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Endothelin system in intestinal villi: A possible role of endothelin-2/vasoactive intestinal contractor in the maintenance of intestinal architecture

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ABSTRACT

The endothelin system consists of three ligands (ET-1, ET-2 and ET-3) and at least two receptors (ETA and ETB). In mice ET-2 counterpart is a peptide originally called "vasoactive intestinal contractor" (VIC) for this reason, this peptide is frequently named ET-2/VIC. In intestinal villi, fibroblasts-like cells express endothelin's receptors and response to ET-1 and ET-3 peptides, changing their cellular shape. Several functions have been attributed to these peptides in the "architecture" maintenance of intestinal villi acting over sub-epithelial fibroblasts. Despite this, ET-2/VIC has not been analyzed in depth. In this work we show the intestine gene expression and immunolocalization of ET-1, ET-2 and the ETA and ETB receptors from duodenum to rectus and in the villus–crypt axis in mice, allowing a complete analysis of their functions. While ET-1 is expressed uniformly, ET-2 had a particular distribution, being higher at the bottom of the villi of duodenum, ileum and jejunum and reverting this pattern in the crypts of colon and rectus, where the higher expression was at the top. We postulated that ET-2 would act in a cooperative manner with ET-1, giving to the villus the straight enough to withstand mechanical stress.

1. Introduction

The three components (ET-1, ET-2 and ET-3) of endothelin system are 21-residues cyclic peptides with two disulfide bridges established between cysteine residues located in position 1–15 and 3–11. ET-2 and ET-3 differ from ET-1 in two and six amino acid residues, respectively. In mice, a homolog of ET-2 that only diverges in one amino acid residue was called vasoactive intestinal contractor (VIC) [1]. Endothelins are important mediators of several physiological processes, mainly in regulation mechanisms of cardiovascular, renal and pulmonary functions [2–4]. Recently, it has been determined that this system would also act in other parts of the body, including reproductive and endocrine systems [5–7]. Furthermore, endothelin axis is implicated in patho-physiological processes including cardiovascular, pulmonary and renal diseases and other important biological processes such as development, cancer, wound healing and even neurotransmission [8–10]. The actions of these peptides are mediated by their interaction with specific receptors that are classified as: ETA, ETB and ETC receptor subtypes [11,12]. The ETA receptor subtype has high affinity for ET-1 and ET-2 and low affinity for ET-3, while the ETB receptor subtype has similar affinities for ET-1, ET-2 and ET-3 [13,14]. The ETC receptor subtype found in *Xenopus* has a higher affinity for ET-3 than for ET-1 and ET-2 [12].

ET-1 is the most potent vasoconstrictor factor known and could be implicated in the maintenance of basal vasomotor tone and blood pressure in humans [15,16]; it also has mitogenic activity acting via receptors and stimulating the production of cytokines and growth factors [17]. ET-1 has also been involved in facilitating several aspects of cancer grow and progression [9]. Even though ET-2/VIC shares many of the biological activities that have been attributed to ET-1, it has been demonstrated to have specific functions. ET-2/VIC is stimulated by hypoxia [18], is a chemoattractant for macrophages [19] and could be implicated in tumor cell invasion [20]. Furthermore, in ovary has been attributed a putative role to ET-2 since elevated ET-2 triggered by Luteinizing Hormone surge and hypoxia may facilitate the corpus luteum formation by promoting angiogenesis, cell proliferation and differentiation [21]. Different gene expression studies in adult mice have shown

Abbreviations: ET, endothelin; ET-1, endothelin 1; ET-2, endothelin 2; ET-3, endothelin 3; ETs, endothelins; VIC, vasoactive intestinal contractor; ETA, endothelin receptor A; ETB, endothelin receptor B; ETC, endothelin receptor C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OCT, optimal cutting temperature; PBS, phosphate buffer solution; IgG, immunoglobulin G; FITC, fluorescein isothiocyanate; DAB, diaminobenzidine tetrahydrochloride.

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that endothelins – given their presence in vascular endothelium – are distributed in virtually all organs [22–24]. ET-1 has the highest expression in lungs [25] while ET-2/VIC reaches highest levels in stomach, ovaries, intestine and lungs [25–28,21]. ET-3 is found in high concentrations in neural tissue [29] where it plays an important role in cellular proliferation and development. It is also produced in renal tubular epithelial cells and intestine [30] where it causes increases the proliferation of epithelial cells and survival of goblet cells [31].

The expression and localization of ET-2/VIC and ET-1 was studied in the whole intestinal tract segments of normal mouse. Gene expression profile of ET-2/VIC was higher than ET-1 except in the colon and rectus [32]. Immunolocalization of ET-2/VIC was observed mainly in epithelial cells concentrated in the vicinity of the basement membrane while ET-1 immunoreactivity was uniformly distributed in epithelial cells. Regarding to the receptors, it is known that ETB is localized mainly closed to the nuclei of villus epithelial cells [33]. Although other studies concerning the ET system in the intestine have been reported, until now, the gene expression and immunolocalization of endothelins on intestine along the villus–crypt and the duodenum–colon axes has not been deciphered altogether. In this study, using real-time PCR immunohistochemistry and immunofluorescence techniques we have elucidated the gene expression levels and the regional localization of endothelin system (ET-1, ET-2/VIC and their receptors ETA and ETB) in mice intestine. The analysis of these findings could highlight a putative key role of ET-2/VIC in maintenance normal functions of intestinal villi.

