The endothelin system in normal human colon

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1Institut National de la Santé et de la Recherche Médicale Unit 36, Collège de France, 75005 Paris, France; and 2Institute of Pathology, Centre Hospitalier Universitaire Vaudois, CH 1011 Lausanne, Switzerland

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Egidy, Giorgia, Lucienne Juillerat-Jeanneret, Petra Korth, Fred T. Bosman, and Florence Pinet. The endothelin system in normal human colon. Am J Physiol Gastrointest Liver Physiol 279: G211–G222, 2000.—Endothelin (ET)-1 is a potent vasoconstrictor and mitogenic peptide that has a variety of biological effects in noncardiovascular tissues. The precise cellular distribution of the ET-1 system in the wall of the normal human colon was studied to identify the physiological role of ET in the gut. In situ hybridization revealed ET-converting enzyme-1 (ECE-1) mRNA in all vessels, the colon epithelium, and macrophages. Prepro-ET-1 (PPET-1) mRNA had a similar distribution except for a scattered signal in mucosal microvessels. ETα and ETβ receptor mRNAs were mainly in the lamina propria, pericryptal myofibroblasts, microvessels, and mononuclear cells, with ETα mRNA more abundant than ETβ mRNA. 125I-ET-1 binding showed ETβ along the crypts and in nerve fibers descending from the ganglionic plexus that contained PPET-1, ECE-1, and ETβ transcripts, whereas glia contained ETα receptors. The finding of the entire ET system in the normal mucosa suggests its implication in some characteristic functions of the colon and its secretion as both a neuroactive and a vasoactive peptide.

in situ hybridization; immunocytochemistry; endothelial cell; smooth muscle cell

THE POTENT VASOCONSTRICTOR peptide endothelin (ET)-1 was identified by Yanagisawa et al. (47). It belongs to a recently discovered family of three 21-amino acid peptides, the endothelins (ETs), which regulate vascular tone (18). The ETs act on two distinct high-affinity ET receptor subtypes, ETα (13) and ETβ (33), which are seven-transmembrane G protein-coupled receptors. ETα receptors bind ET-1 and ET-2, but not ET-3, at physiological concentrations, whereas ETβ receptors bind all three ETs with a similar affinity. ETs are initially synthesized as large precursor polypeptides, prepro-ETs (PPETs), that are cleaved at two pairs of basic amino acids to generate intermediate peptides, the Big ETs. The Big ETs are then cleaved by ET-converting enzyme (ECE) (35) to produce the mature ETs. ECE is a key enzyme in the synthesis of the ETs because Big ETs have negligible biological activities (17). Two ECE-encoding genes have been cloned, ECE-1 (42) and ECE-2 (8). Their sequences are 59% identical, but only ECE-1, the most abundant, has been studied in detail (see Ref. 41 for a recent review). Targeted inactivation studies on ECE-1 in mice showed that although there was still ET-1 in the plasma because of ECE-2, it did not rescue the mutant developmental phenotype, indicating that mature ETs must be produced at specific sites to influence development (46). Also, the cleavage of other substrates, vasoactive intestinal peptide (VIP) and neurotensin, by ECE-1 cannot be excluded, because ECE-1 was recently shown to have a relatively broad specificity (16).

ET-1 was initially believed to be a vasoconstrictor peptide, but it has a variety of other biological activities, such as stimulation of hormone release and regulation of central nervous system activity (26), in nonvascular tissues. ET-1 is also a potent mitogen in many cell types, including vascular smooth muscle cells (11), playing a fundamental role in the development of the cardiovascular system (23). ET-1 and ET-3 peptides are also present in the gastrointestinal tract; they have been found in the rat gut mucosa by immunoassay (27), by in situ hybridization (ISH) (25) and by ET binding (38). ET-3 plays a key role in the development of the mouse enteric nervous system (10). Both ETβ and ET-3 are necessary to prevent the premature differentiation of crest-derived cells, which leads to aganglionosis (45). Shortly after the discovery of ET-1, Whittle and Esplugués (44) reported that ET could be pro-ulcerogenic in the rat, in the pathogenesis of gastric damage and ulceration, and a nonselective ET receptor antagonist was found to reduce injury in a rat model of colitis (12). The pharmacological effects of exogenous ET-1 in the intestine have been studied; the peptide seems to be a potent intestinal secretagogue that increases colon contraction by direct stimulation of smooth muscle (37, 38) and transient transepithelial Cl− secretion mediated by the enteric nerves (2, 19, 28). However, it is now becoming evident that the tissue and cellular distribution of ET receptors is species specific.

