Localisation of endothelin like immunoreactivity in adult and developing human gut

C Escrig, A E Bishop, H Inagaki, G Moscoso, K Takahashi, I M Varndell, M A Ghatei, S R Bloom, J M Polak

Abstract

The distribution of immunoreactivity for the potent vasoconstrictor endothelin-1 was studied in adult and developing human gut using antisera to endothelin-1 (1-21) and the C terminus of big endothelin-1. The coexistence of these peptides with other neuropeptides was investigated using comparative immunocytochemistry. Endothelin-1 like immunoreactivity was detected in extracts of adult (range 20-60 fmol/g wet weight) and fetal (33 fmol/g) gastrointestinal tract and was shown by chromatography to be the predominant isoform of endothelin present in both. It was localised by immunocytochemistry to ganglion cells in the submucous and myenteric plexuses and to scattered nerves, whereas big endothelin-1 like immunoreactivity was found in the submucous plexus only. Colocalisation studies showed immunoreactivity for both endothelin-1 and vasoactive intestinal peptide in the same ganglion cells of the submucous plexus. Although endothelin-1 immunoreactivity was not detected by immunocytochemistry in the fetal human gut until the 32nd week of gestation, big endothelin-1 was found as early as 11 weeks in the developing neural structures and epithelial cells. The latter were shown to be endocrine cells by their immunoreactivity for chromogranin. Our results indicate that endothelin is a neuropeptide found in an adult human gut which shows transient expression in endocrine cells during development.

Endothelin-1, the first member of a newly discovered mammalian family of biologically active peptides, was originally isolated from the supernatant of cultured porcine endothelial cells. It is synthesised as a precursor peptide of 203 amino acids, containing a signal sequence, which is subsequently cleaved proteolytically to produce the 38 (human) or 39 (porcine) amino acid intermediate big endothelin (big endothelin-1). This is processed further to the mature 21 amino acid form, endothelin-1. Several isoforms of endothelin-1 have been isolated – endothelin-2 and endothelin-3. The latest isoform of the endothelin family to be characterised is a 21 amino acid peptide originally isolated from the guinea pig ileum and termed vasoactive intestinal contractor. Vasoactive intestinal contractor differs from endothelin-1 at three positions (positions 4, 6, and 7), and from endothelin-2 at only one position (position 4). In addition, these peptides show structural homology with a group of peptide toxins called sarafotoxins from the venom of a snake, the burrowing asp Atractaspis engaddensis.

Endothelin binding sites are widely distributed, not only in vascular tissues but also in trachea, lung, kidney, gastrointestinal tract, and brain. In addition to its vascular location, there is evidence that endothelin-1 also occurs in nerve tissue. It was detected by immunocytochemistry in the paraventricular and supraoptic nuclei of the hypothalamus and in the hippocampus and the dentate gyrus. Endothelin-1 immunoreactivity was found in the coexistence of the human colon and in the binding sites were localised to nerves, blood vessels, and muscle.

The aims of this study were (a) to determine the pattern of expression of endothelin-1 and its precursor molecule in the developing human gut, and (b) to measure the concentrations and molecular forms of endothelin present using radioimmunoassay and chromatography.

Methods

IMMUNOCYTOCHEMISTRY

Histologically normal samples of adult stomach (n = 5) (body and antrum), duodenum (n = 3), jejunum (n = 2), ileum (n = 1), and pancreas (n = 4) were removed during surgical excision of tumours. Samples of fetal gastrointestinal tract (stomach, small intestine, and large intestine) from fetuses of 8-32 weeks of gestation were obtained after elective terminations of pregnancy or spontaneous abortion. Tissues were fixed immediately in Bouin’s solution for four to 18 hours, washed in 30% (v/v) alcohol, dehydrated through graded alcohols, and embedded in wax. Paraffin sections (3 µm) were mounted on poly-L-lysine coated slides and dried at 37°C overnight.

