Endothelin-B receptor is expressed by neural crest cells in the avian embryo

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ABSTRACT Disruptions of the genes encoding endothelin 3 (EDN3) and its receptor endothelin-B receptor (EDNRB) in the mouse result in defects of two neural crest (NC)-derived lineages, the melanocytes, and the enteric nervous system. To assess the mechanisms through which the EDN3/EDNRB signaling pathway can selectively act on these NC derivatives, we have studied the spatiotemporal expression pattern of the EDNRB gene in the avian embryo, a model in which NC development has been extensively studied. For this purpose, we have cloned the quail homologue of the mammalian EDNRB cDNA. EDNRB transcripts are present in NC cells before and during their emigration from the neural tube at all levels of the neuraxis. At later developmental stages, the receptor remains abundantly expressed in the peripheral nervous system including the enteric nervous system. In a previous study, we have shown that EDN3 enhances dramatically the proliferation of NC cells when they are at the pluripotent stage. We propose that the selective effect of EDN3 or EDNRB gene inactivation is due to the fact that both melanocytes and enteric nervous system precursors have to colonize large embryonic areas (skin and bowel) from a relatively small population of precursors that have to expand considerably in number. It is therefore understandable that a deficit in one of the growth-promoting pathways of NC cells has more deleterious effects on long-range migrating cells than on the NC derivatives which develop close to the neural primordium like the sensory and sympathetic ganglia.

The vertebrate neural crest (NC) is a highly pluripotent structure that gives rise to a large variety of derivatives, such as the neuronal and glial cells of the peripheral nervous system, melanocytes, some endocrine cells, and mesenchymal derivatives that participate in the morphogenesis of the head (1). Soon after the NC is formed, its constitutive cells migrate to a variety of regions of the embryo where they differentiate. By using transplantation and in vitro culture experiments (1, 2), it was shown that the target tissue to which the NC cells home at the term of their migration is of crucial importance in determining their fate. Clonal cultures of NC cells taken at the time they migrate revealed that the population of migrating cells is heterogeneous and composed of cells which exhibit different proliferation and differentiation potentialities (3). Some crest cells are already committed toward a definite phenotype, but most of them are pluripotent. The final differentiation choice results for each NC derivative from the interaction between this heterogeneous cell population and local differentiation cues. Some of the factors able to influence the differentiation of NC cells have been identified. Such is the case for neurotrophins like brain-derived neurotrophic factor (4–6) and NT3 (8), or other factors [e.g., basic fibroblast growth factor (8), glial growth factor (9), retinoic acid (10) and the Steel factor (11)] as shown by in vitro culture experiments or gene targeting (12–18).

Certain mutations in the mouse affect the development of the melanocytic lineage and are characterized by coat color spotting: such is the case for steel (Sl), dominant white spotting (W), lethal spotted (ls), and piebald lethal (sl) mutations. The genes affected in W and Sl mutants have been cloned and turned out to encode, respectively, the c-kit tyrosine kinase receptor and its ligand the Steel factor (19–22). In both the ls and sl mutants pigmentation defects are associated with megacolon resulting from an incomplete innervation of the gut. It was recently demonstrated that the ls phenotype is due to a mutation of the gene encoding endothelin 3 (EDN3) (23), whereas in sl mice the gene encoding EDN3 receptor, endothelin-B receptor (EDNRB), is deleted (24). These data were confirmed by the targeted inactivations of these two genes that reproduced the phenotypes of both ls and sl mutants (23, 24). Recently, a mutation of EDNRB in rat was found in the spotting lethal mutant that also exhibits megacolon and coat color spotting (25). However, no data are available so far about the expression pattern of either EDNRB or EDN3 in the NC and in the structures where NC cells differentiate. If the EDN3/EDNRB signaling pathway acts on NC cell differentiation as does the Sl/c-kit one, it can be proposed that EDN3 is provided to NC cells by the environment, whereas the EDNRB gene product is present in those NC-derived precursors affected in sl and ls mutants. In such a case, EDNRB would act in a cell autonomous manner. Some doubts have recently been shed on this straightforward hypothesis by the fact that in sl/sls/lsls wild-type chimeric mice, sl enteric neuroblasts were reported to be able to colonize the complete gut, including the terminal colon provided that the ratio of normal to sl chimera reaches an appropriate level (26). This suggested that normal NC cells could rescue their sl counterpart, indicating that the EDNRB mutation does not act in a strictly cell autonomous manner. In this context it seemed essential to document the pattern of expression of EDNRB in NC cells and their derivatives to further understand the mode of action of EDN3. Since the development of the NC has been particularly documented in the avian embryo (1, 2) we decided to clone the quail homologue of the EDNRB cDNA and to investigate its expression pattern during avian NC development both in vivo and in vitro, with special emphasis on the melanocytic lineage and the enteric nervous system (ENS) which are affected in EDNRB and EDN3 mutants.

