Reconstruction of central lacteals in the murine jejunum following ischemia-reperfusion injury*

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Summary. The intestinal mucosa is vulnerable to an ischemia-reperfusion (I/R) attendant on some bowel diseases and surgery; thus, the restoration of the mucosal integrity is critical to achieving functional recovery of the intestine injured by I/R. In this histochemical study, we investigated the alteration of the central lacteals — which are essential for the transport of fat, tissue fluid, and immune cells in the intestinal mucosa — in the murine jejunum after I/R. The intestine inflicted with I/R demonstrated mucosal injury involving the inflammatory response, with interstitial edema, disruption of the villous tissue, and subsequent tissue regeneration of the villi. The regenerative villous tissue revealed lymphatic regrowth showing proliferative activity from the residual mucosal lymphatics behind the regenerated blood vasculature. During the regenerative phase, the blood vascular pericytes expressed an intense immunoreaction for VEGF-A, an inducer for monocyte/macrophage recruitment as well as angiogenesis. Also, the F4/80-immunopositive macrophages significantly increased in number in the regenerating villous stroma. Furthermore, the macrophages recruited around the regrowing lacteals expressed the immunoreactivity for VEGF-C, which is a

highly specific lymphangiogenic factor. The present study is first to delineate alterations in the central lacteals in the small intestine following I/R, thereby suggesting that the recruitment of the macrophages induced by upregulation of VEGF-A in the pericytes of regenerative blood vessels might promote reconstruction of the central lacteals through their release of VEGF-C.

Introduction

Ischemia-reperfusion (I/R) injury of the intestine plays a critical role in the pathophysiology of such bowel diseases as strangulated hernia and intestinal intussusception (Stefanutti *et al.*, 2004) as well as in several surgical conditions including small bowel transplantation (Grant *et al.*, 1990; Grant, 1996; Massberg and Messmer, 1998) and cardiovascular surgery (Gennaro *et al.*, 1993; Soong *et al.*, 1994; Parvumus, 1999; Collard and Gelman, 2001). An interruption of the intestinal blood supply progressively damages various cell structures, but restoration of the blood flow to the ischemic tissue (reperfusion) paradoxically intensifies the cell and tissue injury (McCord, 1985; Parks and Granger, 1986; Stallion *et al.*, 2002). The intestinal mucosa is considered to be highly susceptible to I/R because the inflammatory responses develop predominantly in the mucosa to elicit further damage after reperfusion (Granger, 1988; Kong *et al.*, 1998; Yamamoto *et al.*, 2001). Recently, several studies have assessed the regeneration of the intestinal mucosa following I/R from the view of small bowel transplantation and demonstrated that a rapid restoration of the mucosal integrity could help attenuate deleterious disorders including bacterial translocation (Chan *et al.*, 1998; Kozhura *et al.*, 2005; Yoshikawa *et al.*, 2005).

Central lacteal, an important tissue component of the small intestinal mucosa, plays an essential role in the transport of the rich emulsion of fat, tissue fluid, and

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immune cells. The restoration of the central lacteals is therefore assumed to be required for the functional recovery of the intestinal mucosa following I/R. However, no information has been available with respect to alterations of the central lacteals in the small intestine inflicted with I/R, though a number of studies have described the microvascular dysfunction in the intestinal mucosa with I/R injury (Hernandez *et al.*, 1987; Fujimoto *et al.*, 1991; Kvietys and Granger, 1997; Hayward and Lefer, 1998; Davis *et al.*, 1999; Beuk *et al.*, 2000; Cuzzocrea *et al.*, 2002; Cooper *et al.*, 2004). Hence, an investigation of the central lacteals following I/R should be important in order to understand not only the pathophysiology of intestinal I/R injury but also the dynamics of the central lacteals. The present study demonstrates alterations of the central lacteals in the murine jejunum following I/R by immunohistochemistry. In order to gain insight into the mechanism underlying the regrowth of the central lacteals, we further examined the expression of vascular endothelial growth factors (VEGF) in tissue repair of the intestinal mucosa.

Materials and Methods

Adult BALB/cN Sea mice of both sexes weighing 40–50 g were used in this study. They were kept under standard laboratory conditions and received standard animal chow and water ad libitum except for the night before surgery. All procedures were conducted in accordance with the Guidelines for Animal Experimentation of Oita University.

