Differential expression of endothelin-2 along the mouse intestinal tract

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Abstract

Endothelin (ET)-2, an ET family peptide, is highly expressed in intestine. However, the specific distribution and function of ET-2 remain unknown. We elucidated the expression profile and localization of ET-2 in mouse gastrointestinal tract. Real-time PCR analysis revealed that ET-2 gene expression in the gastrointestinal tract of healthy animals was relatively high in the colon. Immunohistochemical analysis revealed ET-2-like immunoreactivity mainly in epithelial cells of the mucosa throughout the intestinal tract of healthy animals. Intracellularly, ET-2 was concentrated close to the basement membrane of intestinal epithelial cells. A weak ET-2-like immunoreactivity was also localized to some neurofibers and the myenteric plexus of the muscle layer, coexpressing with vasoactive intestinal peptide. ET-2-like immunoreactivity was also detected at Brunner's glands of the duodenum and follicle-associated epithelium of Peyer's patch. In contrast, ET-1-like immunoreactivity was uniformly distributed in epithelial cells. In dextran sulfate sodium (DSS)-induced colitis, colonic ET-2 was upregulated during the late stage of DSS treatment. These results suggest that in intestinal epithelial cells ET-2 could be secreted into the lamina propria and the dome region in Peyer's patch, and that it might modulate immune cells in these sites for mucosal defense.

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Introduction

The endothelin (ET) system comprises three ligands, ET-1, ET-2 and ET-3, as well as two G-protein-coupled receptors, the ET A receptor (ETA) and the ET B receptor (ETB). The ligands (ETs), 21-amino acid peptides, have potent vasoconstrictive activity and control many cellular processes including gene expression, differentiation, apoptosis and cell growth (Kedzierski & Yanagisawa 2001). ETs produce their biological effects via activation of the two receptor subtypes. Whereas ETA binds ET-1 and ET-2 under physiological conditions, ETB binds all three ETs with equal affinity.

Mammalian ET-2 has a mouse and rat ortholog known as vasoactive intestinal contractor (VIC). VIC differs from human ET-2 by one amino acid out of 21 amino acid residues: Asn-4 is replaced with Ser-4 in human ET-2 (Inoue *et al.* 1989, Saida *et al.* 1989, 2000, Bloch *et al.* 1991). In this article, we use the term murine ET-2 in place of VIC. Structurally, ET-2 isoforms are highly homologous to ET-1 (Inoue *et al.* 1989, Saida *et al.* 1989). Previous studies showed that the ET-2 gene is strongly expressed in and largely limited to the

gastrointestinal tract, sex organs and pituitary gland (Saida *et al.* 1989, Fang *et al.* 1994, Uchide *et al.* 2000a,b, Masuo *et al.* 2003), whereas ET-1 is expressed in many tissue types. As the receptor selectivity of ET-2 is similar to that of ET-1, many of the biological activities of ET-2 overlap with those of ET-1. However, several recent reports demonstrate ET-2-specific functions distinct from those of ET-1. First, hypoxia upregulates ET-2 mRNA in breast tumor cell lines, and ET-2 acts as a hypoxia-induced autocrine survival factor for breast tumor cells (Grimshaw *et al.* 2002a). Second, ET-2 is a chemoattractant for macrophages and may modulate macrophage distribution in tumors (Grimshaw *et al.* 2002b). Third, ET-2 may be involved in the invasion of tumor cells (Grimshaw *et al.* 2004). Finally, ET-2 is one of four candidate tumor markers in uveal melanoma cell lines and may be involved in uveal melanoma development (Zuidervaat *et al.* 2003).

Several studies concerning the ET system in the intestine have been reported. ET-1 mRNA was detected in the epithelium, macrophages and stromal cells of the lamina propria in human large intestine (Egidy *et al.* 2000, Massai *et al.* 2003). ET-1 and ET-3 peptides

are localized to the mucosa in rat intestine (Takahashi *et al.* 1990). ET-1-like immunoreactivity also was observed in the neurons of human colon, and it has been suggested that ET-1 modulates intestinal motility and secretion, acting as a neuropeptide (Inagaki *et al.* 1991, Escrig *et al.* 1992). Studies with mice deficient in ET-3 have demonstrated that ET-3 is essential for the development of enteric neurons (Baynash *et al.* 1994). Although it is reported that ET-2 mRNA is predominantly expressed in stromal cells of the lamina propria (de la Monte *et al.* 1995), no detailed information on the expression and distribution of ET-2 peptide is available.

