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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′

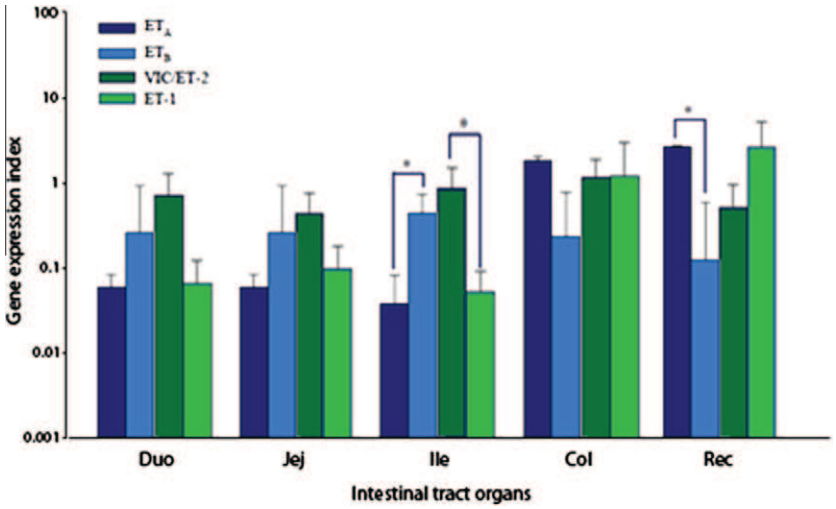

Fig. 1. Gene expression rates of ETA, ETB, ET-2/VIC and ET-1 in duodenum (Duo), jejunum (Jej), ileum (Ile), colon (Col) and rectus (Rec); using real-time PCR and absolute quantification. The gene expression indexes were normalized with GAPDH gene. The results are shown as means ± SD ($n = 4$). Statistically significant differences between groups are shown; $^{*}p < 0.05$.

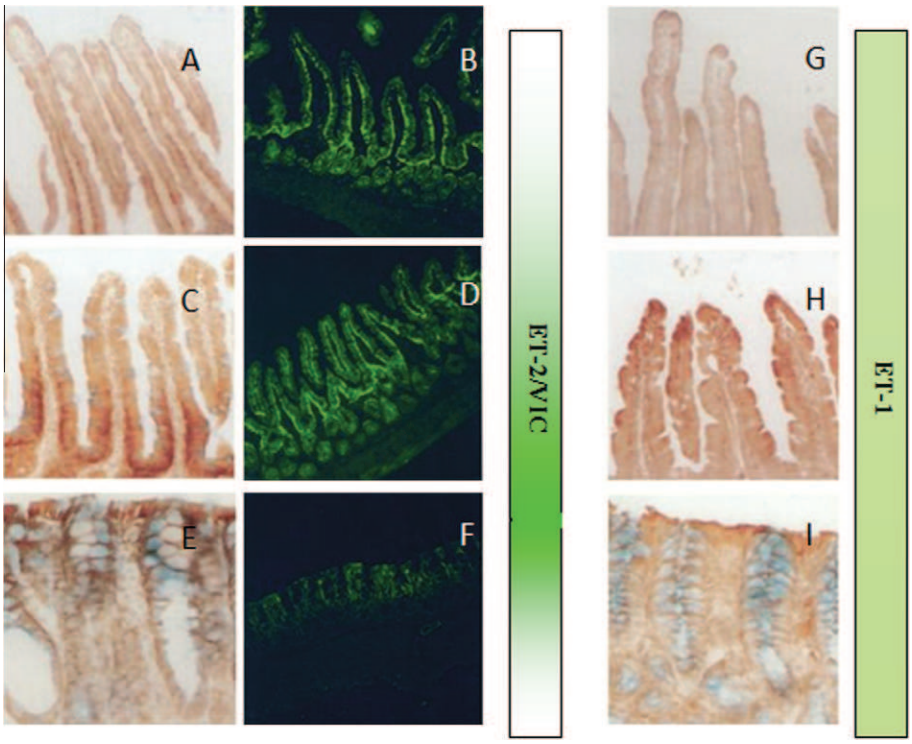

Fig. 2. Immunolocalization of ET-2/VIC and ET-1 peptides in the mouse intestinal tract. Left panel: ET-2/VIC-like immunoreactivity along villus-crypt axis and duodenum-colon axis of (A) duodenum, (B) jejunum, (C) and (D) ileum, (E) and (F) colon. The expression was changing from duodenum to ileum, being higher at the bottom part of the villi to ileum; in colon the pattern inverted it, showing higher expression at the top of the crypts (schematic representation). The immune-detection of ET-2/VIC was increasing from duodenum to colon. Right panel: ET-1-like immunoreactivity along villus-crypt axis and along duodenum-colon axis of (G) duodenum, (H) ileum, (I) colon. ET-1 appeared homogeneously located along intestine. The images (B and D) and (F) of the left were obtained by immunofluorescence whilst the rest were obtained by immunohistochemistry and counterstained with Alcian Blue.