There have been few reports on the distribution of ET in the human colon. Inagaki et al. (14) found ET-like immunoreactivity and binding sites for ET-1 in the human colon, and competition experiments suggested
that there are two populations of ET receptors (9). Mutations in the ETB receptors have been found in some families with Hirschsprung’s disease or aganglionosis (32). Kuhn et al. (22) recently showed that ET-1 was a secretagogue for human colon mucosa in vitro. In a previous study, we (20) showed that ECE-1 mRNA and its protein are present in the adult human colon. We used ISH and immunohistochemistry to demonstrate large amounts of ECE-1 in the epithelium and enteric ganglia of the normal human colon.

However, none of these studies determined the receptor subtype, substrate, and enzyme of the ET system in the same tissue, and most of the studies were carried out at low resolution so that the cellular distribution was not obtained. The present study was therefore carried out to determine the precise cellular locations of all the components of the ET-1 system in the human normal colon and so gain insight into the possible role of ET-1 in gastrointestinal physiology. The distributions of PPET-1, ECE-1, ETA, and ETB receptor mRNAs were studied by ISH. The cells containing the mRNAs were further examined by comparing the distribution of these mRNAs with those markers of endothelial cells, smooth muscle cells, and macrophages. We checked for the presence of specific, functional ET receptors by measuring ET binding in frozen sections after ISH for receptor mRNAs.

**MATERIALS AND METHODS**

**Human tissues.** Normal human colon tissue was obtained from patients undergoing colectomy for cancer, after routine diagnostic examination of the surgical specimen at the Institute of Pathology (Lausanne, Switzerland). Samples from 18 patients (41–84 years, 8 women and 10 men) were examined. Nine samples were from the cecum, and nine were from the sigmoid colon. We examined only tissue that was macroscopically and microscopically normal after routine histological examination. The same protocol was subsequently used for both frozen and paraffin-embedded sections. Sections were incubated overnight at 50°C with the respective antisense and sense riboprobes (3–4 × 105 cpm per section). The slides were washed with increasingly stringent solutions and treated with RNase A (20 μg/ml, Sigma). The sections were dehydrated by 10.220.33.5 on August 28, 2017 http://ajpgi.physiology.org/ Downloaded from |  

**Preparation of radiolabeled probes.** The human PPET-1 partial cDNA (15), corresponding to nucleotide sequence 70–630, was subcloned into pBK-CMV (Stratagene). The recombinant plasmid was linearized by digestion with Sac I to obtain the antisense or Kpn I to obtain the sense RNA probe. Probes for human ECE-1 (35) were prepared as described in Korth et al. (20). Briefly, the recombinant plasmid ECE-1 partial cDNA corresponding to nucleotides 304–1666 was linearized by digestion with Hind III to obtain the antisense or Xba I to obtain the sense RNA. Probes for human ETA (13) and ETB (33) receptors, subcloned into pcDNA3, were prepared as described by Brand et al. (1). Briefly, ETα and ETβ cDNA were linearized by digestion with Xho I and Kpn I, respectively, to obtain the antisense probes. ETα and ETβ cDNA were linearized by digestion with Xba I to obtain the sense probe. In vitro transcription and labeling with [35S]-UTP (Amersham) were carried out with T7 or SP6 RNA polymerase (Boehringer Mannheim). Probes were precipitated with ammonium acetate and ethanol, dried by Speed-vac centrifugation, and dissolved in TE-dithiothreitol (DTT) (in mM: 10 Tris, 1 EDTA, and 20 DTT).