Surgical procedure

The animals were anesthetized with an intraperitoneal injection of a mixture of ketamine (50 mg/kg bodyweight) and xylazine (40 mg/kg) 20 min prior to surgery, and then the epigastric region was opened by a median incision. A segment of the jejunum (5–8 cm in length) was placed on sterilized gauze moistened with a physiological saline solution. The marginal artery at each end of the intestinal segment was ligated and the mesenteric dominant artery was then clamped by a vascular microclip to interrupt the intestinal blood supply. The operated intestine was returned into the abdominal cavity after confirming that it had turned pale, and the abdomen was covered with a polyvinyl film.

Following ischemia for 15 min, the mesenteric vascular clamp was released to reperfuse the blood to the jejunal segment. The intestine was again returned to the abdomen and then the abdominal wall was closed by suturing.

Control animals underwent a laparotomy, but did not receive an operation to halt the intestinal blood flow.

Tissue preparation

After the mice were sacrificed under deep anesthesia with an injection of sodium pentobarbital (50 mg/kg), the operated and control intestines were harvested at 0, 1, 2, 4, 8 and 12 h, and 1, 3 and 7 days after the surgery.

Some tissue pieces of the intestines were immersed in 4% paraformaldehyde dissolved in phosphate buffer saline (PBS; 1/15 M, pH 7.4) for 8 h at 4 °C and embedded in paraffin for light and fluorescence microscopy. They were cut into 5 µm thick sections and processed for hematoxylin-eosin and immunohistochemical staining. For immunoelectron microscopy, some small tissue pieces were fixed in a 4% paraformaldehyde solution for 3 h at 4 °C and immersed in 0.1 M phosphate buffer (pH 7.4) containing 30% sucrose. The tissues were rapidly frozen in liquid nitrogen and sectioned at a 20 µm thickness.

Immunohistochemistry

The deparaffinized tissue sections were incubated in a 0.01 M citrate buffer (pH 6.0) at 121 °C for 15 min to retrieve antigenicity of the objective proteins before immunostaining.

Some deparaffinized tissue sections were immersed in 0.3% H₂O₂ in PBS containing 0.1% sodium azide (Wako Pure Chemical Industries, Osaka) at room temperature for 20 min to block the endogenous peroxidase activity. They were incubated in a 4% block Ace solution (Dainippon Seiyaku, Osaka), and then with an antibody against VEGF-A (11-001, 10 µg/ml, AngioBio, Del Mar, CA, USA), VEGF-C (C-20, 10 µg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or F4/80 antigen (MCA497R, 10 µg/ml, Serotec, Oxford, UK) at 4 °C overnight. Following a rinse in PBS, they were treated with peroxidase (PO)-conjugated goat anti-rabbit or rat IgG or rabbit anti-goat IgG (Histofine Simple Stain Mouse MAX-PO, Nichirei Bioscience, Tokyo) for 1 h at room temperature. The site of the immunoreaction was visualized by the diaminobenzidine (DAB; Dojindo, Kumamoto) reaction, and cellular nuclei were counterstained with hematoxylin.

For electron microscopic observation, the frozen tissue sections were immunostained for VEGF-A as mentioned above and postfixes in 1% OsO₄ for 30 min. They were then dehydrated in a graded ethanol series, embedded in Epon 812, and cut into ultrathin sections for examination in a JEM-1200 EX transmission electron microscope.

Fig. 1. Light micrographs of hematoxylin-eosin staining on tissue sections of intestinal mucosa from the murine jejunum at 0 (a), 2 (b), 4 (c) and 12 h (d), and 3 (e) and 7 days (f) after I/R. **a:** The intestinal mucosa with ischemia for 15 min shows no distinct changes. **b:** Interstitial edema and subepithelial bleb formation (asterisk) are seen in the villi. **c:** The villous tissue is disrupted (arrows), but the cryptal region of the mucosa is preserved. **d:** The injured area of the remaining mucosa is ensheathed by the attenuated epithelial cells (arrows). **e, f:** The regenerative villi extend with central lacteals (L) to be restored by 7 days after I/R. Bar: 100 μ m

(JEOL, Tokyo).

