**INTRODUCTION**

The enteric nervous system (ENS) differs from other regions of the PNS in its structure and unique ability to mediate reflexes independently of CNS input (Furness and Costa, 1987; Gershon et al., 1994). The neural crest origin of the ENS, however, is shared by most of the PNS. Enteric ganglia arise only from vagal (entire bowel) (Le Douarin and Teillet, 1973, 1974), sacral (post-umbilical gut) (Le Douarin and Teillet, 1973, 1974; Pomeranz and Gershon, 1990; Pomeranz et al., 1991a; Serbedzija et al., 1991; Burns and Le Douarin, 1998), and truncal (presumptive esophagus and adjacent stomach) (Durbec et al., 1996) axial levels. The premigratory crest probably contains a subset of cells that are specified (Newgreen et al., 1980; Peters-van der Sanden et al., 1993; Erickson and Goins, 1995; Henion and Weston, 1997); nevertheless, the special properties of the ENS do not arise because particular premigratory crest cells are specified to develop as enteric neurons. The crest-derived émigré population still contains pluripotent precursors when it arrives in the bowel (Rothman et al., 1990; Sextier-Sainte-Claire Deville et al., 1994; Lo and Anderson, 1995), although multiple lineages of such precursors have been identified (Blaugrund et al., 1996). The development of the ENS, therefore, is critically influenced by the enteric microenvironment.

Molecules of the gut wall that have been found to play roles in ENS development by stimulating receptors on crest-derived cells include: (i) glial cell derived neurotrophic factor (GDNF)/GFRα-1/Ret (Pachnis et al., 1993; Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Cacalano et al., 1998); (ii) neurotrophin-3 (NT-3)/TrkC (Chalazonitis et al., 1994); (iii) a still-to-be-identified ligand for the ciliary neurotrophic factor receptor-a (CNTFRα), (Chalazonitis et al., 1998a), (iv) laminin-1/LBP-110 (Kibbey et al., 1993; Kibbey et al., 1995; Rothman et al., 1996; Chalazonitis et al., 1997); (v) 5-HT/5-HT2B (Fiorica-Howells et al., 1997); (vi) endothelin-3 (ET-3)/endothelin-B (ET B) (Baynash et al., 1994; Hosoda et al., 1994; Puffenberger et al., 1994; Ceccherini et al., 1995; Edery et al., 1996; Gariepy et al., 1996; Hofstra et al., 1996; Karaki et al., 1996). These factors differentially affect two sublineages of crest-derived progenitors defined by their expression of the Mash-1 transcription factor (Blaugrund et al., 1996). When GDNF, Ret, CNTFRα, or Mash-1 is lacking,
ganglia are missing or defective in almost the entire bowel. In contrast, when ET-3 or ETB is deleted, only the terminal colon becomes aganglionic, suggesting that the ET-3/ETB effects are fundamentally different from those of the other factors.

Since ET-3/ETB mutations affect melanocyte as well as ENS development, ET-3 was postulated to be an autocrine growth factor required by the precursors of enteric neurons and melanocytes (Baynash et al., 1994). The ET-3/ETB-associated enteric lesion, however, is not neural crest autonomous (Jacobs-Cohen et al., 1987; Kapur et al., 1993; Rothman et al., 1993a,b; Coventry et al., 1994; Kapur et al., 1995); moreover, the loss of an essential autocrine effect on enteric neural precursors would not be expected to be region-specific. It is thus possible that ET-3 affects the enteric mesenchyme as well as crest-derived cells; for example, in ls/ls mice, which are ET-3-deficient (Baynash et al., 1994), smooth muscle development is delayed (Tennyson et al., 1986), and laminin-1 accumulates in the colon (Payette et al., 1988; Rothman et al., 1996).

The current study was undertaken to determine how ET-3 affects the development of crest- and non-crest derived cells of the bowel wall. Experiments were carried out in vitro with isolated crest-derived cells immunoselected from the developing murine gut with antibodies to p75NTR (Pomeranz et al., 1993; Chalazonitis et al., 1998b). ET-3/ETB inhibited development of neurons while promoting that of smooth muscle. We propose that this combination of effects normally prevents crest-derived cells from differentiating prematurely; aganglionosis of the terminal bowel occurs in the absence of ET-3/ETB, because precursors, which proliferate and migrate, give rise to neurons, which neither proliferate nor migrate, before the colonization of the gut has been completed. This hypothesis is consistent with data and conclusions from a study of developing avian ENS, in which ET-3 was found to oppose GDNF-stimulated neuronal development (Hearn et al., 1998).

**MATERIALS AND METHODS**

**Animals**

Female CD mice were obtained from Charles River (Waltham, MA) and lethal spotted mutant (ls/ls) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Gravid mice were killed by exposure to CO2. Pregnancies were timed from the day (E0) a vaginal plug was observed. Mice were killed by cervical dislocation before the colonization of the gut has been completed. This experiment was repeated 2-4 times and each experimental condition was studied in duplicate or triplicate.