**In situ hybridization.** The ISH protocol used for paraffin sections involved microwave pretreatment to enhance the hybridization signal (36). Paraffin-embedded sections (5 μm) were cut, and two adjacent sections were mounted on each silane-coated slide. Deparaffinized sections were immersed in 0.01 M citric acid (pH 6.0) and heated in a microwave oven for 12 min. The sections were then incubated with proteinase K (2 μg/ml, Boehringer Mannheim) for 20 min and dehydrated. ISH on frozen sections used 7-μm sections fixed in paraformaldehyde-PBS and dehydrated without microwaving. The same protocol was subsequently used for both frozen and paraffin-embedded sections. Sections were incubated overnight at 50°C with the respective antisense and sense riboprobes (3–4 × 105 cpm per section). The slides were washed with increasingly stringent solutions and treated with RNase A (20 μg/ml, Sigma). The sections were dehydrated by 10.220.33.5 on August 28, 2017 http://ajpgi.physiology.org/ Downloaded from.

### Table 1. Primers used to amplify ECE-1, PPET-1, ETA, ETB, and GADPH mRNA

<table>
<thead>
<tr>
<th>Gene</th>
<th>Original Sequence</th>
<th>Primer Sequences for RT-PCR</th>
<th>Size of Amplified Fragments, bp</th>
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<td>ECE-1</td>
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<td>Sense: 5’ CGC TGG AAG TTT GTC GTG AGT GAC 3’</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’ GCA CTC GGT CTC ACG 3’</td>
<td></td>
</tr>
<tr>
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<td>341</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>Sense: 5’ ACT TCA GCT TTC AAA TAC ATT AAC 3’</td>
<td>675</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’ CTG CTT AAG ATG TTC AGT GAG GCC 3’</td>
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</tr>
<tr>
<td>ETB</td>
<td>Sakamoto et al. (33)</td>
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<tr>
<td></td>
<td></td>
<td>Reverse: 5’ GTC AAT ACT CAG AGC ACA TAG ACT 3’</td>
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<tr>
<td>GADPH</td>
<td>Tso et al. (40)</td>
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<td></td>
<td></td>
<td>Reverse: 5’ ACC TTC TTG ATG TCA TCA TAT TTG 3’</td>
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ECE-1, endothelin-converting enzyme; PPET-1, prepro-endothelin-1; ETA, ETb, A and B endothelin receptor subtypes; GADPH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair.
drated and placed in contact with Biomax film (Kodak) for 1–3 days. They were then dipped in NTB2 liquid emulsion (Kodak) and exposed for 2 wk with ECE-1 or PPET-1 probes and for 4 wk with ETA and ETB probes. Sections were counterstained with toluidine blue. Figures 1–7 are from ISH performed in paraffin-embedded tissue sections unless otherwise stated.

**125I-ET-1 binding.** Sections were cut using a cryostat (7 μm), thaw-mounted on silane-coated slides, and stored overnight under vacuum at 4°C. Consecutive sections were fixed for 10 min in 4% formaldehyde-PBS and then preincubated for 15 min in 50 mM Tris · HCl buffer, pH 7.5, containing 120 mM NaCl, 5 mM MgCl2, and 40 mg/l bacitracin. Sections were then incubated with 100 pM of 125I-labeled ET-1 (2,125 Ci/mmol) in the previous buffer containing 1% BSA (fraction V, protease free) and 1 mM phosphoramidon for 90 min at room temperature. Sections were given four successive 1-min washes in ice-cold 50 mM Tris · HCl, pH 7.4, dipped in ice-cold distilled water, air dried, and placed in contact with Biomax MR films (Kodak). Nonspecific binding was determined in consecutive sections incubated as described above with 1 μM unlabeled ET-1 (Bachem). The receptor subtypes were identified by incubating consecutive sections as described above with 1 μM BQ-123 (ETα antagonist) and ETβ mRNAs were in smooth muscle cells (small arrows). Scale bar: 50 μm.