Antibodies for immunocytochemistry were raised in New Zealand white rabbits using endothelin-1 or big endothelin-1 (C terminal 22-38) linked to keyhole limpet haemocyanin with glutaraldehyde (peptide/carrier molecular ratio 20:1) (Cambridge Research Biochemicals, Northwich, UK). Primary immunisation (100 µg of endothelin-1 or big endothelin-1 (C terminal 22-38) per ml) was administered in Freund’s complete adjuvant and subsequent immunisations were in Freund’s incomplete adjuvant. The animals were given booster injections eight times and blood was taken seven days after each immunisation.

Immunocytochemistry was carried out using the avidin-biotin complex method. After
dewaxing, the sections were immersed in methanol containing 0-3% (v/v) hydrogen peroxide, rehydrated, and incubated for 30 minutes with normal goat serum (1:20). Antisera to endothelin-1 (RPMS ref no 2020; dilution 1:2000) and big endothelin-1 (RPMS ref no 2051; dilution 1:3000; RPMS ref no 2056:1:1000) (CRB) were applied overnight at 4°C in a moist atmosphere. After washing in 0-01 mol/l phosphatebuffered normal saline, pH 7-2 (PBS), the sections were incubated for 30 minutes with biotinylated goat anti-rabbit IgG (Vectastain Elite Kit, Vector Laboratories, Breton, UK) diluted 1:400 with PBS at room temperature. Following further washing, they were incubated with avidin-biotin peroxidase complex (Vectastain Elite Kit) diluted 1:80 in PBS for 30 minutes at room temperature. The sites of peroxidase activity were visualised by incubation in a solution containing 25 mg of 3-aminio-9 ethylcarbazole (Sigma Chemicals, Poole, UK) dissolved in 10 ml of N,N-dimethylformamide (Sigma Chemicals) and added to 100 ml of 0-02 mol/l acetate buffer, pH 5-2 (sodium acetate 0-2 mol/l, acetic acid 0-2 mol/l), and 40 µl of 30% (v/v) hydrogen peroxide. Sections were counterstained in Mayer’s haemalum (BDH Limited, Eastleigh, UK) washed in tap water, and mounted in Hydromount (National Diagnostics UK Ltd, Bucks, UK). The specificity of the antisera against endothelin-1 was confirmed by incubation of sections with antisera preabsorbed with synthetic human endothelin-1 (CRB), endothelin-2 (Peninsula Labs), endothelin-3 (Nova Biochem, Nottingham, UK), big endothelin-1 (22–38), sarafotoxin S6b, sarafotoxin S6c (Peninsula Labs), and vasoactive intestinal contractor (Peptide Institute, Osaka, Japan) (1 nmol/ml of diluted antisera). Only incubation with endothelin-1 quenched the immunostaining. In addition, endothelin-1 antisera was absorbed with synthetic vasoactive intestinal peptide (Peninsula Labs) (10 nmol/ml) to test for possible cross-reactivity. No diminution of immunostaining intensity was seen. The specificity of the antisera against big endothelin-1 was determined by incubation of sections with antisera preabsorbed with synthetic human big endothelin-1 (Peninsula Labs).

Immunostaining with antisera 2051 could be quenched by addition of big endothelin-1 at a concentration of 0-1 nmol/ml diluted antisera but only partial absorption of the immunostaining with antisera 2056 could be obtained. Neither antisera was affected by addition of endothelin-1 at a concentration of 10 nmol/ml. Negative controls included the use of non-immune serum instead of the primary antisera or omission of one of the steps in the avidin-biotin complex procedure.

In the adult gut, the possible coexistence of endothelin with neuropeptides was determined using pairs of serial wax sections (3 µm) immunostained with antisera to endothelin-1 and the major gut neuropeptides – vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), calcitonin gene related peptide (CGRP), and Substance P (SP) (see Table I for details). The specificities of the antisera to VIP, NPY, CGRP, and SP, have been reported previously. In order to identify cells in the developing gut, a similar procedure was followed using antisera to chromogranin and protein gene product 9-5, gastrin, glucagon, gastric inhibitory polypeptide, secretin, somatostatin-14 and 5-hydroxytryptamine (see Table I).