**Immunocytochemistry**

Neurons were identified by using the immunoreactivity of a neuronal ubiquitin hydrolase (PGP 9.5; diluted in 1:500; Biogenesis Ltd., Poole, UK) (Wilkinson et al., 1989). Smooth muscle cells were identified with monoclonal antibodies against desmin (diluted in 1:100; Sigma, St. Louis, MO) (Debus et al., 1983) or a smooth muscle actin (diluted 1:100; Sigma, St. Louis, MO). Crest-derived cells were identified with a 1:2000 dilution of antibodies to mouse p75NTR (Huber and Chao, 1995). Neuronal/glial precursors (Hockfield and McKay, 1985; Friedman et al., 1993; Lendahl et al., 1990) were identified by their nestin immunoreactivity (diluted 1:500; Pharmingen, San Diego, CA). Other antibodies included polyclonal antibodies to laminin (diluted 1:100; Sigma), monoclonal antibodies to bromodeoxyuridine (BrDU; Boehringer Mannheim, Germany; diluted 1:2.5; 30 minutes), polyclonal antibodies to the cell-surface laminin binding protein, LBP110 (diluted 1:100; donated by Hynda Kleinman, NIH) (Jucker et al., 1993; Kibbey et al., 1993; Chalazonitis et al., 1997), polyclonal antibodies to ETα (Immunob-logical Laboratories Co., Fujiooka-city, Gunma Prefecture, Japan) (Yamamoto et al., 1997), and monoclonal antibodies to ETB (Immuno-Biological Laboratories Co., Fujioka-city, Gunma Prefecture, Japan) (Yamamoto et al., 1997), and monoclonal antibodies to the enteric smooth muscle actin (generously contributed by Dr. James Lessard of Cincinnati Children’s Hospital; diluted 1:100) (Qian et al., 1996). To detect BrdU, DNA was denatured with 4 N HCl for 10 minutes and neutralized prior to immunostaining.

**Immunoselection**

Crest-derived cells within the fetal gut were separated from cells that were not of neural crest origin by immunoselection (Pomeranz et al., 1993; Chalazonitis et al., 1998a) with antibodies to mouse p75NTR (Huber and Chao, 1995), (no. 9561, contributed by Dr. Moses Chao of Cornell Medical College; diluted 1:1000). p75NTR is expressed only by crest-derived cells within the developing bowel (Baetge et al., 1990). Positive and negative immunoselection with this antibody separate crest- and non-crest-derived cells very effectively (Chalazonitis et al., 1998b). The positively immunoselected population consists largely of p75NTR-immunoreactive cells, and mRNA encoding neurofilament protein-M, can be detected by reverse transcription-polymerase chain reaction (RT-PCR) only in the positively selected set of cells. For immunoselection, gastrointestinal tissue from 25-30 fetal mice (E15) was pooled, dissociated into a suspension of single cells by incubation with collagenase A, and finally immunoselected with magnetic beads (Chalazonitis et al., 1998a,b).

**Tissue culture**

Crest-derived (immunoselected) and non-crest-derived cells were each plated (6.0x10⁴ cells/well) and grown on 12 mm glass coverslips, which were coated with poly-D-lysine (20 μg/ml; Sigma) and laminin (10 μg/ml; Collaborative Research) or fibronectin (50 μg/ml; Boehringer Mannheim). The cells were cultured in a serum-free defined medium (Anderson et al., 1991), modified for use in an atmosphere of 5% CO₂. For analysis of bromodeoxyuridine (BrDU) incorporation, cells were cultured in the presence of BrdU (1 μg/ml) for 48 hours and maintained for an additional 5 days. Experimental conditions were studied in quadruplicate and experiments were repeated 3 times.

When organotypic cultures of control and ls/ls gut were analyzed, explants of gut (<1 mm²) were plated on 12 mm coverslips coated with NH₄OH-polymerized rat tail collagen (Upstate Biotechnology; Lake Placid, NY) (Coulter et al., 1988). Cultures were maintained in serum-free defined medium, as described above, for 7 days. Each experiment was repeated 2-4 times and each experimental condition was studied in duplicate or triplicate.

**[³H]Thymidine incorporation**

Cells were plated at a density of 2x10⁴ cells/well in 24-well culture plates. After 4 hours in culture, ET-3 (0.1-1000 nM), IRL 1620 (2 nM), or BQ 788 (100 nM) were added. Twenty hours later, [³H]thymidine ([³H]Tdr; NEN-Dupont, Boston, MA) was added at a final concentration of 1.0 μCi/ml and incorporation was allowed to continue for an additional 4 hours. Cultures were washed with ice 10% trichloroacetic acid (TCA). The TCA-precipitable material was then extracted with 0.3 N sodium hydroxide. Radioactivity was analyzed with a liquid scintillation spectrometer. Eight culture wells were studied for each set of conditions and experiments were repeated 5-6 times.

**RT-PCR**

Total RNA was isolated from cultured cells or fetal tissues by using...
ET-3/ET_B inhibits enteric neuronal development

1163

a commercial kit (RNAgents Total RNA Isolation System; Promega Corp., Madison, WI). Genomic DNA was removed by treating samples with DNase I (Gibco) for 15 minutes. For first-strand cDNA synthesis, the DNA-free total RNA (1.0 μg) was reverse transcribed (SUPERSCRIPT Preamplification System; Gibco BRL) and then amplified by incubating with sequence-specific primers and Taq DNA polymerase (Clontech, Palo Alto, CA) for 25-32 cycles. The PCR conditions were: 1 start cycle: 94°C 1 minute; subsequent cycles: 94°C 30 seconds, 68°C 2 minutes. The primers and expected sizes of the PCR products were: prepro ET-3, upstream: 5′-A TGGAGCCGGGGCTGTGGCTC-3′; downstream: 5′-CTAAGGCCGGTGGGCTTTA TCTGT-3′ (640 bp). Laminin α1, upstream: 5′-ACTTGGTGCTCCCTCTGAA TC-3′; downstream: 5′-AGCGG TGTTGGTCTTGTTGGG-3′ (799 bp) (Sasaki et al., 1988). ET_B, upstream: 5′-TCCACAATGAGGAGCATGAG-3′; downstream: 5′-CAGTTTCTACTTCTGTTGCC-3′ (536 bp). β-actin, upstream: 5′-CTCCTTTGTGATGTCAACGACAATTTC-3′; downstream: 5′-GTGG GCCGCTCTAGGCACCAA-3′ (508 bp). Each experiment was replicated.