**Fig. 1.** Components of the endothelin (ET) system in human colon submucosa. In situ hybridization was performed in consecutive sections of normal human colon tissue with the antisense probes for ET-converting enzyme (ECE-1; A), prepro-ET-1 (PPET-1; C), ETA (G) and ETB (I) receptors and the sense probes for ECE-1 (D), PPET-1 (F), ETA (H), and ETB (not shown) receptors. The photographs are presented in dark-field illumination. Immunohistochemistry was performed to identify endothelial cells using anti-CD-31 (B) and smooth muscle cells with anti-α-smooth muscle actin (SMA) (E) antibodies. PPET-1 and ECE-1 mRNAs were mainly present in endothelial cells (arrows), and ETA and ETB mRNAs were in smooth muscle cells (small arrows). Scale bar: 50 μm.
ET-3 (natural ET\textsubscript{B} agonist), or 0.2 \textmu M sarafotoxin 6c (S6c, selective ET\textsubscript{B} agonist). The sections were then air dried, fixed in paraformaldehyde at 80°C for 2 h, dipped in NTB2 photographic emulsion (Kodak), exposed for 4 days, and counterstained with toluidine blue.

Immunohistochemistry. Paraffin-embedded sections (5 \textmu m) were incubated with xylene (to remove paraffin) and rehydrated in a graded ethanol series, and their endogenous peroxidase was inactivated by incubation with 3% hydrogen peroxide in methanol for 10 min. They were then washed in water and incubated with monoclonal antibodies to CD31, CD68 (both from Dako), \alpha-smooth muscle actin (\alpha-SMA, Sigma), or MIB-1 (Dianova) according to the manufacturers’ instructions. The antiserum 473–17-A (21) was used to stain for ECE-1. The bound anti-CD31 and anti-\alpha-SMA antibodies were reacted with avidin-biotin complex (Dako), and those for CD68 and ECE-1 were reacted with peroxidase-antiperoxidase (Dako). Sections were then treated with 0.035% diaminobenzidine (Fluka) for 30 min, counterstained with hematoxylin (according to Mayer), and mounted. Control reactions without the primary antibody showed no nonspecific staining (not shown).

ISH and immunohistochemistry labeling were evaluated by different investigators (G. Egidy, L. Juillerat-Jeanneret, and F. Pinet), each blind to the others’ assessment. Results were summarized on a four-point scale (as shown in Table 2), although the techniques used provided only semiquantitative evaluation.

RT-PCR analysis. Total RNA was isolated from primary culture of human dermal fibroblast cells (a gift from M. Benathan, CHUV, Lausanne, Switzerland) and from normal human neonatal colon fibroblasts, CCD-18Co (ATCC CRL1459) using the protocol of Chomczynski and Sacchi (3). cDNA was prepared with 1 \textmu g of total RNA and 10 pmol of oligo(dT) using Moloney murine leukemia virus reverse transcriptase (Gibco BRL) according to the manufacturer’s instructions. PCR was performed using 3 \textmu l of cDNA solution and 1.25 U of Taq polymerase (Boehringer Mannheim) ac-

**Fig. 2.** ET system components in the myenteric plexus. In situ hybridization performed in adjacent sections of myenteric plexus of cecum colon with the antisense probe for PPET-1 (A), ECE-1 (B), and ET\textsubscript{A} (E) and ET\textsubscript{B} (F) receptors are presented in bright-field illumination. Immunohistochemistry was performed with anti-CD31 (arrow on endothelial cell; C) and anti-\alpha-SMA (D), staining longitudinal and circular smooth muscle cells. Neurons (arrows) were labeled by PPET-1, ECE-1, and ET\textsubscript{B} probes. Glial cells (arrowheads) were labeled with antisense probe for ECE-1 and ET\textsubscript{A} and ET\textsubscript{B} receptors. Scale bar: 20 \mu m.