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the intestine that is considered to arise from dysregulation of mucosal immunity. In human IBD, the tissue ET-1 level was found to be elevated (Murch *et al.* 1992). A recent report showed that ET-2 gene expression was more predominant than ET-1 in human colon, although the ET-2 mRNA level was not significantly changed in human IBD (McCartney *et al.* 2002). In an animal model of IBD, 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in rat, the gene expression of ET-1 was predominant over ET-2 gene expression (McCartney *et al.* 2002). An ET antagonism study showed that the ET system is involved in tissue injury due to TNBS-induced colitis (Hogaboam *et al.* 1996). Although it has been shown that ET-1 is involved in the pathogenesis of IBD, little is known regarding the pathological role of ET-2.

In this study, in an attempt to elucidate the specific physiological and pathophysiological roles of ET-2 *in vivo*, we determined the localization of ET-2 in mouse intestinal tract and compared it with that of ET-1. In addition, we elucidated the differences in colonic ET-2-like immunoreactivity between normal mice and mice with dextran sulphate sodium (DSS)-induced colitis. The implication of these observations is discussed.

Materials and methods

Animals

Male ICR mice (7–12 weeks old) were purchased from Japan Clea (Tokyo, Japan) and were housed under conventional conditions. This study was approved by the Animal Care Committee at the National Institute of Advanced Industrial Science and Technology, Japan.

Quantitative real-time PCR

Total RNA was isolated from six segments of the mouse gastrointestinal tract (stomach, duodenum, jejunum, ileum, colon and rectum) with Isogen (Nippon Gene, Tokyo, Japan). cDNA was synthesized using an RNA PCR kit (AMV ver.2.1; TaKaRa Bio, Shiga, Japan). Real-time PCR was performed as previously described

(Uchide *et al.* 2000b). Real-time PCR for ET-1, ET-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed using the TaqMan PCR Core Reagent Kit (Applied Biosystems, Foster City, CA, USA). Composition of the forward and reverse primers and the TaqMan probe for ET-1, ET-2 and GAPDH are as follows: ET-1 forward primer, 5'TTC CCG TGA TCT TCT CTC TGC T3'; ET-1 reverse primer, 5'TCT GCT TGG CAG AAA TTC CA3'; ET-1 probe, 5'-FAM-ACA AGG AGT GTG TCT ACT TCT GCC ACC TGG-TAMRA-3'; ET-2 forward primer, 5'CTG CGT TTT CGT CGT TGC T3'; ET-2 reverse primer, 5'TGC AGC TCA TGG TGT TAT CTC TTC3'; ET-2 probe, 5'-FAM-CTG CAA CTC CTG GCT TGA CAA GGA A-TAMRA-3'; GAPDH forward primer, 5'CTT CAC CAC CAT GGA GAA GGC3'; GAPDH reverse primer, 5'GGC ATG GAC TGT GGT CAT GAG3'; GAPDH probe, 5'-FAM-CCT GGC CAA GGT CAT CCA TGA CAA CTT T-TAMRA-3'. Real-time PCR was monitored on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Reaction conditions were 95 °C for 10 min followed by 50 cycles of the amplification step (95 °C for 20 s and 60 °C for 2 min). Gene-expression levels of ET-1, ET-2 and GAPDH were calculated using standard curves. ET-expression levels were normalized to GAPDH and presented as gene-expression rates:

ET expression = (amount of ET-1 or ET-2 mRNA) / (amount of GAPDH mRNA) × 100.

Induction of colitis

Male 7-week-old ICR mice were used in this study. Experimental colitis was induced by oral administration of 5% (w/v) DSS (molecular mass, 40 000 Da; ICN Biomedicals, Aurora, OH, USA) in distilled water *ad libitum* for 7 days.