Competitive PCR

Competitive PCR was utilized to investigate the effects of ET-3 on the transcription of laminin α1 in cultures of non-crest-derived cells. A DNA fragment (competitor), containing the same primer binding sites as the template, was constructed using a commercial kit (PCR MIMIC construction kit, Clontech). The competitor DNA was added to each PCR reaction tube (Siebert and Larrick, 1992). After PCR reactions were completed, the amplified products of template and competitor were distinguished by size in 1% agarose gels and visualized by staining with ethidium bromide (EtBr). The amount of template was...
RESULTS

Isolated crest- and non-crest-derived cells differ in their in vitro developmental capacities

Crest- (immunoselected) and non-crest-derived cells (those not selected by antibodies to p75\textsuperscript{NTR}) were cultured for 12 hours (Fig. 1A-D), fixed, and prepared for the immunocytochemical demonstration of type-specific cell markers. Both p75\textsuperscript{NTR}, (Fig. 1A) and PGP 9.5-immunoreactive cells (Fig. 1B) were present in the cultures of crest-derived, but not the non-crest-derived cells (Fig. 1C). The non-crest-derived cultures, however, contained many desmin-immunoreactive cells (Fig. 1D), which were not seen in the 12-hour cultures of crest-derived cells. Crest-derived precursors and neurons thus survive their immunoisolation and can be identified solely in cultures of positively immunoselected cells. In contrast, smooth muscle can only be detected in the cultures that were depleted of crest-derived cells by negative immunoselection.

Neurons were still abundant in cultures of immunoselected cells maintained for 6-7 days in vitro (Fig. 1E). These cultures now also contained glia, marked by S-100 immunoreactivity (Fig. 1F). There were no ICCs (Kit-immunoreactive cells) in the immunoselected cultures; however, Kit-immunoreactive cells (Fig. 1G), as well as smooth muscle (Fig. 1H) developed in sister cultures of non-crest-derived cells. The non-crest-derived cells that expressed Kit formed densely interwoven clusters of very fine cellular processes (Fig. 1G). Neurons and glia thus selectively develop and/or survive for a least a week in cultures of crest-derived cells, while ICCs arise in the cultures of non-crest-derived cells.

Although antibodies to desmin did not react with immunoselected cells up to 12 hours after their isolation, desmin-immunoreactive cells appeared in cultures of crest-derived cells maintained in vitro for ≥6 days (Fig. 1I,J). Coincident expression of desmin- and PGP 9.5 immunoreactivities was not observed (Fig. 1J). Most of the p75\textsuperscript{NTR}-immunoreactive cells that were present in cultures of immunoselected cells exhibited a neuronal morphology similar to that of cells immunostained with antibodies to PGP 9.5 (Fig. 1K, compare with Fig. 1E); nevertheless, these cultures also contained smaller numbers of p75\textsuperscript{NTR}-immunoreactive cells with a mesenchymal appearance (Fig. 1K). Virtually no cells were immunostained with antibodies to p75\textsuperscript{NTR} in cultures of non-crest-derived cells, even after culture for >6 days. Nestin-immunoreactive intracellular filaments were detectable in many of the cells of crest-derived cultures (Fig. 1L). Most of these cells were large and flat and were not immunostained with antibodies to PGP 9.5; however, coincident expression of nestin and PGP 9.5 immunoreactivities was found in occasional small cells with neurites. These observations are consistent with the ideas that p75\textsuperscript{NTR}-immunoreactive crest-derived cells may remain mesenchymal or give rise to smooth muscle in vitro. The subset that develops as neurons is probably derived from cells that first express nestin. The development of smooth muscle from Ret-immunoselected enteric crest-derived cells has previously been reported (Lo and Anderson, 1995).

ET-3 inhibits the development of neurons from enteric crest-derived cells

After immunoselection, crest-derived cells were cultured in serum-free defined media for 6-7 days in the presence or absence of ET-3, IRL 1620 (an ET\textsubscript{B} agonist) (Takai et al., 1992), or BQ 788 (an ET\textsubscript{B} antagonist) (Ishikawa et al., 1994). Neurons (PGP 9.5-immunoreactive) were found to develop in vitro in the absence of ET-3 (Figs 2A, 3). Neuronal development, furthermore, was also unaffected by the addition of BQ 788 (100 nM; Fig. 3). ET-3, moreover, decreased the number of neurons in the cultures (compare Fig. 2B with Figs 2A, 3). The ET-3-induced decrease in neuronal development was prevented by the addition of IRL 1620 (2.0 nM; Fig. 3). This concentration-dependent effect of ET-3 was mimicked by IRL 1620 (2.0 nM; Fig. 3). The ET-3-induced decrease in neuronal development was prevented by the addition of IRL 1620 (2.0 nM; Fig. 3).
ET-3 does not induce enteric crest-derived cells to proliferate

ET-3 has been demonstrated to stimulate the proliferation in vitro of primary neural crest cells, an effect that leads to the development of large numbers of melanocytes (Lahav et al., 1996). If ET-3 were to exert a similar effect on enteric crest-derived cells, then it might prevent the appearance of terminally differentiated neurons by preventing precursor cells from leaving the cell cycle. The ability of ET-3 to influence the proliferation of enteric crest-derived cells in vitro was therefore investigated.