2. Materials and methods

2.1. Animals

Adult male ICR mice ($n = 5$) between eight and thirteen weeks old and 10–30 g body weight, were purchased from Japan Clea (Tokyo, Japan). Mice were killed by cervical dislocation. Segments of intestine (duodenum, jejunum, ileum, colon and rectum) were removed. Our experimental procedures were in accordance with the Guidelines on Handling of Laboratory Animals for our institution.

2.2. Quantitative real-time PCR

Total RNA was isolated from the intestine segments with Isogen (Nippon Gene, Tokyo, Japan). The cDNA was synthesized using an RNA PCR kit AMV (Takara Biomedicals, Japan). Real-time PCR was performed as previously described [34]. Real-time PCR for ET-1, ET-2, ETA, ETB and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed using the TaqMan PCR Core Reagent kit (Perkin-Elmer, Applied Biosystems, Foster City, USA). Composition of the forward and reverse primers for ET-1, ET-2/VIC, ETA, ETB and GAPDH are listed in Table 1. The process was performed on an ABI Prism 7700 (Perkin-Elmer-Applied Biosystems, Foster City, CA, USA). Reaction conditions were 95 °C for 10 min followed by 50 cycles of the amplification step (95 °C for 20 s and 62 °C for 2 min). Gene expression levels were calculated using standard curves, normalized to GAPDH and presented as gene expression rates as previously described [32].

2.3. Immunohistochemistry

Intestinal 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 immunoreactions were made on 8 μm-thick cryostat (HM500-OM, Microm, Germany) sections. To avoid nonspecific reactions, sections were blocked with heat-inactivated normal goat serum/0.1% sodium azide/PBS. Immunostain was carried out as previously described [35]. The antibodies used were rabbit polyclonal immunoglobulin G (IgG) antibodies (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan) against ET-1 (diluted 1:50), ET-2/VIC (diluted 1:64), ETA (diluted 1:10), and ETB (diluted 1:50). All reactions were visualized by diaminobenzidine tetrahydrochloride (DAB). Sections were counterstained with Alcian Blue. Control staining was carried out jumping the primary antibody to the dilution buffer.

2.4. Immunofluorescence

The procedure was similar to the applied for immunofluorescence but the secondary antibodies were donkey IgG antibodies FITC-conjugated (Chemicon, Temecula, CA, USA) diluted 1:200, to 37 °C for 30 min. After reaction, the sections were mounted using Vectashield, fluorescence mounting medium (Vector Laboratories).

2.5. Statistical analysis

The results are presented as means ± SD and analyzed by non parametric Mann–Whitney test to determine significant differences among data groups. p values lower than 0.05 were considered statistically significant.

3. Results

In the present report, the expression of ET-2/VIC, ET-1 and their receptors ETA and ETB was regionally discriminated in the mouse intestine. Several studies have shown that in this system ET-3 is also expressed [30,31,36]. Expression rates of ET-2/VIC exceeded those of ET-1 in duodenum, jejunum and ileum, it was similar in colon although in rectus was lower. ET-2/VIC only was significantly higher than ET-1 in ileum ($p < 0.05$). Expression rates of ETA were higher than ETB in colon and rectus, but only in the last, these percentages were statistically significant ($p < 0.05$). The expression rates in the remaining segments analyzed were the opposite way, being the differences statistically significant only in ileum ($p < 0.05$) (see Fig 1).

The ET-2/VIC peptide was mainly distributed in mucosal epithelial cells and weakly in myenteric plexus. The signal was of higher intensity closer to the basement membrane than in the apical border of duodenal and ileum villi, reversing this pattern in the colon.

Table 1
Optimal primers for real-time PCR of murine endothelin system and GAPDH.

Gene	Sense	Antisense
ET-2/VIC	5′-CTGCGTTTTCGTCGTTGCT-3′	5′-TGCAGCTCATGGTGTTATCTCTTC-3′
ET-1	5′-TTCCCGTGATCTTCTCTCTGCT-3′	5′-TCTGCTTGGCAGAAATTCCA-3′
ETA	5′-GCTGGTTCCCTCTTCACTTAAGC-3′	5′-TCATGGTTGCCAGGTTAATGC-3′
ETB	5′-TGTGCTCTAAGTATTGACAGATATCGAG-3′	5′-GGCTGTCTTGTAAAACTGCATGA-3′
GAPDH	5′-CTTCACCACCATGGAGAAGGC-3′	5′-GGCATGGACTGTGGTCATGAG-3′