higher than that for the other groups ($P < 0.01$; Fig. 5).

Discussion

The small intestine is innervated by extrinsic and intrinsic nerves, and small bowel transplantation inevitably causes complete disruption of the extrinsic nerves to the graft. This change may adversely alter intestinal physiology. Some unexpected changes in the autonomic nervous system in the transplanted intestine have been reported. Our previous studies have indicated that both the excitatory and inhibitory neural systems might be modified and that the NANC component was the dominant intrinsic neural component after transplantation, with substance P playing an important role in excitatory NANC transmission [6, 8]. Taguchi et al. have demonstrated the absence of extrinsic adrenergic inhibitory innervation in small bowel grafts [14].

Recently, much attention has been given to NO as a potent muscle NANC relaxant. NO-synthase has been detected throughout the gastrointestinal tract and has been shown to mediate the neural inhibition of intestinal smooth muscle contraction. However, the possibility that NO plays an important role in small bowel grafts has not been studied previously. Therefore, we investigated whether NO production in the intrinsic neural system was changed by small bowel transplantation.

Based on an electrophysiological study, Lefebvre et al. have proposed that relaxation and contraction are respectively mediated by NO and acetylcholine [9]. The possibility that potentiation of the excitatory response to transmural stimulation by inhibitors of NO biosynthesis is related to the inhibitory action of endogenous NO on the release of substance P or acetylcholine has been suggested by Gustafsson et al. [4] and Lefebvre et al. [10]. Boechxstaens et al. recently demonstrated that the response to low-frequency stimulation (2–5 Hz) was mainly mediated by NO, whereas peptidergic neurotransmitters were released at higher frequencies (10–50 Hz) [2].

We assessed the effect of the NO synthesis inhibitor L-NNA on NANC relaxation produced by ETS in longitudinal muscle strips from each group. In our study, NO production significantly increased in the rat intestine 4 weeks after transplantation. L-NNA inhibited ETS-mediated relaxation and enhanced contraction in the presence of atropine and guanethidine. When compared with the control value, L-NNA increased contraction by 22 % and reduced relaxation by 43 %. The identification of neurons that may utilize NO for various cellular activities was achieved through the localization of NO synthase (NOS), the enzyme that activates the synthesis of NO from its precursor, L-arginine. The NADPH-diaphorase technique was utilized to label neurons containing NOS-like immunoreactivity in the enteric nervous system. Since neuronal NADPH-diaphorase is a form of NO-synthase, NADPH-diaphorase histochemistry provides a specific histochemical marker for neurons producing NO, and such neurons were observed in all groups. Positive fibers were increased in the myenteric plexus of the transplanted intestine at 4 weeks, a finding in agreement with our physiological observations. Neither cholingeric nor adrenergic blockers altered the effect of NO on the motility of jejunal strips, indicating that NO acted via the NANC pathway. An increase in both NO-induced motility and NO staining of the strips was observed at 4 weeks after transplantation, which is when NANC neural components reach their maximum level [6].

Our preliminary observations suggested that substance P was a candidate transmitter for causing increased contraction [8]. An increment in NO production might play a significant role in controlling neural mechanisms after small bowel transplantation. Inhibition of acetylcholine release by NO has been reported by Hryhorenko et al. [5]. Both the number and physiological activity of NANC nerves were enhanced at 4 weeks after transplantation, suggesting the establishment of a new intrinsic neural network in the transplanted intestine. It would be interesting to know how NO-related neural components are modified over the long term after small bowel transplantation.

In conclusion, the presence of NO after small bowel transplantation was demonstrated in this study using histochemistry. However, additional studies on inhibitory neural components after syngeneic transplantation are necessary to unravel the complexities of graft neural control.

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