Immunohistochemistry

Gastrointestinal segments were fixed with 2% paraformaldehyde/15% saturated picric acid in 0.15 M sodium phosphate buffer (pH 7.3, 4 °C, 2 h) and embedded in Optimal cutting temperature (OCT) compound (Tissue-Tek, Torrance, CA, USA). The immunohistochemistry was performed as follows. For ET-2 detection, the 8 µm-thick sections were blocked with 5% heat-inactivated normal goat serum/0.1% sodium azide/PBS; the primary antibody was rabbit anti-ET-2 antibody (Yanaihara Institute, Shizuoka, Japan; diluted 1:64; 37 °C, 2 h) and the secondary antibody was biotin-labeled goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA; diluted 1:250; 37 °C, 30 min). The sections were treated with 0.3% hydrogen peroxide in methanol (room temperature, 30 min) after the primary antibody reaction. Subsequent

immunostaining procedures were performed using the peroxidase Vectastain *Elite* ABC Kit (Vector Laboratories) and the DAB Substrate Kit (Vector Laboratories) as a substrate, according to the manufacturer's recommendations. For ET-1 detection, the sections were blocked by the same procedure used for ET-2; the primary antibody was rabbit anti-ET-1 antibody (code 18201; Immuno-Biological Laboratories, Gunma, Japan; cross reactivity, ET-1, 100%; ET-2, 1%; ET-3, 0.4%; big ET-1, 100%; big ET-2, 0.2%; big ET-3, 0.4%; VIC, 0.2%, 1:10; room temperature, 1 h) and the secondary antibody was the same as that used for detection of ET-2 (room temperature, 30 min). The sections were treated with 0.3% hydrogen peroxide in methanol (room temperature, 30 min) before the blocking reaction. The avidin-biotin complex method was performed with the peroxidase Vectastain ABC Kit, and the substrate was the same as that used for ET-2 detection. Sections were counterstained with Alcian Blue. To demonstrate that the immunohistochemical reaction was specific, the diluted antibody was absorbed onto VIC or ET-1 peptide-immobilized Affi-Gel 10 agarose beads (Bio-Rad, Hercules, CA, USA) prior to immunostaining.

Immunodoublefluorescence

Sections were incubated with rabbit anti-ET-2 antibody (1:64) and guinea pig anti-vasoactive intestinal peptide (VIP) serum (Euro-Diagnostica, Medeon, Sweden; diluted 1:1000; 4 °C, overnight). After washing with PBS, fluorescein isothiocyanate (FITC)-labeled donkey anti-rabbit IgG (Chemicon, Temecula, CA, USA; diluted 1:200) and rhodamine isothiocyanate (RITC)-labeled donkey anti-guinea pig IgG (Chemicon; diluted 1:200) were applied (37 °C, 30 min). After staining, sections were mounted with Vectashield Mounting Medium (Vector Laboratories). Confocal microscopy was performed on a Zeiss LSM410 microscope (Carl Zeiss, Oberkochen, Germany).

Lectin staining

For detection of M-cells in Peyer's patch, we performed lectin staining of Peyer's patch by the method of Giannasca *et al.* (1994) with minor modification. Cryosections were blocked with 1% BSA/PBS (room temperature, 2 h) and incubated with 50 µg/ml RITC-labeled *Ulex europaeus* agglutinin type I (UEA-I; EY Laboratories, San Mateo, CA, USA; room temperature, 30 min). The specificity of lectin binding was assessed by preincubation of RITC-labeled UEA-I with its cognate monosaccharide, α -L-fucose at a concentration of 0.2 M for 2 h before reaction with sections. After washing with PBS, immunofluorescence for ET-2 was performed by the method described above.

In situ hybridization

In situ hybridization using digoxigenin-labeled riboprobes was performed with an ISHR Starting Kit (Nippon Gene). The cloned ET-2 cDNA plasmid (pCR-Script SK+; Stratagene, La Jolla, CA, USA) was used as a template for synthesizing the riboprobes. Digoxigenin-labeled riboprobes were synthesized using the DIG RNA Labeling Kit (Boehringer Mannheim, Mannheim, Germany). Excised intestinal (duodenal) tissue was fixed with 4% paraformaldehyde/PBS (4 °C, overnight) and embedded in OCT compound. The 10 µm-thick cryosections were treated with 0.2 M HCl (room temperature, 20 min), 5 µg/ml proteinase K (Boehringer Mannheim) in PBS (37 °C, 10 min) and 2 mg/ml glycine in PBS (room temperature, 15 min), and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine hydrochloride (pH 8.0; room temperature, 15 min). After prehybridization with 2 × SSC containing 50% formamide (50 °C, 30 min), the sections were hybridized with hybridization buffer (50% formamide, 2 × SSC, 1 µg/µl tRNA, 1 µg/µl sonicated salmon sperm DNA, 1 µg/µl BSA and 10% dextran sulfate) containing 1 µg/µl digoxigenin-labeled riboprobe (50 °C, overnight). The sections were incubated with alkaline phosphatase-labeled anti-digoxigenin antibody (Boehringer Mannheim; 1:500), and visualized with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolylphosphate. Sections were counterstained with Methyl Green.