**Table I. Characteristics of antisera used in colocalisation studies**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Ref no</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vasoactive intestinal peptide</td>
<td>652</td>
<td>HH</td>
<td>1:10000</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>1101</td>
<td>HH</td>
<td>1:2000</td>
</tr>
<tr>
<td>Calcitonin gene related peptide</td>
<td>1204</td>
<td>HH</td>
<td>1:2000</td>
</tr>
<tr>
<td>Substance P</td>
<td>1651</td>
<td>HH</td>
<td>1:5000</td>
</tr>
<tr>
<td>Chromogranin</td>
<td>1981</td>
<td>Hybritech</td>
<td>1:1000</td>
</tr>
<tr>
<td>Protein gene product 9-5</td>
<td>1648</td>
<td>Ultraltrac</td>
<td>1:4000</td>
</tr>
</tbody>
</table>

HH = Hammersmith Hospital; Ultraclone, Cambridge, UK; Hybritech, Bingham, Nottingham, UK.

**Figure 1: Endothelin-1 like immunoreactivity in the submucous plexus of human adult duodenum. (Original magnification ×500.)**
coefficients of variation were 12% (n=9) and 18% (n=8). No radioimmunoassay for big endothelin-1-like immunoreactivity was available.

Fractionation of the endothelin like immunoreactivity was carried out in extracts of stomach by fast protein liquid chromatography (FPLC) using a high resolution reverse phase 5/5 (Pep Rpe HR 5/5) C-18 column (Pharmacia, Uppsala, Sweden), with a gradient of acetonitrile from 0 to 50% (v/v) in water (both acetonitrile and water with 0.1% trifluoroacetic acid) over one hour at 1 ml per minute per fraction. Samples of each fraction were dried in a Savant vacuum centrifuge, reconstituted with buffer and assayed as described previously."

Results
IMMUNOCYTOCHEMISTRY

Adult gut
In the adult gut, numerous endothelin-1-immunoreactive nerve cell bodies were seen in both submucous (Fig 1) and myenteric ganglia (Fig 2) in all regions examined. The immunoreactivity, localised to the cytoplasm, had a granular appearance. In the submucosa, groups of immunoreactive ganglion cells were mainly found just under the muscularis mucosae or close to the circular muscular layer. In the myenteric ganglia, almost all of the reactive cell bodies were large and ovoid and were at the periphery of the ganglion. Few, scattered immunoreactive nerve fibres were seen. No glial cells showed positive immunostaining and no immunoreactivity was detected in cells of the mucosal epithelium. About 80% of neuronal cells of the submucous plexus and 10% of the myenteric plexus showed endothelin-1 immunoreactivity. Only weak, inconsistent immunostaining of endothelium was obtained. Immunostaining with both antisera to big endothelin-1 gave positive results only in the submucous plexus, where about half of the ganglion cells were immunostained. No immunoreactive nerve fibres could be detected.

Pairs of serial sections immunostained for endothelin-1 and VIP, NPY, CGRP, or SP showed positive immunostaining in both plexuses, but only VIP was found to be colocalised with endothelin-1 (Fig 3). Most endothelin immunoreactive nerve cell bodies showed colocalisation with VIP in the submucous plexus.

Developing gut
In the developing gut, immunoreactivity for endothelin-1 could not be shown until the 32nd week of gestation, when it was found only in neural structures. In contrast, big endothelin-1 (ref no 2056) was localised to the submucous and myenteric plexuses of the stomach and small (Fig 4) and large intestine in fetuses at 11 weeks of gestation. These developing structures were identified as neural by their immunoreactivity for protein gene peptide 9-5 in serial sections. A second antisem against big endothelin-1 (ref no 2051), raised in a different rabbit from 2056 with the same antigen and immunisation methods, showed the presence of big endothelin-1 like immunoreactivity in cells of the mucosal epithelium of the small intestine only (Fig 5) from 11 weeks of gestation. These cells
Localisation of endothelin-like immunoreactivity in adult and developing human gut

Radioimmunoassay and chromatography
Endothelin-like immunoreactivity was detected in adult human stomach (60 fmol/g wet weight) and rectum (20 fmol/g) and fetal small intestine (33 fmol/g). FPLC of adult stomach extracts showed three peaks in the positions of the three isoforms of endothelin (Fig 6). The peak for endothelin-1 was slightly greater than that for endothelin-2 while the smallest peak corresponded to the position of endothelin-3. The chromatograph of extracts of fetal small intestine showed only one major peak for endothelin-1 and a much smaller one at the position of endothelin-3 (Fig 7).