For double immunostaining for LYVE-1 and CD34 or proliferating cell nuclear antigen (PCNA), some tissue sections were incubated with an antibody for CD34 (MEC 14.7, diluted 1: 10, Abcam, Cambridge, UK) or PCNA (M0879, diluted 1: 300, DAKO Cytomation, Glostrup, Denmark) at 4 °C overnight, followed by the alkaline phosphatase (AP) -conjugated streptavidin-biotin method

(Vecstain ABC-AP kit, Vector Laboratories, CA, USA) or a PO-bound secondary antibody (Histofine Mouse Stain Kit, Nichirei Bioscience). The immunoreaction was detected by AP (Vector red substrate kit, Vector Laboratories) or DAB reaction. The sections were then immersed in a citrate buffer at 95–99 °C for 10 min to remove the precipitated antibodies from the tissues. They were thereafter incubated with an antibody to LYVE-

Fig. 2. Light micrographs of double immunostaining for CD34 (red) and LYVE-1 (brown) on tissue sections at 4 (a) and 12 h (b), and 3 days (c) after I/R. **a:** The upper portion of the villous tissue involving LYVE-1-immunopositive lacteal (L) and CD34-immunoreactive blood vessels is disrupted. **b:** The residual portion of LYVE-1-immunopositive lymphatic (L) and CD34-immunoreactive blood vessels are seen in the remnant villus. **c:** The dilated lacteal (L) with LYVE-1-immunoreactivity is seen in the regenerating villous core, whereas CD34-immunopositive blood vessels are distributed beneath the regenerative epithelium. Bar: 50 μ m

Fig. 3. Light micrograph of double immunostaining for LYVE-1 (blue) and PCNA (brown) on a tissue section of murine jejunum 3 days after I/R. Several LYVE-1-immunopositive endothelial cells of a regrowing lacteal show immunoreaction to PCNA (arrows) in their nuclei. Bar: 50 μ m

1 (11-034, 10 μ g/ml, AngioBio) at 4 $^{\circ}$ C overnight, followed by PO- or AP-conjugated anti-rabbit IgG (Simple Stain MAX-PO or -AP, Nichirei Bioscience), and the immunoreaction site was visualized by DAB or AP (Vector blue substrate kit, Vector Laboratories) reaction. Some sections were further stained with hematoxylin for nuclear counterstaining. All the immunostained sections for light microscopy were examined with a BX-60 light microscope (Olympus, Tokyo).

For fluorescence microscopy, the other tissue sections were incubated in 10% normal donkey serum, and then in a mixture of an antibody to VEGF-C, together with an antibody to F4/80, at 4 $^{\circ}$ C overnight. After washing with PBS, they were treated with a mixture of indocarbocyanine (Cy3)-conjugated donkey anti-goat IgG (Jackson Immuno Research, West Grove, PA, USA) and fluorescein isothiocyanate (FITC)-conjugated donkey

Fig. 4. Light micrographs of immunostaining for VEGF-A on tissue sections of intestinal mucosa from the murine jejunum at 4 (a) and 12 h (b), and 3 days (c) after I/R. **a:** No immunoreaction products are seen in the injured villi. **b, c:** Intense immunoreaction products are seen in a subpopulation of stromal cells, some of which show a tubular (arrows in b) or vascular (arrows in c) configuration, in the regenerating villus. Bar: 50 μ m

anti-rat IgG (Jackson Immuno Research) for 1h at room temperature. For nuclear counterstaining, 4',6-diamidino-2-phenylindole (DAPI; Molecular Probe, Eugene, OR, USA) was included in the secondary antibody solution. The sections were rinsed in PBS, mounted in a fluorescent mounting medium (DAKO Cytomation), and then examined by fluorescence microscope (Olympus, BX-60).

Control immunostaining was carried out by the same procedure, except for utilization of a non-immunized serum or an antibody absorbed with an excess amount of antigens (50–100 μ g/ml diluted antibody) instead of the corresponding antibodies; completely negative results were observed.

Count of F4/80-immunopositive cells and statistical analysis

Using a calibrated ocular grid in a microscope, nuclei of the F4/80-immunopositive cells were counted in fifty microscopic fields of the intestinal villi at a magnification of $\times 400$ on each tissue section, and the mean count of the cells per unit of intestinal villous field (field of view, 0.0175 mm²) was determined by averaging the findings.