Statistical analysis

The results are shown as means $\pm$ S.D. and analyzed by Scheffé's *F* test to identify significant differences among the data. *P* values lower than 0.05 were considered significant.

Results

Gene-expression profiles of ET-2 and ET-1 in normal mouse gastrointestinal tract

To assess the expression of the ET-2 gene, we performed real-time PCR analysis of ET-2 in normal mouse gastrointestinal tract, and compared the results with those for ET-1 (Fig. 1). While the gene expression of ET-1 was higher than that of ET-2 in the stomach, ET-2 gene expression exceeded that of ET-1 in the ileum ($P < 0.05$). Among the tissues of the gastrointestinal tract, the level of ET-2 was significantly higher in the colon than in the stomach ($P < 0.05$).

Localization of ET-2 in normal mouse intestinal tract

To determine the localization of ET-2, we examined the immunohistochemistry of ET-2 in normal mouse

Mouse Gastrointestinal Tract

Figure 1 Gene-expression profiles of ET-2 and ET-1 in normal mouse gastrointestinal tract. The gene-expression levels of ET-2 (■) and ET-1 (□) were quantified by real-time PCR. St, stomach; Du, duodenum; Je, jejunum; Il, ileum; Co, colon; Re, rectum. The results are shown as means±S.D (n=4). Statistically significant differences between groups are shown; **P*<0.05.

intestinal tract: colon (Fig. 2A and C), ileum (Fig. 3A, B and C), Peyer's patch (Fig. 3D and E) and duodenum (Fig. 3F and G). Within the intestinal tract, most of the ET-2-like immunoreactivity was localized to the mucosal layer, observed mainly in epithelial cells (Figs 2A, 3A and 3F). Most epithelial cells showed intracellular ET-2 to be concentrated in the vicinity of the basement membrane throughout the intestinal tract: colon, ileum, Peyer's patch and duodenum (Figs 2A, 3A, 3D and 3F). However, ET-2-like immunoreactivity was very weak at the lamina propria (Fig. 3A and F). Compared with the mucosal layer, ET-2-like immunoreactivities of the submucosa and muscle layers were weak (Figs 2C, 3B and 3F). In contrast, colonic ET-1-like immunoreactivity was uniformly distributed in the epithelial cells, and surface epithelial cells showed a higher concentration of ET-1 (Fig. 2B). In addition, ET-1-like immunoreactivity was also observed in scattered neurofibers and myenteric plexus of the muscle layer (Fig. 2D). At other intestinal sites (ileum, Peyer's patch and duodenum), ET-1 showed a similar localization pattern as in the colon (data not shown).

Immunohistochemical analysis of the colon revealed that the highest ET-2-like immunoreactivity was located at the tips of surface epithelia (Fig. 2A), as was observed for ET-1 (Fig. 2B). Along the bottom-up axis of colonic crypt, a gradient-like expression of ET-2 was observed (Fig. 2A). Whereas the top of colonic crypt showed higher expression of ET-2, the bottom of colonic crypt showed lower expression of ET-2 (Fig. 2A). In the ileum, it was more obvious than in the colon (Fig. 2A) that the

highest concentration of ET-2 occurred in the vicinity of the basement membrane in epithelial cells (Fig. 3A). Along the villus-crypt axis in the ileum, a gradient-like expression of ET-2 was observed (Fig. 3A). However, the gradient-like pattern of ileal ET-2 was different from that of colon. Whereas ileal ET-2 was expressed more strongly at the bottom of the villus-crypt axis than at the top, colonic ET-2 was expressed higher at the top of the crypt than at the bottom (Figs 2A and 3A). Immunohistochemical studies along the transverse section also supported this finding (Fig. 3C). In Peyer's patch, ET-2 immunostaining showed that whole cells of the follicle-associated epithelium (FAE) were stained at the highest level, whereas the lymphoid follicle was not stained (Fig. 3D). FAE consists of two kinds of cells, absorptive enterocytes and M-cells. M-cells are the center of mucosal immunity and are specifically stained by the α -L-fucose-binding lectin UEA-I (Giannasca *et al.* 1994). To confirm distribution of ET-2 in M-cells, we stained M-cells with RITC-labeled UEA-I and analyzed the immunofluorescence of ET-2. Immunofluorescence analysis revealed the presence of ET-2 in M-cells (Fig. 3E). In FAE, ET-2 was localized to the vicinity of the basement membrane, as observed in other intestinal tissues. In the duodenum, villous epithelial cells showed the highest ET-2-like immunoreactivity, and Brunner's glands showed the second highest (Fig. 3F and G).