DNA of cultured crest-derived cells was stained with bisbenzamide to facilitate accurate counting of cells (Fig. 2E,F). Exposure of cultured crest-derived cells to ET-3 (30-300 nM) for 7 days did not affect the total number of cells found in the cultures (compare Fig. 2E [control] and Fig. 2F [ET-3]; Fig. 4A). Since the measurement of the absolute number of cells might not reveal the proliferation of a small subset of the populations, the incorporation of BrdU and [3 H]TdR by neuronal precursors was studied. Since neurons are postmitotic, BrdU-labeled neurons reflect the incorporation of BrdU by neuronal precursors. The numbers of BrdU-labeled PGP 9.5 immunoreactive cells were thus counted in cultures grown ± ET-3 (Fig. 2C,D). The BrdU-labeling of neurons was not affected by ET-3 (100-1000 nM), the ET_b antagonist, BQ 788, or the ET_b agonist IRL 1620 (Fig. 4B). ET-3 also failed to influence the uptake of [3 H]TdR by the crest-derived cell population (Fig. 4C). Since neither ET-3 nor an ET_b antagonist promoted the proliferation of enteric crest-derived cells in vitro, the effects of GDNF, which is known to be a mitogen for enteric crest-derived neuronal precursors (Chalazonitis et al., 1998b; Hearns et al., 1998; Heuckerth et al., 1998), were investigated as a positive control. In contrast to ET-3, GDNF (10 ng/ml) stimulated the uptake of [3 H]TdR (Fig. 4C). It is thus unlikely that the observed inhibitory action of ET-3 on the in vitro development of enteric neurons is due to a mitogenic action of the peptide.

ET-3 promotes the in vitro development of enteric smooth muscle

The development of smooth muscle has been found to be abnormal in the gut of ET-3-deficient (ls/ls) mice (Tennyson et al., 1986). We therefore investigated the effects of ET-3 on the in vitro development of smooth muscle, using desmin immunoreactivity as a marker. For this purpose, crest-derived cells were removed by negative immunoselection with antibodies to p75NTR. Desmin immunoreactivity was found to be expressed by a subset of cells as soon as 12 hours following plating (Fig. 1D). Since ET-3 was itself found to be expressed simultaneously addition of BQ 788 (Fig. 3). These observations indicate that stimulation of ET_b by ET-3 is not essential for the development of enteric neurons. Instead, the effect of ET-3 on the in vitro development of enteric neurons is inhibitory.

**Fig. 3.** The development of enteric neurons is inhibited by ET-3. Neurons develop in the absence of ET-3 and even in the presence of BQ 788. ET-3 inhibits neuronal development; this effect is concentration-dependent ($P<0.05$ [control vs. 100 nM ET-3]; $P<0.01$ [control vs. 300 nM ET-3]; $P<0.001$ [control vs. 1000 nM ET-3]). IRL 1620 (2 nM) also inhibits neuronal development ($P<0.001$). The inhibition of neuronal development by ET-3 is blocked by BQ 788 ($P<0.001$ [BQ 788 + 300 nM ET-3 vs. 300 nM ET-3 alone]; $P$ is not significant [BQ 788 + ET-3 vs. control or BQ 788 alone]).

**Fig. 4.** ET-3 does not promote the proliferation of enteric crest-derived cells in vitro. (A) Total cell numbers (bisbenzamide-fluorescent nuclei) were not affected by exposure to ET-3. (B) The ratio of BrdU-labeled/total neurons (PGP 9.5-immunoreactive) was not significantly affected by BQ 788, ET-3, IRL 1620, or the combination of BQ 788 plus ET-3. (C) The uptake of [3 H]TdR by crest-derived cells was not affected by ET-3; however, the uptake of [3 H]TdR was increased by GDNF ($P<0.001$).
by non-crest-derived cells (see Fig. 9 below), the analysis of the effects of ET-3 on this population could, potentially, be confounded by the endogenous production of ET-3. This possibility was supported by the observation that the ET-3 antagonist, BQ 788 (100 nM), significantly decreased the number of desmin-immunoreactive smooth muscle cells developing in culture (to 83.7±3.7% of control; \( P<0.01 \)). The development of smooth muscle in ET-3- or IRL 1620-containing media was therefore normalized to that observed in media supplemented with BQ 788 (100 nM). ET-3 promoted smooth muscle development in a concentration-dependent manner (\( P<0.01 \) [100 nM ET-3 vs. BQ 788]; \( P<0.01 \) [1000 nM ET-3 vs. BQ 788]). IRL 1620 (2 nM) also promotes smooth muscle development (\( P<0.05 \) [IRL 1620 vs. BQ 788]).

The expression of laminin \( \alpha_1 \) by cultured enteric smooth muscle cells is decreased by ET-3

The extracellular matrix protein, laminin-1, has been found to accumulate in the aganglionic bowel of ET-3-deficient mice (\( {ls/ls} \)) (Payette et al., 1988; Rothman et al., 1996) and human patients with Hirschsprung’s disease (Parikh et al., 1992). The \( \alpha_1 \) subunit of laminin-1, moreover, promotes the development of enteric neurons through an action that is independent of the function of laminin as an adhesion molecule (Chalazonitis et al., 1997). We therefore determined which cells from the fetal bowel express laminin in vitro and investigated the effects of ET-3 on laminin \( \alpha_1 \) expression. Mixed cultures, containing crest- and non-crest-derived cells from the E11 fetal bowel and cultures of non-crest-derived cells obtained by negative immunoselection at E13 were studied.