All values were expressed as means $\pm$ SE. Comparisons were made by an unpaired t-test, and a two-tailed value of <0.01 was considered to indicate statistical significance.

Results

Histological changes

The murine jejunum inflicted only with ischemia for 15 min revealed no significant histopathological findings in this study (Fig. 1a). In contrast, the intestinal villi demonstrated progressive histological changes following reperfusion (Fig. 1b–f). No histological damage was detected in any other intestinal regions or internal organs.

The villous tissue inflicted with I/R displayed interstitial edema, inflammatory cell infiltration, and subepithelial bleb formation (2 h after I/R, Fig. 1b). Portions of the upper half or beyond of the villous tissue were subsequently disrupted (4 h after I/R, Fig. 1c), and the degenerative tissue was eliminated from the mucosa into the intestinal lumen. This experimental period is therefore termed the "degenerative phase" in this study.

At 12 h after reperfusion, the injured area of the residual mucosal tissue was often covered with attenuated regenerative epithelial cells (reepithelization), the injured villi becoming dramatically shortened in height (Fig. 1d). Thereafter, the surviving intestinal villi lined with renewed epithelium extended through regeneration of the tissue elements involving stromal cells and blood and lymphatic vasculature to be restored to a configuration similar to that of the control intestinal villi by 7 days after reperfusion (Fig. 1e, f). This latter experimental period is accordingly referred to as the "regenerative phase".

Fig. 5. TEM view of immunostaining for VEGF-A in intestinal villus from the murine jejunum 1 day after I/R. The immunoreaction products are seen in the cytoplasm and on the cell membrane of a pericyte (P) around the regenerating blood capillary (BC) but not in the blood vascular endothelium (E). Bar: 500 nm

Alteration of central lacteal

Immunohistochemistry for LYVE-1 clearly defined the endothelium of lymphatic vessels including central lacteals in all tissue sections at each experimental time point (Fig. 2, 3), whereas immunostaining for CD34 and PCNA exclusively demonstrated significant reaction products in the blood vascular endothelial cells (Fig. 2) and the cellular nuclei of proliferative cells (Fig. 3), respectively.

During the degenerative phase, the villous tissue demonstrated the dilatation of the central lacteals with interstitial edema after reperfusion, and consequently portions beyond the upper half of the lacteals collapsed along with the other tissue components, including blood vessels (4 h after I/R, Fig. 2 a).

At 12 h following reperfusion, blood vessels with CD34-immunoreactivity were observed beneath the newly formed epithelial lining at the tips of the primarily repaired villi, whereas the surviving portions of the LYVE-1-immunopositive lymphatics in the cryptal region remained relatively short (Fig. 2b).

The regenerating villi immediately restored the rich supply of the renewed blood vessels beneath the epithelium (3 days after I/R, Fig. 2c). On the other hand, the lymphatics extended into the regenerating villous tissue behind the regenerated blood vasculature and reformed central lacteals surrounded by a newly formed blood vascular network (Fig. 2c). The regrowing lymphatic vessels often exhibited significant proliferative activity — evidenced by an immunoreaction to PCNA — in the lymphatic endothelial cells during the regenerative phase (Fig. 3).

Immunohistochemistry for VEGF-A

Immunostaining for VEGF-A demonstrated no significant reaction products in the control or degenerative intestinal villi (4 h after I/R, Fig. 4a). In contrast, the surviving and regenerating villi displayed an intense immunoreaction to VEGF-A in a subpopulation of the stromal cells (12 h and 3 days after I/R, Fig. 4b and c).

The VEGF-A-immunopositive cells were spindle or irregular in shape and often disposed in tubular or vessel-like configurations beneath the epithelium (Fig. 4b, c). Immunoelectron microscopic examination further disclosed that VEGF-A was preferentially immunolocalized in the cytoplasm and cell membrane of the pericytes of the regenerating blood capillaries but not in the blood vascular endothelial cells (Fig. 5).