MATERIALS AND METHODS

JA57 strain chicken and quail (Coturnix coturnix japonica) eggs were obtained from commercial sources and incubated in a

Abbreviations: E, embryonic day; DRG, dorsal root ganglion; EDN, endothelin; EDNR, endothelin receptor; ENS, enteric nervous system; NC, neural crest; ss, somite stage; Sl, sympathetic ganglion.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. X99295).

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humidified atmosphere at 38°C. The embryos were staged by the number either of somite pairs formed or of days of incubation (E).

Cloning of the quail EDNRB was achieved by reverse transcription–PCR and subsequent cDNA library screening. Total RNA from a variety of chicken tissues was extracted as described (27). Ten micrograms of RNA was reverse-transcribed with 200 units of Moloney murine leukemia virus reverse transcriptase (BRL). The reaction was primed with 50 pmol of 3'-oligonucleotide (see below), PCR (1 min at 94°C, 1 min at 50°C, 1 min at 72°C, 25 cycles) was performed in 100 µl of a buffer containing 10 mM Tris (pH 8.3), 50 mM potassium chloride, 2.8 mM magnesium chloride, 0.01% gelatin, 0.25 mM dNTPs, 100 pmol of each oligonucleotide, and 2.5 units of Taq polymerase (Perkin–Elmer). The oligonucleotides used were 5'-oligonucleotide: 5'-CTANATTGGNATCAACATG-3', and 3'-oligonucleotide: 5'-AAGTGTGTCNTATNCGTGATC-3' (nucleotides 1103–1123 and 1278–1298, respectively, in human EDNRB cDNA). A PCR product of the expected size (200 bp) was amplified from kidney RNA, purified from a 1% agarose gel, and ligated to a pCR II vector (Invitrogen).

For isolation of cDNA clones, we used an E4 quail cDNA library (Invitrogen) as described (28). Approximately 3 x 10⁶ recombinants were screened with 32P-labeled probes prepared from the PCR fragment using a random-prime labeling kit (Promega). High stringency hybridization was performed at 42°C in buffer containing 50% formamide followed by washing at 65°C in 0.5x standard saline citrate (SSC)/0.1% SDS. The 492-bp EcoRI fragment located at the 5' end of the longest clone isolated in the first screen (clone b) was used in a subsequent screen. Nucleic acid sequences were determined on both strands by the dideoxynucleotide chain termination method. Sequence alignments were performed using the GENEWORLD program (IntelliGenetics).

In Situ Hybridization. Antisense and sense RNA probes were prepared from the EDNRB cDNA clones a and b by in vitro transcription using SP6 and T7 RNA polymerase with either 32P-UTP (Amersham, 1000 Ci/mm; 1 Ci = 37 GBq) or dig-UTP (Boehringer Mannheim). Whole mount nonradioactive in situ hybridization was performed according to Henrique et al. (29). Radioactive probes were prepared as described (28). Radioactive hybridizations were performed on paraffin sections (5 µm) prepared from quail and chicken embryos fixed in Carnoy's solution (60% EtOH/30% CHCl3/10% acetic acid) and on cell cultures fixed in 4% paraformaldehyde in PBS. The procedures applied for slide treatment, hybridization, and washing were already described (28). After hybridization, the washed sections and cultures were either dehydrated and autoradiographed (NTB-2 emulsion; Kodak) or treated with antibody (see below). Exposure time was 8–10 days.

Antibody Staining. The HNK1 monoclonal antibody (mAb) recognizes an epitope present on most migrating NC cells (30). At later stages, it stains most neuronal and nonneuronal cells of the peripheral nervous system, while mesectodermal derivatives and melanocytes are unlabeled (31). After hybridization, the sections were incubated for 1 h at room temperature with HNK1 mAb used as undiluted hybridoma culture supernatant. Subsequent treatments were performed as described (28).

Cell Cultures. Briefly, the trunk neural tube was isolated from embryos at 20- to 25-somite stage (ss) and cultured in collagen-coated Lab-Tek (Miles) chamber slides. The neural tube was removed from the cultures 48 h after explantation, when crest cells had migrated on the substrate as described in ref. 32. DMEM (GIBCO) with 10% fetal calf serum and 2% chick embryo extract was used during the first 48 h and replaced by DMEM, 10% fetal calf serum, and 10% chick embryo extract after removal of the tube. The percentage of EDNRB-expressing cells in NC cultures was evaluated by counting at least 1500 cells in randomly chosen fields.