All of the cells that were immunolabeled with antibodies to \( \alpha \) smooth muscle actin were co-labeled with antibodies to laminin (Fig. 6A-C). Rare cells were also found that either were laminin-immunoreactive but lacked \( \alpha \) smooth muscle actin immunoreactivity (Fig. 6A,D) or which expressed neither antigen. Intracellular laminin immunoreactivity was found in an anastomosing endoplasmic reticulum-like network that was most concentrated in the perinuclear cytoplasm and did not extend into lamellipodia (Fig. 6A-C). These observations suggest that laminin accumulates intracellularly in developing cells of the smooth muscle lineage. LBP 110, the receptor putatively activated by laminin-1 to stimulate neuronal development (Chalazonitis et al., 1997; Howard and Gershon, 1998) was not found in cultures of non-crest-derived cells, but was expressed in the cultures of p75NTR-immunoselected crest-derived cells (Fig. 6E).

To investigate the effects of ET-3/ET\( _B \) on the expression of laminin \( \alpha_1 \), mRNA encoding laminin \( \alpha_1 \) was measured in cultures of non-crest-derived cells (1x\( 10^6 \) cells/flask). Cells were exposed either to ET-3 (10 nM-1 \( \mu \)M), or the ET\( _B \)
ET-3/ETB inhibits enteric neuronal development

antagonist, BQ 788 (30-300 nM). RNA was extracted, reverse transcribed, and the resulting cDNA encoding laminin α1 was quantified by competitive PCR. ET-3 decreased (Fig. 7A), and BQ 788 (Fig. 7B) increased the expression of laminin α1. In both cases the effects were concentration-dependent. The ability of BQ 788 to increase expression of laminin α1 in the absence of added ET-3 suggests that there is an endogenous source of ET-3 (the effects of which are inhibited by BQ 788) in cultures of non-crest-derived cells. The observations that laminin is produced by smooth muscle, and that its expression is affected by ET-3 and BQ 788 support the idea that ETB receptors are expressed by smooth muscle cells (Fig. 6F).

The effect of ET-3 on neuronal development is substrate-dependent

Laminin and fibronectin are each constituents of the extracellular matrix of the fetal gut (Simon-Assmann et al., 1995) that support the in vitro development of enteric neurons from crest-derived precursors (Chalazonitis et al., 1997). Laminin α1 also promotes enteric neuronal development and its expression is regulated by ET-3/ETB (Fig. 7). Conceivably, therefore, laminin and/or fibronectin might influence the ability of ET-3 to inhibit enteric neuronal development. To investigate the putative interaction of laminin and fibronectin with ET-3, crest-derived cells were immunoselected from the E13 fetal bowel and cultured ± ET-3 (30-300 nM) on coverslips coated either with laminin-1 (10 μg/ml) or fibronectin (50 μg/ml). The number of PGP 9.5-immunoreactive cells developing in cultures exposed to ET-3 was determined and normalized to the average number of neurons developing on the same substrate in cultures grown in the absence of ET-3. ET-3 inhibited the in vitro development of neurons, no matter whether cells were grown on laminin or fibronectin (Fig. 8); however, ET-3 was significantly less effective when cells were grown on laminin (Fig. 8).

Although cultures of crest- and non-crest-derived cells each contain mRNA encoding ETB, only cultures of non-crest-derived cells contain mRNA encoding prepro ET-3

The hypothesis that ET-3 is an autocrine growth factor essential for the formation of enteric neurons (Baynash et al., 1994) predicts that crest-derived cells produce both ET-3 and ETB. mRNA encoding ETB and prepro ET-3 were therefore analyzed by RT-PCR in cultures of crest- and non-crest-derived cells, isolated respectively by positive and negative immunoselection from the E13 fetal mouse gut. Transcripts encoding ETB were detected in cultures of both crest- and non-crest-derived cells, although more ETB mRNA appeared to be present in the crest-derived cells. In contrast, mRNA encoding prepro ET-3 could only be detected in cultures of non-crest-derived cells. These observations are consistent with the ideas that ETB is expressed in the E13 gut both by crest- and non-crest-derived cells, while ET-3 is primarily a product of the non-neuronal cells of the enteric mesenchyme.

ET-3 promotes the development of neurons in cultured explants of terminal colons of ls/ls mice

Neurons developed well in explants of terminal colon removed at E12 from normal mice (Fig. 10A) and in explants of small intestine from E12 ls/ls mice (Fig. 10B). In contrast, no neurons could be detected in explants of terminal colon removed at E12 from ls/ls mice (Figs 10C, 11A-C). These observations confirm observations that have been reported previously (Rothman and Gershon, 1984). Even when the terminal ls/ls bowel was explanted together with pelvic p75NTR-immunoreactive crest-derived cells, none of these cells entered the gut, which remained aganglionic in culture (Fig.
of derived cells were present in explanted gut, p75 NTR -explants of ls/ls
Fig. 9. mRNA encoding prepro ET-3 is detectable only in cultures of non-crest-derived cells, while that encoding ETβ is detectable both in cultures of crest- and non-crest-derived cells. Cells were isolated by positive and negative immunoselection from fetal mouse gut at E13. Sel, crest-derived cells; Res, non-crest-derived cells; Ac, β-actin; et-3, endothelin 3; ETβ, endothelin B receptor.