Fig. 6. Light micrographs of immunostaining for F4/80 on tissue sections of intestinal mucosa from the murine jejunum at 4 h (a), and 1 (b) and 3 days (c) after I/R. **a:** Disrupted intestinal villi show few immunoreaction products. **b, c:** Many immunopositive cells are seen around regrowing lacteals (L) and at the villous tips in regenerative villous stroma. Bar: 50 μ m

Immunohistochemistry for F4/80 antigen

Immunostaining for F4/80 antigen demonstrated the distribution of macrophages in the intestinal villi (Fig. 6). In control and degenerative conditions, the intestinal villi showed a poor supply of the F4/80-immunopositive macrophages (4 h: Fig. 6a and Fig. 7). In contrast, during the regenerative phase, the macrophages significantly increased in number in the intestinal villi except on the 7th day after I/R, and then tended to be distributed around the regrowing lacteals and/or at the tips of the regenerating stroma (1 day: Fig. 6b, 3 day: Fig. 6c and Fig. 7).

Immunohistochemistry for VEGF-C

With immunostaining for VEGF-C, no reaction products were detected in the intestinal mucosa of either the control or degenerative condition (4 h after I/R, Fig. 8a), whereas the regenerative villi showed many stromal cells intensely immunoreactive for VEGF-C around the regrowing lacteals and/or at the villous tips (1 day: Fig. 8b, 3 day: Fig. 8c).

The distribution of the VEGF-C-immunopositive cells displayed a tendency similar to that of the

No. of F4/80-immunopositive cells

Fig. 7. Changes in mean numbers of F4/80-immunopositive macrophages in murine jejunal villi after I/R. * $P < 0.01$, statistically significant compared with the control group.

Fig. 8. Light micrographs of immunostaining for VEGF-C on tissue sections of intestinal mucosa from the murine jejunum at 4 h (a), and 1 (b) and 3 days (c) after I/R. **a:** A disrupted intestinal villus shows no immunoreaction products. **b, c:** Intense immunoreaction products are seen in a subpopulation of stromal cells in regenerative villi. Bar: 50 μ m

macrophages during the regenerative phase (Fig. 6, 8). Double immunostaining for VEGF-C and F4/80 further demonstrated that VEGF-C was preferentially immunolocalized in the F4/80-immunopositive macrophages in the villous stroma during the regenerative phase (Fig. 9).

Discussion

The present study clearly demonstrated morphological alterations of the central lacteals concomitant with mucosal injury and regeneration in the murine jejunum following I/R. The mucosal injury in our murine I/R model displayed an inflammatory reaction and denudation of the villous tissue, as previously described in other animal models (Chan *et al.*, 1998; Noda *et al.*, 1998; Beuk *et al.*, 2000; Xia *et al.*, 2003; Wu *et al.*, 2004; Kozhura *et al.*, 2005; Yoshikawa *et al.*, 2005). Adjusted to the histological grading of mucosal I/R injury in the previous report (Quaedackers *et al.*, 2000), our experimental model is thus presumed to provoke mild to moderate mucosal damage showing interstitial edema, a disruption of the villi, preservation of the cryptal region, and subsequent tissue regeneration. Therefore, the present murine model

is considered useful for investigating not only ischemia-reperfusion injury but also intestinal mucosal tissue regeneration.

Alteration of central lacteals

The central lacteals exhibited a pronounced dilatation concomitant with edema of the lamina propria mucosae at the early stage of the degenerative phase after reperfusion in this study. The reperfusion of ischemic tissue is known to enhance the extravasation of fluid containing plasma protein and leukocytes (Carden and Granger, 2000). This increased vascular permeability perhaps contributes to the mucosal edema involving subepithelial bleb formation, inflammatory cell infiltration, and the dilatation of the central lacteals.

Several reports have described apoptosis to be a major mode of cell death in I/R mucosal injury of the small intestine (Ikeda *et al.*, 1998; Noda *et al.*, 1998; Wu *et al.*, 2004). Our preliminary immunohistochemical examination demonstrated DNA fragmentation not only in a number of epithelial cells but also in some endothelial cells of the central lacteals in the murine jejunum inflicted with I/R during the degenerative phase (data not shown), thus implying that the disruption of the central lacteals

Fig. 9. Fluorescence micrographs of double immunostaining for VEGF-C (**a**, red) and F4/80 (**b**, green) on a tissue section of intestinal mucosa from the murine jejunum 1 day after I/R. Figure **c** shows a merged image. The immunoreaction products for VEGF-C are selectively distributed in F4/80-immunopositive macrophages (yellow in **c**). Bar: 50 μ m

evoked by I/R is also — or at least partly — attributable to the apoptosis of the endothelial cells.