This situation differed to ET-1-like immunoreactivity which appeared uniformly distributed in epithelial cells along intestinal villus axis (see Fig 2). The cellular types that exhibit ET-1 immunoreactions are intestinal epithelial cells, sub-mucosal ganglion cells, myenteric plexus cells, mast cells, macrophage and vascular endothelial cells.

The ETA-like immunoreactivity was widely and uniformly distributed both in the mucosal and muscular layers. However ETB receptor showed remarkable peculiarities. In the mucosal layer, villus epithelial cells, crypt cells, and stromal cells of the lamina propria were immunostained. In agreement with Takizawa and et al. [33], ETB was localized to the nuclei in villus epithelial cells.

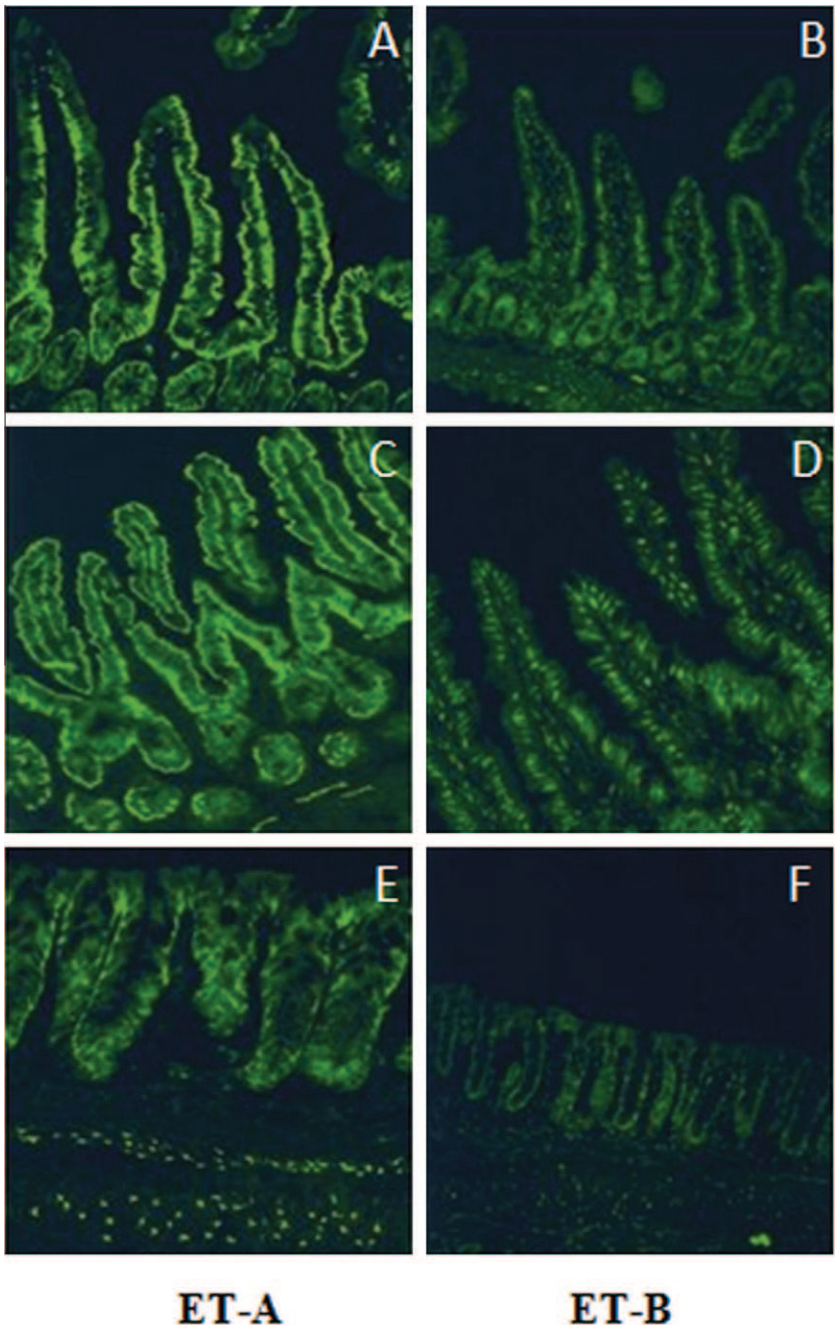

Fig. 3. Immunofluorescence detection of ETA and ETB receptors. (A) Duodenum, (B) jejunum, (C and D) ileum, (E and F) colon. Both receptors were homogeneously distributed in intestinal tract. ET_B was particularly located to the nuclei.

In muscular layer, ETB was found in circular muscle cells and myenteric plexus (see Fig 3).

4. Discussion

The structure and function of the intestine vary along the villus–crypt and the duodenum–colon axes. In the small intestine, sub-epithelial fibroblasts change their cellular shape in three regions: the crypt, the upper and the lower area of the villus. In the last one, cells are flat with broad cell processes, but in the upper area, cells are stellate with several thin processes, suggesting different functions. Numerous slender processes contact with each other, and form a cellular sieve which becomes more porous to the top of the villus [37]. This configuration allows water, nutrients and immune cells penetrate through these pore structures [38]. According with functional and structural differences verified and on the intestinal tract wall, it was observed a marked regionalization of immunolocalization of ET-2/VIC.