10C,D). Although crest-derived cells were absent from explants of ls/ls terminal colon, they were highly abundant in explants of ls/ls small intestine (Fig. 10E). Whenever crest-derived cells were present in explanted gut, p75NTR, immunoreactive crest-derived cells migrated out of the explants (Fig. 10E) and gave rise to satellite ganglia several mm away from the explants (Fig. 10F). Neurites from these satellite ganglia projected to other such ganglia and back to neurons remaining within the parental explant (Fig. 10E,G).

When the entire ls/ls colon was explanted, neurons developed well in the proximal region, but the terminal zone remained aneuronal (Fig. 10H). In fact, a sharp line of demarcation divided the innervated from the non-innervated region. When ET-3 (100 nM) was added to these long explants of colon, crest-derived cells appeared in the presumptive aganglionic region of the bowel (Figs 10I, 11). The population of crest-derived cells reaching the anal end of the bowel (Fig. 11). The boundary between the innervated and aganglionic zones of the explants of ls/ls colon that develops in the absence of ET-3 is quite sharp (Fig. 10H) (Jacobs-Cohen et al., 1987). No such boundary is detectable when ls/ls colon in cultured in the presence of 100 nM ET-3 (Fig. 11C′′).

Co-culture experiments were carried out to analyze further the effect of ET-3 on the ability of crest-derived cells from extrinsic sources to colonize the presumptive aganglionic ls/ls terminal colon. Proximal bowel was used as a source of enteric crest-derived cells, because previous studies have established that crest-derived cells migrating out of such explants in vitro (see Fig. 10E) will colonize a co-cultured recipient segment of normal bowel (Jacobs-Cohen et al., 1987; Couthier et al., 1988). For these experiments, care was taken, while explanting the ls/ls terminal bowel to exclude adherent pelvic tissue, which might have contained crest-derived cells (see Fig. 10C above). When grown by itself, the ls/ls terminal gut was indeed found to be free of p75NTR-immunoreactive cells (Fig. 12A). Culture of these segments of terminal colon with ET-3 (100 nM) failed to cause p75NTR-immunoreactive cells or neurons to develop in these explants (Fig. 12B,C). As a positive control, segments of ls/ls stomach or small intestine were co-cultured together in the absence of ET-3. When this was done, crest-derived cells migrated out of each explant, formed satellite ganglia and established a continuous ganglionated plexus connecting the two segments of gut (Fig. 12D). In contrast, when either stomach or small intestine were cultured with segments of terminal colon, again in the absence of ET-3, no neurons were found in the terminal bowel (Fig. 12E). Crest-derived cells migrated out of the donor stomach and/or small intestine and formed satellite ganglia, as they did when these tissues were explanted alone or co-cultured with each other; however, the crest-derived cells did not migrate from the explants of proximal bowel into the terminal colon. In contrast, when the same experiments were carried out in the presence of ET-3 (100 nM), crest-derived cells did enter the explanted segments of ls/ls terminal colon and give rise to neurons (Fig. 12F,G) that innervate colonic smooth muscle (Fig. 12G).

DISCUSSION

During development, enteric neurons and glia are formed by crest-derived precursor cells that migrate into the developing bowel (Le Douarin and Teillet, 1973, 1974). This process depends on the presence of specific signaling molecules that must be present at the correct time and in the right location. Since the terminal colon is completely aganglionic when ET-3 or ETβ is absent (Baynash et al., 1994; Hosoda et al., 1994), this ligand and its receptor are each essential in order to complete the formation of the ENS; nevertheless, the molecular and cellular mechanisms by which ET-3 influences enteric neuronal development have been unclear.

The failure of neurons to develop in the terminal bowel of ET-3/ETβ-deficient animals, led to the suggestion that ETβ receptors on crest-derived cells have to be stimulated by ET-3 (secreted by the cells themselves) in order to express an enteric neuronal phenotype (Baynash et al., 1994; Hosoda et al., 1994; Puffenberger et al., 1994). This idea did not explain why neurons continue to develop normally in the proximal gut, even when ET-3 or ETβ is absent. An alternative hypothesis, based on the strong mitogenic effect exerted by ET-3 on avian crest cells in vitro (Lahav et al., 1996), was proposed subsequently: that ET-3 might be required to stimulate the proliferation of vagal crest cells to build up a population large enough to colonize the entire bowel. Neither of these hypotheses is compatible with the repeated observations that the ET-3/ETβ-associated aganglionosis of the terminal colon is not neural crest autonomous (Jacobs-Cohen et al., 1987; Kapur et al., 1993; Rothman et al., 1993a,b; Coventry et al., 1994; Kapur et al., 1995). Furthermore, neither hypothesis is supported by the current study, which suggests that: (i) ET-3 is synthesized by non-crest- rather than crest-derived cells, and thus is not an autocrine factor; (ii) ETβ is expressed both by crest- and non-crest-derived cells; (iii) ET-3/ETβ inhibits enteric neuronal differentiation by an action that cannot be explained as a mitogenic effect; (iv) ET-3/ETβ promotes smooth muscle development and downregulates smooth muscle production of laminin α1; (vi) the bowel is itself the site where signaling by ET-3 plays its critical role in ENS development. Expression of mRNA encoding ET-3 by enteric mesenchymal cells has previously been observed in chick embryos (Nataf et al., 1998). We propose that the physiological role of ET-3/ETβ in the
development of the ENS is to prevent the premature differentiation of enteric neurons; the effects of ET-3 on smooth muscle and crest-derived neural precursors, reinforce one another in enabling crest-derived precursors to finish colonizing the gut before they differentiate and consequently cease migrating and proliferating. The earlier observation that ET-3 inhibits the GDNF-driven differentiation of avian enteric neurons in vitro also led to the idea that ET-3 might prevent premature development of enteric neurons (Hearn et al., 1998).