**Fig. 3.** ET system components in longitudinal and transverse sections of human colon mucosa. In situ hybridization performed with the antisense probes for PPET-1 (A, B), ECE-1 (C, D), ET\textsubscript{A} (E, F), and ET\textsubscript{B} (G, H) in serial sections are presented in dark-field illumination. Photographs of longitudinal sections (A, C, E, G, I) are from consecutive sections, whereas transverse sections (B, D, F, H, J) are from different fields of distal colon in both cases. Immunohistochemistry with anti-MIB-1 (proliferation marker; I) and anti-\alpha-SMA (smooth muscle cell marker; J) antibodies demonstrates the absence of pathology and the integrity of the regions studied. Small arrows indicate the immunohistochemistry signal. Arrow in B indicates Peyer’s patch. PPET-1 and ECE-1 mRNA were found in the crypts; ET\textsubscript{A} receptor mRNA was mainly in the core of the crypts, whereas ET\textsubscript{B} was only faintly detected on paraffin sections. Scale bar: 100 \mu m.
According to the manufacturer’s instructions. Control reactions for RT-PCR analysis were carried out with non-reverse-transcribed RNA samples. No amplification was observed for any of the RNA samples tested (not shown). Specific primers (10 pmol, Table 1) for ECE-1 (35), PPET-1 (15), ETA (13), and ETB (33) receptors and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (40) were added. The primers chosen were designed to avoid false positive reactions from genomic DNA contamination. Thirty cycles were carried out, consisting of denaturation at 94°C (30 s), annealing at 58°C (PPET-1, ETA, and ETB) or 55°C (ECE-1 and GAPDH) (30 s), and extension at 72°C (30 s) with a final extension step of 10 min at 72°C. Amplified products were analyzed on a 1.5% agarose gel.

RESULTS

ET system mRNAs in human colon. Tissue samples from 18 patients were analyzed, and the results shown are representative of all the cases. Samples collected from the cecum and distal colon were also not significantly different in their distributions of the ET system. All the components of the ET system were first looked for in the large vessels of the human colon submucosa (Fig. 1). ECE-1 (Fig. 1A) and PPET-1 (Fig. 1C) mRNAs were detected in CD31-positive endothelial cells (Fig. 1B). Smooth muscle vascular cells surrounding the vessels, which were immunostained for α-SMA (Fig. 1E), also contained ECE-1 mRNA but at a lower concentration than in endothelial cells. Smooth muscle cells were labeled for ETₐ (Fig. 1G) and ETₐ (Fig. 1I). Receptor mRNA was not clearly detected in the endothelium of these vessels (Fig. 1, G and I). Submucosa connective tissue was not labeled by any probe. None of the sense probes hybridized specifically with any structure, as shown for ECE-1 (Fig. 1D), PPET-1 (Fig. 1F), ETₐ (Fig. 1H), and ETₐ (not shown) receptors.

Myenteric ganglia also contained the ET system. PPET-1 mRNA was clearly present in neurons (Fig. 2A). ECE-1 mRNA (Fig. 2B) and ETₐ mRNA (Fig. 2F) were found in neurons and glial cells, and ETₐ mRNA was present only in glial cells (Fig. 2E); all cells showed no immunostaining for CD31 (Fig. 2C) and α-SMA (Fig. 2D). The circular and longitudinal muscle layers (Fig. 2D) were labeled more faintly with all the antisense probes.