Colocalization of ET-2 with neuronal marker VIP in myenteric plexus and muscular neurofibers

To confirm ET-2-like immunoreactivity in the intestinal muscle layer, we performed immunodoublefluorescence of ET-2 and VIP in the colonic muscle layer. VIP is a neuropeptide that expresses in enteric neurons including scattered neurofibers and the myenteric plexus (Sundler *et al.* 1988). We used this peptide as a neuronal marker and compared its localization with that of ET-2. ET-2-like immunoreactivity was found in some scattered neurofibers and the myenteric plexus of the colonic muscle layer (Fig. 2E), although immunoenzymatic detection of ET-2 showed very weak immunoreactivity in the intestinal muscle layer (Figs 2C and 3B). In some ET-2-positive scattered neurofibers and the myenteric plexus, VIP was colocalized (Fig. 2 G).

Localization of ET-2 mRNA in mouse duodenum

To determine the cellular distribution of ET-2 mRNA, we performed *in situ* hybridization for ET-2 in mouse duodenum. ET-2 mRNA was located predominantly in epithelial cells (Fig. 3H). ET-2 mRNA was also observed at the myenteric plexus (Fig. 3H). However, ET-2 mRNA was not detected in stromal cells of the lamina propria (Fig. 3H). No signal was detected in the sections by a sense probe (data not shown). Together with the

Figure 2 Immunohistochemistry of ET-2 and ET-1 in normal mouse colon. (A–D) Immunohistochemistry of ET-2 and ET-1. The longitudinal sections of colonic mucosa (A, B) and colonic muscle (C, D) were immunostained with an anti-ET-2 antibody (A, C) or an anti-ET-1 antibody (B, D). Blue color shows staining with Alcian Blue. Scale bars, 100 µm (A, B) and 50 µm (C, D). (E–G) Immunofluorescence of ET-2 and VIP. The longitudinal sections of colonic muscle (E–G) were double-stained with an anti-ET-2 antibody (E, G; green) and an anti-VIP antiserum (F, G; red). The merged images of each co-staining are depicted in panel G (yellow). Some scattered neurons (arrows) and myenteric plexus (arrowheads) were stained with ET-2 and VIP. Scale bars, 100 µm.

immunohistochemical studies (Fig. 3F), we conclude that duodenal epithelial cells highly express ET-2 mRNA and peptide.

Upregulation of ET-2 during the late stage of DSS treatment

To assess whether the localization pattern of intestinal ET-2 changes under pathological conditions, we analyzed the localization pattern of colonic ET-2 during DSS-induced colitis. Downregulation of ET-2 peptide was noted at the early stage (day 1) of DSS-induced colitis (Fig. 4C), compared with a normal control (day 0,

Fig. 4A). The ET-2 peptide level increased in colonic epithelial cells during the late stage (days 3–7) of DSS treatment (Fig. 4E, G and I), compared with a normal control (day 0, Fig. 4A). From day 1 to day 5 inflammation was not detected clearly (Fig. 4D, F and H). At day 7, evidence of inflammation – infiltration of inflammatory cells and focal disappearance of mucosal crypts – was observed (Fig. 4J).

Discussion

We have determined for the first time the cellular distribution of ET-2 mature peptide in mouse intestinal