To avoid the potentially confounding effects of cellular interactions in analyses of the actions of secreted factors on the development of enteric crest-derived cells in vitro, it is necessary to separate them from their non-crest-derived neighbors. In the current study this separation was accomplished by positive and negative immunoselection with antibodies to p75NTR. Markers for crest-derived cells, neural/glial precursors, and determined neurons and glia (p75NTR, nestin, PGP 9.5, and S-100 respectively) were found almost exclusively in cultures of positively immunoselected cells, while muscle (desmin, \(\alpha\) smooth muscle actin) and ICC (Kit) markers were found in the cultures of negatively immunoselected cells. The effectiveness of immunoselection enabled the technique to be used to determine which populations of enteric cells are affected by ET-3, the nature of

**Fig. 10.** Although neurons do not develop in explants of terminal gut from \(ls/ls\) mice, they can be made to do so by providing a source of crest-derived cells and adding ET-3 to the medium. (A) Terminal colon from a control mouse. PGP 9.5 immunoreactivity. Ganglia (arrow) and connectives (arrowhead) have formed a neural plexus within the explant. (B) Small intestine from an \(ls/ls\) mouse. PGP 9.5 immunoreactivity. A large ganglion has formed within the explant; neurons and varicose neurites are abundant. (C-D) Terminal colon from an \(ls/ls\) mouse (same field). (C) p75NTR immunoreactivity; (D) desmin immunoreactivity. The explant (E) contains no p75NTR-immunoreactive cells; however, small clusters of such cells (C; arrows), which were explanted with the gut, do not enter it. The crest-derived cells do not use the outgrowth of smooth muscle (D) that has migrated away from the bowel as a bridge over which to migrate to the explant (compare with C). (E) Small intestine from an \(ls/ls\) mouse. p75NTR immunoreactivity. Many crest-derived cells leave the explant and disperse on the surrounding substrate where neurons develop and connect to the explant via thick nerve bundles (arrowheads). (F) Small intestine from an \(ls/ls\) mouse (ET-3 present, 100 nM). PGP 9.5 immunoreactivity. Neurons (arrow) form satellite ganglia in the zone of outgrowth far from the explant proper. (G) Small intestine from an \(ls/ls\) mouse (ET-3 absent). PGP 9.5 immunoreactivity. A ganglion has formed within the explant; neurons (arrows) and tangled nerve bundles are abundant. (H-I) Whole colon from an \(ls/ls\) mouse. Explants include both the presumptively aganglionic terminal colon and the more proximal bowel. P75NTR immunoreactivity. In the absence of ET-3 (H), crest-derived cells are present in the proximal bowel and form ganglia (arrows) and connectives (arrowheads) but p75NTR-immunoreactive cells do not enter the terminal colon (TC). In the presence of ET-3 (I), crest-derived cells enter the terminal colon (TC); some retain a mesenchymal appearance (arrowhead), while others extend neurites (arrows). The inset in I illustrates where in the explant of the whole colon the crest-derived cells illustrated in I are located; P, proximal; D, distal. Bars (A-H), 50 \(\mu\)m, (F), 100 \(\mu\)m, (I inset), 500 \(\mu\)m.
those effects, and the identity of the sets of enteric cells that express ET-3 and ET-B.

ET-3 was observed to inhibit, not to stimulate, the in vitro development of enteric neurons. In fact, crest-derived cells not only developed in the absence of ET-3, they even appeared normally in the presence of the ET-B antagonist, BQ 788. Clearly, therefore, ET-3 is not, as originally postulated, a factor that is essential for the formation of enteric neurons. ET-3, moreover, did not induce enteric crest-derived cells to proliferate. Since GDNF, an authentic mitogen, did so, the isolated crest-derived cells were capable of dividing. The response to ET-3 of crest-derived cells that have migrated to the bowel, therefore, may be different from that of their premigratory predecessors, which are induced by ET-3 to proliferate and develop as melanocytes (Lahav et al., 1996). Differences between primary crest and enteric crest-derived cells have previously been reported. These differences include expression of plasmalemmal receptors (Pomeranz et al., 1991b) and developmental potential (Rothman et al., 1990; Sextier-Sainte-Claire Deville et al., 1994; Lo and Anderson, 1995). Responses of enteric crest-derived cells to ET-3 and other factors are probably modified by the age and experience of the responding cells. NT-3, for example, exerts no effect on enteric crest-derived cells isolated from the gut at E12, but promotes neural and glial development at E14 (Chalazonitis et al., 1998b). TrkC expression is also induced by GDNF. The sequence in which crest-derived cells encounter particular growth factors may thus be significant. Alternatively, it is possible that truncal crest-derived cells are selectively induced to proliferate and develop as melanocytes because vagal crest cells have been reported not to respond to ET-3 (Hearn et al., 1998).