RESULTS

Cloning of a Partial Quail EDNRB cDNA. A fragment of the EDNRB mRNA was amplified by reverse transcription–PCR from adult chicken RNA using oligonucleotides located in conserved regions of the EDNRB sequence of three mammalian species [rat (33), human (34), and bovine (35)]. To clone the EDNRB but not the EDNRA cDNA, the 3' oligonucleotide was located in the cytoplasmic tail of the EDNRB, which is distinct from the EDNRA sequence. The 5' oligonucleotide was located in the 7th transmembrane domain. A 200-bp fragment was amplified, cloned, and sequenced. Comparison of the deduced amino acid sequence showed 92% homology to the corresponding region of the human EDNRB and only 51% to human EDNRA.

To isolate longer cDNA clones, the EDNRB PCR product was used to screen an E4 quail cDNA library. Two overlapping clones of 1.1 kb (clone a) and 2.1 kb (clone b) were isolated. Restriction mapping and sequence analysis of these two clones demonstrated that both contain the sequence originally amplified by reverse transcription–PCR and that clone b encompassed the 1.1-kb clone (Fig. 1A). Both clones contained a stop codon 29 bp downstream from the sequences amplified by PCR. Clone b did not show a polyadenylation site at its 3' end and contained 466 bp of additional 5' sequences spanning transmembrane domains V and VI of the EDNRB.

Rescreening of the cDNA library with a 494-bp EcoRI fragment isolated from clone b led to the isolation of a 4-kb clone, clone c (Fig. 1A). This clone contained 2.8 kb of 3' untranslated sequences devoid of a polyadenylation site and an additional 5' stretch corresponding to transmembrane domains I to IV. The entire coding region of both clones b and c was sequenced on both strands. The deduced amino acid sequence of clone c is 92% identical to EDNRB and 63% identical to EDNRA of both human and rat (Fig. 1B).

Expression Pattern of EDNRB in Avian Embryo. The expression of EDNRB was investigated by in situ hybridization using radioactive and nonradioactive riboprobes. Most of the experiments were carried out in quail embryos. Observations in chicken were similar to those obtained in quail.

Whole-mount in situ hybridization showed that, as early as 4 ss, when the neural tube begins to close at the level of the presumptive mesencephalon, cells of the neural epithelium express EDNRB (Fig. 2A). Expression is particularly intense in the neural folds that later on yield the NC cells. EDNRB expression was never detected in the anteriormost region of the neural folds, which does not yield NC cells (36) (Fig. 2B). At 10 ss, NC cells migrating from the mesencephalon contained EDNRB transcripts. At 14 ss, two streams of positive rhombencephalic NC cells migrating rostral and caudal to the otic vesicle were strongly labeled. At that stage, in the trunk region, expression was found in the neural folds (Fig. 2C).

Radioactive in situ hybridization on sections confirmed the labeling of NC cells migrating at the cephalic level (Fig. 3A and B). In the trunk region, at 27 ss, EDNRB transcripts were present in the neural folds before crest cell emigration and in migrating cells as well, depending on the anteroposterior level considered (Fig. 3 C–F).

Expression of the EDNRB gene in NC derivatives was studied on transverse sections of E3 to E6 quails. At the trunk level, dorsal root ganglia (DRG), peripheral nerves (Fig. 3 G and H), sympathetic ganglia (SG), and NC-derived cells migrating ventrally to form the aortic plexus are positive from E3. The adrenal gland at E6 is EDNRB+ (not shown). At the cephalic level, E3–E6 cranial nerves and associated sensory ganglia strongly express EDNRB. At E5, cephalic NC cells invade the branchial arches; these cells comprise mesectodermal cells and neural type cells. Only the latter express the HNK1 epitope (31). We found that only HNK1+ cells con-
Fig. 1. Restriction map of the quail EDNRB cDNA (A) and amino acid alignment to mammalian EDNRB and EDNRA (B). (A) The PCR clone is indicated (solid box). The cDNA clones isolated in successive screens are shown below (clone a, clone b, clone c). The stop codon (*) and restriction sites (B, BglII; E, EcoRI; H, HindIII) are indicated. (B) The deduced amino acid sequences of EDNRB clone c (qB), human and rat EDNRB (hb, rb), and EDNRA (ha, tA) were compared. Identical amino acids are boxed. Putative transmembrane domains I to VII are indicated.

EDNRB transcripts in E3 branchial arches, indicating that mesenchymal cells of NC origin are EDNRB⁺ (not shown).