ISH for PPET-1 mRNA gave a diffuse signal in the crypt epithelium of normal intestinal mucosa (Fig. 3A). A few crypts were strongly labeled with the PPET-1 probe (Fig. 3B), as were the epithelial cells within one crypt. ECE-1 mRNA was found in crypt epithelial cells (Fig. 3, C and D) at a similar intensity from one crypt to another and an apparent gradient toward the lumen.
in the opposite sense to the proliferation marker MIB-1 (Fig. 3I). Unlike PPET-1 and ECE-1, ET₄ mRNA was detected only in the lamina propria in α-SMA-positive cells (Fig. 3J), at the top of the crypts (Fig. 3E) and deeper in the mucosa (Fig. 3F). In contrast, ET₃ transcripts were detected only by ISH using frozen sections with no consistent labeling in paraffin sections (Fig. 3, G and H). Although we did not intend to quantify the ISH signals, receptor mRNA seemed much less abundant than mRNA for PPET-1 and ECE-1, with ET₃ mRNA being the weakest, considering that ET₄- and ET₃-hybridized sections were exposed for twice as long as PPET-1 and ECE-1 probes.

Examination at higher magnification showed PPET-1 mRNA in tall columnar cells along the crypts (Fig. 4A) and in CD68-positive lamina propria macrophages. Immunohistochemistry was performed with anti-α-SMA (smooth muscle cell marker) (B) and anti-CD68 (panmacrophage marker) (E) antibodies in consecutive sections to identify the cells labeled with ET₄ and ET₃ probes. ET₄ mRNA was strongly present in myofibroblasts (arrows) (A, D). The few cells containing ET₃ mRNA were identified as epithelial cells (small arrow), myofibroblasts (arrow), and endothelial cells (arrowhead) (C, F). Scale bar: 20 μm.

Fig. 5. Cellular distribution of ET₄ and ET₃ receptor mRNAs in human colon mucosa. In situ hybridization with the antisense probes for ET₄ (A, D) and ET₃ (C, F) receptors carried out on embedded paraffin (A, C) and frozen (D, F) sections from a sigmoid colon sample. Immunohistochemistry was performed with anti-α-SMA (smooth muscle cell marker) (B) and anti-CD68 (panmacrophage marker) (E) antibodies in consecutive sections to identify the cells labeled with ET₄ and ET₃ probes. ET₄ mRNA was strongly present in myofibroblasts (arrows) (A, D). The few cells containing ET₃ mRNA were identified as epithelial cells (small arrow), myofibroblasts (arrow), and endothelial cells (arrowhead) (C, F). Scale bar: 20 μm.
phages (Fig. 4F). Lymphocytes and some fibroblasts were labeled with the PPET-1 probe (Fig. 4D). Contrary to the endothelial cells in the submucosa, the microvascular endothelium of the mucosa was rarely positive for PPET-1 mRNA. ECE-1 mRNA was abundant in the crypt epithelium and in the endothelium of stromal microvessels (Fig. 4B), which were immunostained for CD31 (Fig. 4C). The enterocytes at the tips of crypts were also labeled for ECE-1 (Fig. 4E), as were resident macrophages (Fig. 4F). ECE-1 was detected immunohistochemically in crypts, with the signal on the luminal surface of the crypt and cuff epithelium and in vessels of the mucosa and submucosa (not shown).

ET\textsubscript{A} mRNA was confined to \(\alpha\)-SMA-positive cells (Fig. 5B) adjacent to the basal membrane of enterocytes (Fig. 5, A and D). Some ET\textsubscript{A}-labeled cells corresponded to CD68-immunoreactive resident macrophages (Fig. 5E). There was little ET\textsubscript{B} mRNA in paraffin-embedded specimens; it was confined to scattered endothelial cells, myofibroblasts, and mononuclear cells (Fig. 5C) but was clearly detected in frozen sections (Fig. 5F). A diffuse signal over the epithelium apparent in some sections was not considered to be specific because it also covered the lumen of the crypts, although there was localized labeling in a few columnar cells per section (Fig. 5, C and F).