In addition to contraction-relaxation functions, and given the particular distribution of endothelin peptides and their receptors [32,33,39,40], it has been postulated that this system would be implicated in the regulation of sieve/barrier function, in the mobility and mechano-sensitivity of intestinal villi. This regulation would be carried out targeting sub-epithelial and lamina propria fibroblasts (see Fig 4). This hypothesis strengthens it because

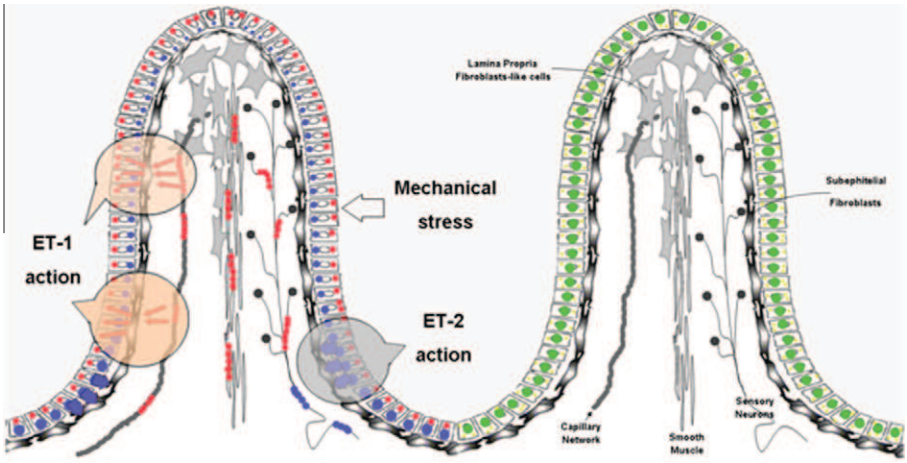

Fig. 4. On the left villus, a schematic representation of ET-2/VIC (blue circles) and ET-1 (red circles) are depicted. Also is represented the possible roles exercised on it. ET-2/VIC and ET-1 would regulate the inter-conversion of the fibroblastic cellular shape, the size of sieve of the filtration barrier, the tension, the contractility and the mechanical sensitivity. As observed, the fibroblastic phenotype changes along baso-apical axis of the villus, in line with the expression of ET-2/VIC. On the right villus: schematic representation of the ETA receptor (yellow circles) and ETB receptor (green circles). It is remarkable the particular distribution of ETB receptor at cells nuclei while ETA receptor is widely distributed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

recently it has been demonstrated that ETs mediate morphological changes in sub-epithelial fibroblasts modulating the mentioned functions [40]. Furthermore, it has been found that fibroblasts possess endothelin receptors [33,37,39–41].