Figure 3 Localization of ET-2 mRNA and peptide in normal mouse small intestine. (A–C) Immunohistochemistry of ET-2 in normal mouse ileum. The longitudinal section of ileal mucosa (A), ileal muscle (B) and transverse sections of the villus in ileum (C) were immunostained by an anti-ET-2 antibody. Blue color shows staining with Alcian Blue. Scale bars, 50 µm (A) and 25 µm (B, C). (D, E) Localization of ET-2 in Peyer's patch. (D) Immunohistochemistry of ET-2. Blue color shows staining with Alcian Blue. FAE, Follicle Associated Epithelium; LF, lymphoid follicle. (E) Double-staining of ET-2 and UEA-I-binding sugar (M-cell marker). Peyer's patch was double-stained with anti-ET-2 antibody (green) and RITC-labeled UEA-I (red). Scale bars, 100 µm (D) and 25 µm (E). (F–H) Distribution of ET-2 mRNA and peptide in duodenum. (F, G) Immunohistochemistry of ET-2. Blue color shows staining with Alcian Blue. BG, Brunnel's gland. (H) *In situ* hybridization of ET-2. In the section hybridized with an antisense probe, ET-2 mRNA signals (dark brown, arrow) were found predominantly around the nuclei (green) of epithelial cells. ET-2 mRNA was also localized in the myenteric plexus (arrowhead). Scale bars, 100 µm (F) and 50 µm (G, H).

tract. ET-2 peptide is predominantly localized to the epithelial cells of the mucosal layer in the intestinal tract (Figs 2 and 3). This finding corresponds to the localization of ET-2 mRNA (Fig. 3H). In particular, intestinal ET-2 is concentrated in the vicinity of the basement membrane in epithelial cells. On the contrary, ET-1 is distributed widely in many cells of both the mucosal and muscle layers at all intestinal sites. The distribution of ET receptors in intestine was analyzed

by ^{125}I -ET-1 ligand-binding assay (Koseki *et al.* 1989, Inagaki *et al.* 1991, Okabe *et al.* 1995). These reports revealed that ET-1-binding sites are localized mainly in the mucosal layer and the myenteric plexus. These findings suggest that both ET receptors, ETA and ETB, are localized in these regions of the intestine. We also analyzed the distribution of ETA and ETB in intestine by immunohistochemistry. Whereas ETA is widely and uniformly distributed in both the mucosal and muscle

Figure 4 Immunohistochemistry of ET-2 in normal and DSS-treated colon. Sections were treated with anti-ET-2 antibody (A, C, E, G, I). Consecutive sections were stained by hematoxylin and eosin (HE; B, D, F, H, J). (A, B) Normal mouse colon (day 0; without DSS treatment). (C–J) DSS-treated mouse colon at day 1 (C, D), day 3 (E, F), day 5 (G, H) and day 7 (I, J). In all cases ET-2 was detected exclusively in epithelial cells. The expression of ET-2 increased in epithelial cells during late stages of DSS treatment. Representative images are shown ($n=5$). Scale bars, 50 μ m.

layers (Satoshi Takizawa, Javier Adur & Kaname Saida, unpublished observations), ETB is expressed in the nuclei of epithelial cells, stromal cells in the lamina propria and neurons in the muscle layer (Takizawa *et al.* 2004). A recent report showed that ETB is localized in nuclei and may function as an 'intracrine' receptor for intracellular ET ligands (Boivin *et al.* 2003). These results

demonstrate that the cellular distribution of ET-2 corresponds to the distribution of its receptors in the intestinal tract. Thus it is suggested that ET-2 functions in an intracrine/autocrine/paracrine fashion in the intestinal tract and may modulate intestinal function.

Previous reports (de la Monte *et al.* 1995, Liu *et al.* 1998) presented localization patterns for rat ET-2 mRNA and precursor peptide (big ET-2) in the gastrointestinal tract that differed from the patterns we observed for mouse (this study). These earlier studies revealed that rat ET-2 mRNA and big ET-2 were expressed mainly in stromal cells of lamina propria. On the contrary, we found that mouse ET-2 mRNA and mature peptide were expressed predominantly in epithelial cells of intestinal tract, and displayed very weak signals in stromal cells of lamina propria. For both mouse and rat, the correspondence between the distribution of mRNA and peptide is consistent. Moreover, de la Monte *et al.* (1995) determined the gene-expression profiles of rat ET-2 and ET-1 in gastrointestinal tract by RNase-protection assay (de la Monte *et al.* 1995). We analyzed the same expression profiles using real-time PCR (Uchide *et al.* 2000a), and our rat data are largely consistent with those of de la Monte *et al.* Earlier we had determined the gene-expression pattern of mouse ET-2 in various tissues by semi-quantitative reverse transcriptase PCR and showed that the gene expression of mouse ET-2 in the intestine was higher than in the stomach (Uchide *et al.* 1999). These data also support our current data. However, the gene-expression profiles of mouse ET-2 and ET-1 in gastrointestinal tract are somewhat different from those of rat (Fig. 1). We speculate that the differences in expression profiles between rat and mouse may arise from species differences or depend on unidentified physiological conditions. Further analysis is needed to determine the reasons for these differences.