**Localization of ET binding sites in the human colon by autoradiography.** The presence and distribution of ETA and ET\textsubscript{B} receptors in human colon were determined by autoradiography of \(^{125}\text{I}\)-labeled ET-1 bound to frozen samples from eight patients. All eight cases gave identical results (Fig. 6). Panels A and E of Fig. 6 show total \(^{125}\text{I}\)-ET-1 binding, and panels D and H show the nonspecific binding that remained after displacement with BQ-123 plus S6c (or ET-1, not shown), which was uniformly low. \(^{125}\text{I}\)-ET-1 bound specifically to the colon mucosa rather than to the submucosa, where only the vessels were labeled (Fig. 6E). Binding was very high in the lamina propria of the mucosa and
Receptor subtypes were identified by competition with an ET A-selective antagonist (BQ-123) and two ETB agonists, S6c (ET B analog) and ET-3 (natural ligand with high affinity for ET B and low affinity for ETA). Total binding was partially displaced by S6c (Fig. 6, B and F) and by BQ-123 (Fig. 6, C and G), indicating the presence of both receptor subtypes. Figure 6B shows ETA binding in which the selective ET B ligand S6c competed for a small proportion of 125I-ET-1 binding; only residual binding was left when the competing agent was BQ-123 (Fig. 5C), confirming a higher proportion of ETA than ETB receptors. However, longitudinal sections showed 125I-ET-1 binding along the crypts and at their base to be mainly displaced by S6c (Fig. 6F) as well as 10 nM ET-3, together with weak competition by BQ-123 (Fig. 6G), indicating ETB receptor subtypes in this location. Most of the 125I-ET-1 binding in the periphery of large-caliber vessels was displaced by BQ-123 (Fig. 6G), and the residual signal was displaced by S6c, demonstrating that there are ETB receptors in the media and adventitia of vessels (Fig. 6, F and G).

ET-1 system in human fibroblasts. The unexpected presence of both ET receptors in fibroblasts was checked by analyzing their presence in isolated human fibroblasts by RT-PCR. Two types of human fibroblasts were used, primary cultures of dermal fibroblasts and the intestinal subepithelial myofibroblast cell line CDD-18Co. RT-PCR was performed on total RNA from both cell types. The amplification primer sequences and expected sizes of the amplified fragments are shown in Table 1. Both dermal and intestinal human fibroblasts contained the whole ET system, with a higher concentration in the CDD-18Co cells (Fig. 7) than in the dermal cells (not shown).

### DISCUSSION

We have determined the distribution of the ET system in the normal human colon. Evidence is accumulating that ETs act locally rather than as circulating peptides, so that ECE-1 must be present in the same cell or close to its substrates for ETs to be effective local mediators (47). This study has examined the distribution of the ET system in 18 human colon samples by ISH, 125I-ET-1 binding, and immunohistochemistry. The data presented are representative of all cases, whether the samples were collected from the cecum or distal colon. They indicate that PPET-1, ECE-1, and both ET-1 receptors are synthesized in situ in the normal human colon, which is consistent with ET-1 acting locally rather than as a hormone. The relative cellular distributions of ET components are summarized in Table 2.

### Table 2. Relative cellular distributions of endothelin components in normal human colon

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<tr>
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<th>PPET-1 mRNA</th>
<th>ECE-1 mRNA</th>
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<th>ETA mRNA</th>
<th>Protein</th>
<th>ETB mRNA</th>
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mRNA expression was evaluated by in situ hybridization studies, ECE-1 protein by immunocytochemistry, and ET receptors from binding autoradiography. –, No labeling; +, weak labeling; ++, moderate labeling; ++++, strong labeling in the vast majority of cells in the designated population; s indicates labeling in only scattered cells; ND, not determined. The results were consistent from specimen to specimen (18 samples) when comparisons were carried out separately for each probe. No significant differences were found in the ET system in the cecum and distal colon. Evaluation was performed by different investigators (G. Egidy, L. Juillerat-Jeanneret, and F. Pinet), each blinded to the others’ assessments.
Distribution of ET system components. The morphology of the colon mucosa is heterogeneous (30), but although the mRNA whose content varied most was PPET-1, this did not correlate with the source of the material (cecum or sigmoid colon). The overall distribution of PPET-1 is in good agreement with the published ET-1 immunohistochemistry in the human colon (14), which has shown that PPET-1-containing cells at the base of the crypts may be enterochromaffin cells, which contain Big ET-1 in the fetal human gut (9). Although there is not a great deal of ECE-1 in the colon epithelium compared with that in the lung epithelium (20), it was reliably detected in vessels of all calibers, including the mesenteric artery, mucosal capillaries, and lymphatic capillaries, both by ISH and immunohistochemistry. This confirms previous results (20). There was mRNA for ECE-1 but not for ET-1 in the microvascular endothelial cells of the stromal mucosa; this reflects the presence nearby of other ET precursors, mainly ET-2 (5) or other substrates such as VIP or neurotensin (9), recently demonstrated to be cleaved by ECE-1 (16).