Discussion
This study aimed to determine the presence and localisation of endothelin-1, a newly discovered polypeptide, in the adult and developing human gut using immunocytochemical and biochemical techniques and, with immunocytochemistry, to...
localise its precursor form, big endothelin-1.

Endothelin-1 is one of the most potent vasoconstrictors known and, as well as being present in vascular endothelium, its immunoreactivity and binding sites has been detected recently in the adult human colon. The results of the present study show that endothelin-1 is the major isoform of endothelin in the adult and fetal human gut and that the peptide is widely distributed in the innervation of most regions of the adult and developing human gut along with its precursor form, big endothelin-1. The findings provide further evidence that endothelin may be a neuropeptide. Only weak immunoreactivity for endothelin-1 was detected in the endothelium. This is probably because of the selection of a tissue processing method that was optimal for demonstration of neural rather than endothelial endothelin-1 immunoreactivity. The same antibody has been found to give satisfactory immunostaining of endothelium in tissue processed in a different way.

Immunocytochemistry detected endothelin-1 like immunoreactivity in most ganglion cells of the submucous plexus in all regions of the adult human gastrointestinal tract and in about 10% of those of the myenteric plexus. As shown by the authors in a previous study, most submucosal ganglion cells that are immunoreactive for endothelin also contained VIP, a major gut neuropeptide. Since VIP is a potent vasodilator, this coexistence of peptides suggests a possible antagonistic action in the control of local blood flow in the gut.

Big endothelin-1 like immunoreactivity was detected by immunocytochemistry in the developing human gut as early as 11 weeks of gestation. Although radioimmunoassay showed the presence of endothelin-1 like immunoreactivity in the fetal small intestine at 17 weeks' gestation, it was not detected in nerves until the 32nd week of gestation. Radioimmunoassay detected both endothelial and neural endothelin-1 like immunoreactivity but, for immunocytochemistry, the tissue processing method selected provided optimal preservation of endothelin-1 in nerves rather than endothelium. Thus, endothelin-1 may occur in endothelium earlier than in nerves. This may explain the apparent discrepancy between the results obtained by radioimmunoassay and immunocytochemistry. Either way, it seems that the precursor of endothelin-1 is widely expressed in the developing human gut and only to a lesser extent in the adult gut. The change in the predominant form of endothelin immunoreactivity in the fetus, from the precursor to mature endothelin-1, may reflect a change in the post-translational processing of the peptide during development. The exact mechanism causing the switch, possibly the change in production or activity of a particular converting enzyme, remains to be explained.

Immunoreactivities for both endothelin-1 and big endothelin-1 were localised to neural structures in the adult human gut but no immunoreactivity was observed in the mucosal layer. This contrasted with the results obtained in the fetal gut where immunostaining for big endothelin-1 was seen both in neural plexuses and, with antisera no 2051, in the mucosal epithelium. An epithelial localisation for both endothelin immunoreactivity and mRNA has been reported in the adult rat ileum and colon. The presence of endothelin immunoreactivity in the epithelium may be related to the reported role of the peptide in stimulation of mitogenesis in vitro, which has been shown previously in different systems.

In conclusion, endothelin-1 is a neuropeptide which, in addition to its endothelial localisation,
has an extensive distribution in most regions of the human gastrointestinal tract. Furthermore, in humans, certain isoforms of endothelin may operate as specific growth factors in the epithelium of the developing gut in early fetal life.


Localisation of endothelin like immunoreactivity in adult and developing human gut.

C Escrig, A E Bishop, H Inagaki, G Moscoso, K Takahashi, I M Varndell, M A Ghatei, S R Bloom and J M Polak

Gut 1992 33: 212-217
doi: 10.1136/gut.33.2.212