4.1. Possible role of ET-2/VIC in the control of intestinal villi permeability

The physical distribution of fibroblasts is tightly correlated with gradient expression of ET-2/VIC found in this work, where the major expression levels were found on the basal region, decreasing to the apical part of the villus. This distribution strongly suggests a relationship between fibroblasts and enterocytes. This last type of cells through the ET-2/VIC production may mediate a paracrine mechanism to regulate the fibroblast network. *In vitro* studies have demonstrated that cultured fibroblasts undergoing different ETs doses change their morphology. In line with increased cAMP levels, these change their geometry from flat to stellate reversing this process after ET-1 or ET-3 are added, with consistent decrease of cAMP [40]. Studies using ETA and ETB specific antagonists (BQ123 and BQ788, respectively) suggest that this switch is achieved via both receptors. In the previously mentioned work, it was verified that endothelial cells adjacent to the fibroblast network have ET-1-like immunoreactivity, allowing postulating that the regulation of fibroblastic shape would be mediated by these peptides [40]. Additionally, it has been shown that ET-1 is synthesized and released by intestinal epithelial cells when they are stimulated by interleukin 2 (IL-2); which can be secreted by fibroblasts [42]. Therefore, given the localization of ET-1 [32] in mouse and having in mind the above results, Furuya and Furuya propose that ET-1 released for endothelial cells and enterocytes would regulate, via receptors, the size of sieve of fibroblast network, changing their cellular morphology [37]. However, since we and also Furuya have found that the ET-1 expression is uniform along intestinal villi, we are proposing that the differential changes in fibroblastic morphology must be attributed to ET-2/VIC differential gradient more than the ET-1 one (see Fig 4).

4.2. Possible role of ET-2/VIC in mechanical properties of intestinal villi

Previous studies have reported that the immunosignal of proteins linked to contractile cytoskeleton of intestinal villi is more intense in the fibroblasts located near the crypts than that located in the apical border of the villi and very weak in the lamina propria fibroblasts [43]. In duodenum, the fibroblasts at the bottom of the villi exhibit a high signal from α-actin while this is weak or null at the upper portion of them. This declination of the immunosignal could be due to the decrease of α-actin expression or its depolymerization [37]. These results suggest that flat fibroblasts are favored in their cytoplasm contractibility and the maintaining of the tension while the stellate fibroblasts are disfavored in that processes [37]. With the contributions of this work, it is possible postulate a correlation between the expression gradient of VIC/ET-2 and α-actin in the villi as the expression of both molecules is highest at the bottom, decreasing to the apical border. This gradient also suggests a connection between the ETs levels and the mobility of the villi. This last property is exerted in part by the smooth muscle contraction at the center of the villi and, also by the fibroblasts contraction. The contraction of the longitudinal axis could be induced by VIC/ET2. This hypothesis seems highly plausible given that, besides to induce morphological changes it has been demonstrated sub-epithelial fibroblasts can contract when are exposed to ETs and ATP [40]. Both the ATP (0.1–100 μM) and ETs (0.1–10 nM) application induce an increase of cytoplasmatic Ca^{2+}, followed by contraction of the villi [40]. *In vitro* studies showed that colon fibroblasts exhibit a persistent contraction (10–15 min) when they are exposed to ET-1 [44], although in the duodenum the contraction only takes a few seconds [40]. This difference could be in part explained for the major expression of the receptors and ligands found in this work in colon, respect the other segments of the intestine.

As main remarks, we can conclude that the fibroblastic phenotype does change along the baso-apical axis of the villus establishing a gradient of flattened–stellated fibroblast in this direction. This will give to the villi a mechanical differential sensitivity along its major axis. The cell shape also do changes temporarily by the specific expression gradient of ETs, regulated by several cell types of the villi. Therefore, the mechanical sensitivity of the villi would change locally, temporally and dynamically based on cellular environmental conditions, determining the absorptive and defensive functions of the intestine. Based on the background discussed above, we can conclude that ET-2/VIC and ET-1are key factors in the complex control of the permeability of the intestinal villi. ETs would regulate the mechanical properties of contractility and

tension of these villi, acting on their fibroblasts. Given the distribution gradient of ET-2/VIC peptide and the role postulated for the endothelin system in the mechanics of the villi, we can propose that ET-2/VIC also participate in this function like a paracrine factor that assists in the maintenance of greater mechanical stress and/or the maintenance of contraction in fibroblasts from the bottom of these structures, in a cooperative manner with the ET-1.

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