Receptors for ET-1 were widely detected in the colon lamina propria as reported by Inagaki et al. (14). To our knowledge this is the first study of an ET-1 receptor subtype in human colon at the mRNA or protein level. ETA receptors were mainly expressed in pericryptal myofibroblasts. By contrast, ETB receptors were present heterogeneous. The discrepancy in the ETB mRNA between the signals observed by ISH in frozen and paraffin-embedded sections may reflect differences in mRNA stability during tissue processing (J. D. Aubert, personal communication). The higher signal of ETB-specific binding at the serosal side of the mucosa compared with ETB mRNA by ISH could be accounted for by the presence of the receptor protein in ganglion nerve fibers. We have clearly shown the presence of ETB mRNA in neurons and associated cells of myenteric plexus.

Possible roles of ET-1 system in normal gastrointestinal tract. In addition to the well-characterized neural components of ET-1 action in the rat gut (6), the role of ET-3 and ETB in the formation of mouse enteric neurons (45) and the presence of ET receptors in ganglia, pericryptal, vascular, immune, and some epithelial cells suggests several possible functions for this peptide.

The colon is an important site of water absorption and ion transport, and ET-1 has been shown to stimulate mitogenesis and survival of Swiss 3T3 fibroblasts (39) and rat endothelial cells (34) as well as the migration of endothelial cells (48). These data suggest that ET-1 might be a colon survival factor that acts on stromal cells. The presence of both ETA and mainly ETB mRNAs in human fibroblasts from dermal and colonic origin corroborates the experiments performed by Wu et al. (45), who elegantly demonstrated the proliferative effect of ET-3 on smooth muscle cells from mouse gut embryonic mesenchyme. In fact, the critical developmental role of ET-3/ETB in the innervation of the distal colon seems to involve fetal smooth muscle cells; this would prevent the premature differentiation of crest-derived precursors, favoring their migration to colonize the distal bowel (45).

The ET system may also be involved in the immune response in the colon. Ehrenreich et al. (7) demonstrated the production of ETs by human macrophages, suggesting a role for ETs in the microenvironment of tissue macrophages. Because macrophages are found in close proximity to vascular smooth muscle cells and fibroblasts that possess ET receptors, these cells are potential targets for the actions of macrophage-derived ETs. This hypothesis is strengthened by the findings that bosentan, a mixed antagonist for ET receptors, reduces injury in a rat model of colitis (12) and that in Crohn’s disease ET-1 immunoreactivity is increased in colon tissue (29).

In conclusion, the finding of the ET system in the human enteric nervous system makes it possible to consider ET as a neuropeptide in the human intestine (14). The presence of ETB in neurons and glial cells enables us to hypothesize that they are also involved in the formation of enteric neurons, as has been shown for the mouse (45). In the submucosa, the ET-1 system seems to be a “classical” type, playing a paracrine role between endothelial cells and smooth muscle cells in the control of vascular function. The main local source of ET-1 seems to be the epithelial cells, colocalized with ECE-1, suggesting that ET could be a survival factor implicated in epithelial restitution. The presence of ET receptors in myofibroblasts suggests a role in the contractility of smooth muscle cells. The various cellular localizations of ET components suggest that this system is also implicated in the modulation of intestinal motility, defense, and secretion.

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