The localization of epithelial ET-2 leads us to a hypothesis for ET-2-specific functions in the intestine. First, basal localization of ET-2 indicates that ET-2 may be secreted from the basement membrane of epithelial cells into the lamina propria where it could modulate the mucosal immune system. Takahashi-Iwanaga *et al.* (1999) reported that the basement membrane of epithelial cells has many small pores running along the entire length of the villus in mouse intestine, and that the epithelial cells interact with macrophages in the lamina propria through these pores. In addition, a recent report demonstrated that ET-2 is a chemoattractant for macrophages (Grimshaw *et al.* 2002b). Moreover, ETs have immunomodulating activity and control Th1/Th2 cytokine balance in mast cells (Coulombe *et al.* 2002). These findings suggest that ET-2 could be secreted from colonic epithelial cells and FAE in Peyer's patch into the lamina propria and lymphoid follicle, respectively, where it might modulate the immune system for mucosal

defense. As M-cells in Peyer's patch are the center of mucosal immunity, ET-2 in M-cells might play a crucial role in antigen presentation. Second, ET-2 might be involved in the apoptosis of colonic surface epithelial cells. Interestingly, ET-2 and ET-1 are expressed most highly in these cells, and it is well known that these cells are apoptotic. In addition, Lauber *et al.* (2003) showed that apoptotic cells secrete the chemoattractant factor lysophosphatidylcholine, which stimulates the attraction of phagocytes. This study suggests that apoptotic cells present attraction signal to ensure their efficient removal after cell death. It is possible that ET-2 could be secreted from apoptotic cells and might function as chemoattractant signal for phagocytes. Third, ET-2 might be involved in the differentiation of epithelial cells. It is well known that intestinal epithelial cells migrate toward the intestinal lumen, and the differentiation of epithelial cells in the small intestine and the colon depend on the position along the villus-crypt axis of the small intestine and the bottom-up axis of the colonic crypt, respectively. In addition, we observed that the intestinal ET-2-like immunoreactivity exhibits a gradient-like pattern along the villus-crypt axis of the small intestine and also along the bottom-up axis of colonic crypt (Figs 2A and 3A). These observations suggest that ET-2 might control the differentiation of intestinal epithelial cells through its gradient-like expression.

In the muscle layer, ET-2 is localized to some VIP-positive scattered neurons and the myenteric plexus. Previous reports showed that ET-2 has contractile activity in the ileum (Ishida *et al.* 1989) and in cultured human aortic smooth muscle cells (Iwashima *et al.* 1997). Thus, it is suggested that ET-2 in the muscle layer acts as a neuropeptide and controls smooth muscle contraction.

We further speculate on other ET-2 functions in the intestinal tract. In the duodenum, ET-2 is observed at the highest level in epithelial cells, and at the second highest in Brunner's gland (Fig. 3F and G). Brunner's glands secrete alkaline mucus to neutralize acidic gastric juice. Thus, it is suggested that ET-2 plays a role in the neutralization function of Brunner's glands.

The expression of ET-2 during DSS-induced colitis was also determined. DSS-induced colitis is an experimental animal model of human IBD and resembles ulcerative colitis (Okayasu *et al.* 1990). Colonic ET-2 was upregulated during the late stage of DSS treatment, although the localization pattern of ET-2 was basically the same in the DSS-treated mouse colon as in normal colon (Fig. 4). As described above, it is suggested that upregulated ET-2 is secreted from the basement membrane into the lamina propria during DSS-induced colitis, and that it attracts macrophages to the lamina propria for mucosal defense. This study also showed that ET-2 was expressed in morphologically preserved epithelial cells during DSS-induced colitis, and we can

use ET-2 as an epithelial marker for scoring histological damage.

In conclusion, we have revealed that ET-2-like immunoreactivity is localized mainly to epithelial cells of the mucosal layer in the intestinal tract. Intracellularly, intestinal ET-2 is concentrated in the vicinity of the basement membrane. These findings suggest that ET-2 may be secreted from the basement membrane of epithelial cells. Furthermore, ET-2 may act as a chemoattractant for macrophages to the lamina propria and might modulate the function of immune cells for mucosal defense. Further study is needed to confirm this premise and support ET-2 as a potential therapeutic target for intestinal disorders such as IBD.

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