The terminal colon-restricted nature of the effect of deleting ET-3/ET-B on ENS development is strikingly different from that of deletions of other factors or their receptors. When GDNF/GFRα-1/Ret, Mash-1, LIFRβ or CNTFRα are deleted, the entire territory of the vagal and sacral derivatives of the crest are uniformly affected (Gershon, 1998). Any explanation of the actions of ET-3, therefore, has to account for their geographic restriction. The terminal colon, moreover, receives crest-derived precursors from two sources, the sacral as well as the vagal (Le Douarin and Teillet, 1973; Le Douarin and Teillet, 1974; Pomeranz et al., 1991a; Serbedzija et al., 1991). To reach the terminal bowel, vagal crest-derived emigrés have to descend the entire gut, and sacral cells, which begin migrating later than their vagal counterparts, have to traverse the pelvic mesenchyme. While the crest-derived cells that colonize the bowel are able both to migrate and proliferate (Teitelman et al., 1981), terminally differentiated neurons can do neither; therefore, if neurons in the vagal and sacral

**Fig. 11.** Exposure to ET-3 enables crest-derived cells to migrate to the anal end of an explanted colon. (A) The entire length of colon was explanted from an E12 ls/ls mouse and cultured in the presence of ET-3 (100 nM). The preparation was triply labeled with bisbenzamide to show the location of all nucleated cells (A–C), antibodies to desmin to show the location of cells developing in a muscle lineage (B′, C′), and antibodies to p75NTR to show the locations of crest-derived cells (B″, C″). The bisbenzamide fluorescence of the entire explant is illustrated in A. P, proximal; D, distal. The proximal field encompassed within the horizontal double headed arrow is shown at higher magnification in B, B′, and B″, while that encompassed within the vertical double headed arrow is illustrated in C, C′ and C″. Note that both p75NTR-immunoreactive cells (B″) and desmin-immunoreactive cells (B′) are abundant at the proximal end of the explant. Cells have grown out of the anal end of the explant (C). This outgrowth contains many desmin-immunoreactive cells (C′) and also a smaller, separate set of cells that are p75NTR-immunoreactive (arrows: C″). Bars, 100 μm.
ET-3/ETB inhibits enteric neuronal development

1171

ET-3/ETB inhibits enteric neuronal development

Distributions differentiate before they have finished colonizing the gut. The remaining bowel will become aganglionic. The observation that ectopic pelvic ganglia, not present in normal mice, develop along the sacral pathway in ET-3-deficient animals supports this idea (Rothman and Gershon, 1984; Payette et al., 1987); these ganglia have been postulated to arise from sacral crest-derived cells that have stopped migrating short of the gut.

Both of the actions of ET-3 observed in the present investigation would seem to work to delay the differentiation of crest-derived cells. Neuronal development is inhibited directly, via ETB expressed by crest-derived cells, and also indirectly, via ETB expressed by cells in the smooth muscle lineage. The ET-3-enhanced maturation of smooth muscle, and associated downregulation of laminin α1 expression, should diminish the exposure of crest-derived cells to an environment that would favor their neuronal differentiation. Laminin α1, which promotes neuronal development in vitro (Chalazonitis et al., 1997), was found, in the current study, to oppose (relative to fibronectin) the inhibitory effects of ET-3 on the development of neurons. Decreasing the concentration of laminin in the extracellular matrix would thus be expected to potentiate the in vivo restraining influence of ET-3 on the differentiation of crest-derived cells. The observation that laminin α1 expression is upregulated in ET-3 deficient mice (Payette et al., 1988; Rothman et al., 1996), suggests that the in vitro actions of ET-3 on laminin expression reflect an in vivo phenomenon.

If the hypothesis that ET-3 prevents premature differentiation is correct, then the critical site of action of ET-3 in the formation of the ENS would be the gut, not the premigratory or early-migrating neural crest. Current observations strongly support this idea. The addition of ET-3 to explants of whole colon from ls/ls mice was found to enable crest-derived cells to enter the terminal presumptively aganglionic zone. ET-3 also enabled exogenous sources of crest-derived cells to enter co-cultured explants of terminal bowel. According to our hypothesis, the in vitro effectiveness of ET-3 would be explained by a dual action, one to accelerate the development of smooth muscle with a concomitant decrease in the biosynthesis of laminin α1 within the explants, and the other to delay the differentiation of crest-derived cells. The decrease in laminin α1 would make the microenvironment less conducive to neuronal differentiation, which together with the anti-differentiative effect exerted by ET-3 on the crest-derived cells themselves, would enable them to remain migratory long enough to enter the otherwise forbidden zone of the terminal colon. Experiments with transgenic mice have
also suggested that the gut is the critical locus of ET-3’s action in the formation of the ENS (R. Kapur, personal communication). The presence of mRNA encoding ETB in both the crest- and the non-crest-derived cell populations isolated by immunoselection provide further support for the idea that ET-3 exerts an action within the gut. The idea that cells in the smooth muscle lineage, as well as crest-derived cells, express functional ETB receptors is supported by the coincident localization of ETB and desmin immunoreactivities in the same cells, in addition to effects of ET-3 on smooth muscle development and laminin α1 expression. ETB is also expressed by adult intestinal smooth muscle (Okabe et al., 1995).

This work was supported by NIH grants NS15547 and NS 07062 to M. D. G. and HD21032 to T. P. R.

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demonstrate that the primary defect responsible for aganglionic megacolon in lethal spotted mice is not neuroblast autonomous. Development 117, 993-998.


**ET-3/ETb inhibits enteric neuronal development**