Since null mutations of the EDNRB gene in the mouse affect the ENS and the melanocytic lineage, we investigated in more detail the expression of this gene in NC cells which colonize the developing quail gut and skin.

The NC-derived cells that form the ENS were identified by their immunoreactivity for the HNK1 mAb. Virtually all the HNK1⁺ cells destined to yield Auerbach’s and Meissner’s plexuses of the gut wall as well as the ganglion of Remak (located in the dorsal mesentery) express EDNRB at all the stages observed (E3–E8). At E3, HNK1⁺ cells expressing EDNRB were found only in the rostral region of the digestive anlage. At E4, the gizzard contains cells double labeled with HNK1 mAb and EDNRB riboprobe. In the post-umbilical gut
caudal to the ileocecal junction, NC-derived cells start to express EDNRB from E6 onward. Thus, both the HNK1 epitone and the EDNRB transcripts were simultaneously detected in the NC-derived cells during the rostrocaudal colonization of the gut which proceeds as described by Le Douarin and Teillet (37) (Fig. 2 D–G). No messenger was ever found in the gut wall mesenchyme and endoderm.

No EDNRB⁺ cells were detected dorsolaterally to the neural tube at E3, when melanocyte precursors migrate in the subectodermal mesenchyme along a dorsolateral pathway. Moreover, no EDNRB expression was found in the epidermis at E5, E6, and E8. Melanoblasts and pigment cells in epidermis and in the feather at E10–E14 do not express EDNRB.

In addition to the neural folds, the early migratory NC cells and certain of their derivatives, the developing kidney was strongly labeled at the stages observed in this study (E3–E6) (Fig. 3G and H). EDNRB is already expressed in the Wolffian duct at 14 ss. The heart and liver of E5 and E6 quails were weakly labeled.

Expression of EDNRB in NC Cultures. Cultures of trunk NC cells were obtained by isolating the neural primordium from 20 to 25 ss quail embryos. After 2 days, NC cells had migrated in vitro from the neural tube explants. About 65% of these NC cells showed a positive signal after in situ hybridization with 3⁵-S-EDNRB probe (Fig. 4 A and B). In 5-day-old cultures,
melanin-containing cells were differentiated. These pigmented cells did not express EDNRB mRNA whereas about 55% of the unpigmented NC cells showed an hybridization signal (Fig. 4 C and D).

DISCUSSION AND CONCLUSIONS

We have cloned a partial quail EDNRB cDNA with the aim of studying its expression pattern during NC ontogeny. We find that the lateral ridges of the neural primordium called neural folds, which are at the origin of the NC, strongly express the EDNRB gene as soon as the early somitic stages. Interestingly, the anteriormost region of the cephalic neural fold that does not yield NC cells and remains epithelial (36) does not contain EDNRB transcripts. At the level of the spinal cord, expression of the gene is well visible in the dorsal aspect of the neural tube as it closes and before NC cells have started to migrate. The early steps of NC cell migration are characterized by a strong EDNRB gene expression at both the cephalic and trunk levels.

In the head, the NC cells migrate superficially, between ectoderm and paraxial cephalic mesoderm (1, 38) and give rise to melanocytes and to neural (the cranial sensory and autonomic ganglia) and mesectodermal derivatives. The latter form most of the skull and facial bones and cartilages (39). Notably, the cephalic NC cells invade the branchial arches and colonize the space lying between the forebrain and the superficial ectoderm later differentiating into membrane bones and cartilages. We have shown that, once NC-derived mesenchymal cells have reached the branchial arches, they do not express EDNRB, while the cephalic neural derivatives of the crest remain EDNRB+ as do their truncal counterpart. Interestingly, null mutants of a EDN3-related peptide, EDN1, have morphological defects in hypobranchial and craniofacial structures that are mostly made up of mesenchymal NC cells (40). The effect of EDN1 on the development of these structures is therefore likely to be mediated by a non-EDNRB endothelin receptor.

At the trunk level, the NC cells exiting the neural fold after neural tube closure first take the so-called dorsoventral pathway by migrating (i) within the rostral moiety of each somite where most of them aggregate, in contact with the neural tube, to form the DRG, and (ii) between two consecutive somites, to form the SG chain and, at the brachial level, the adrenal medulla (1). It is only later (at E2.5–E3 depending on the anteroposterior level considered) that the dorsolateral pathway of migration becomes available for NC cells migrating to the skin. Then NC cells penetrate into the subectodermal mesoderm and finally home to the epidermis where they differentiate into melanocytes (41–43).