The regrowing lacteals, which extended from the lymphatics saved in the cryptal region, often displayed PCNA-immunoreactivity in the nuclei of the endothelial cells during the regenerative phase. This indicates a highly regenerative activity of the injured lacteals, which may be due to vigorous proliferative potentials of the lymphatic endothelial cells.

Mechanism of regeneration of lymphatic lacteals

At the early stage of the regenerative phase in our experiment, significant immunoreaction products for VEGF-A, a representative angiogenesis factor (Shibuya, 1995; Ferrara and Davis-Smyth, 1997), were found in a subpopulation of the stromal cells in the regenerating villi. The present immunohistochemical results further demonstrated that the VEGF-A was preferentially immunolocalized in the pericytes of the regrowing blood capillaries in the regenerative villous stroma. The upregulated VEGF-A in the blood vascular pericytes during the regenerative phase may effect the regeneration of the intravillous vascular beds in this I/R model through its angiogenic action.

On the other hand, our immunohistochemical findings demonstrated that macrophages, which presumably release mediators to amplify any inflammatory reaction to I/R (Carden and Granger, 2000; Mallick *et al.*, 2004; Cerqueira *et al.*, 2005), significantly increased in number during the early regenerative phase in comparison with those in the control and degenerative state. Since VEGF-A is also considered to be a potent chemoattractant for monocytes/macrophages (Shen *et al.*, 1993; Clauss *et al.*, 1996; Hiratsuka *et al.*, 1998), the upregulation of VEGF-A in the regrowing blood vessels presumably contributes to the enhanced recruitment of macrophages as well as angiogenesis in the regenerative villi.

The present study further showed that the intense immunoreactivity of VEGF-C, which is a primary lymphangiogenic factor (Jeltsch *et al.*, 1997; Veikkola *et al.*, 2001), was exclusively expressed in F4/80-immunopositive macrophages in the villous tissue during the regenerative phase, but not under control or degenerative conditions. This corresponds with the recent findings that a subfraction of circulating monocytes and peritumoral macrophages express VEGF-C (Schopmann *et al.*, 2001, 2002). Since the recruited macrophages were often distributed around the regenerative central lacteals in the present study, they might thus facilitate the

regrowth of the injured central lacteals by the paracrine action of their released VEGF-C in the regenerative phase following I/R.

Cursiefen *et al.* (2004), using a corneal vascularization model with a neutralization technique for VEGF-A in VEGF-A transgenic mice, noted that the recruitment of monocytes/macrophages by VEGF-A plays a crucial role in inflammatory lymphangiogenesis. Our data obtained in the intestinal I/R model support their suggestion regarding the significance of VEGF-A and its mediated macrophage recruitment for lymphangiogenesis. Regarding the reconstruction of the central lacteals after I/R, the present study consequently implies a possible mechanism in which VEGF-A, being upregulated in the regenerating blood vessels prior to lymphatic regrowth, induces the recruitment of monocytes/macrophages, and that the macrophages, which are associated with the central lacteals, release VEGF-C to promote thereby lymphatic regeneration during the regenerative phase. It is thus likely that reconstruction of the central lacteals is intimately associated with blood vascular restoration through the macrophage-mediated molecular signaling in the reorganization of I/R-injured villous tissue.

Recently, VEGF-A has been reported to induce lymphangiogenesis by directly activating lymphatic endothelial cells (Hirakawa *et al.*, 2003; Hong *et al.*, 2004) and to introduce hematopoietic progenitor cells into the blood vascular formation (Carmeliet, 2003). Conversely, VEGF-C is thought to play dual lymphangiogenic and angiogenic roles under certain conditions (Cao *et al.*, 1998; Carmeliet, 2003). It remains for future studies to investigate whether the molecules exert such actions on lymphangiogenesis and angiogenesis in villous tissue restoration following I/R.

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