During their dorsolateral progression NC cells start to express the c-kit receptor while the c-kit ligand, the Steel factor, is expressed at that time in the developing skin (44). Our observations of EDNRB gene expression during this highly dynamic process have revealed that NC cells exhibit a strong level of transcription of this receptor at E2 and E3, when they are invading the dorsoventral pathway. The cells that form the DRG, SG, and adrenal medulla as well as Schwann cells, lining the peripheral nerves, maintain high levels of EDNRB expression over the whole developmental period screened in this study (up to E8). In contrast, the cells that are in the skin pathway and express c-kit at E3.5–E4 do not show EDNRB transcripts. Those cells are likely to be derived from EDNRB+ precursors that have switched off expression of this gene when penetrating this pathway.

One of the main derivatives of the NC, the ENS, arises essentially from the “vagal” NC, the level of somite 1–7, with also a modest contribution of the “sacral” NC, in both quail and chicken embryo (37).The wave of migration of these cells has been followed over its anteroposterior progression along the gut through either the quail-chicken chimera technique (37) or by means of the HNK1 mAb (45). We show here by double labeling with the HNK1 mAb and in situ hybridization with the EDNRB probe that the “vagal” NC cells express the EDNRB gene during their migration and after they have formed the Auerbach’s and Meissner’s plexuses as well.

Fig. 3. (A–F) EDNRB expression by migratory and premigratory NC cells as shown by radioactive in situ hybridization on transverse sections of quail embryos. (A and B) Transverse section in mesencephalic region of a 10 ss. (A) Bright field. (B) Dark field showing expression of EDNRB gene by NC cells migrating under the ectoderm (arrows). At this stage, the ventral part of the neural tube is also EDNRB+. (C and F) Trunk level at 27 ss. Section in C and D is more dorsal than in E and F. NC cells express EDNRB when they are still in the neural folds (D) and when they migrate in the dorsoventral pathway (arrows) (F). (Bar = 60 μm.) (G and H) In situ hybridization on transverse sections of an E5 quail in trunk region. Strong expression of EDNRB mRNA in DRG, SG, and, in peripheral nerve (PN). The kidney (K) is also labeled. Signal in blood cells is not specific. (Bar = 120 μm.)

Fig. 4. EDNRB expression in cultures of trunk quail NC cells. In situ hybridization was carried out on 2- and 5-day cultures. Bright fields are shown in A and C and corresponding epipolarization views are shown in B and D. In 2-day cultures (A and B), about 65% of the cells express EDNRB. In 5-day cultures (C and D), melanocytes are EDNRB+ (arrows) while about 55% of unpigmented cells are EDNRB+ (double arrows). (Bar = 60 μm.)
In view of these findings we wish to speculate on the mechanisms through which EDN3 influences the development of NC derivatives. First, expression of EDNRB by virtually all NC cells during the migration process is compatible with the deleterious effect on NC derivatives development of EDN3 and EDNRB knock-out experiments carried out in the mouse (23, 24). Another observation from our laboratory is also in complete agreement with the results of knock-out experiments. It was shown that EDN3 increases dramatically the proliferation of cultured NC cells whether they are of trunk (46), vagal or mesencephalic origin (C. Ziller and E. Dupin, personal communication). In presence of EDN3 and chick embryo extract, which is well known to favor melanocytic differentiation (ref. 10 and references therein), most (but not all) of the cultured cells differentiate into melanocytes. This concurs with the observation reported here that in vivo and in vitro, most NC cells express the EDNRB gene before and at the onset of their migration. It was previously shown that, at the time they migrate, most NC cells are pluripotent (3, 47). One can therefore assume that EDN3 plays a crucial role in expanding the population of the NC cells as they exit from the neural folds. We suggest that this early growth of the NC cell population is crucial to ensure the survival of a sufficient number of NC precursors for them to be able to colonize the entire skin area and the entire bowel. Melanocytes and enteric plexuses are actually the two NC derivatives, the constitutive cells of which have to undergo the more extended migration and numeric expansion. Therefore it is understandable that a deficit in cell proliferation resulting from inactivation of the EDN3/EDNRB pathway leads to incomplete colonization of both skin and bowel; hence the white spots on the coat color and the aganglionic posterior gut of s^1 and ls mutants.

Other observations support an early role for EDNRB in the differentiation of the melanocytic lineage. By explantation, Mayer (48, 49), in experiments of embryonic skin and NC cells from s^1 and normal mice, showed that the expression of s^1 mutation is cell autonomous. Moreover, NC cells expressing tyrosinase related protein 2 (TRP2), a lineage-specific marker for melanoblasts, are lacking in s^1 while they are detectable as early as E10 in wild-type embryos (50). This shows that the EDNRB mutation affects melanocyte precursors at a very early stage of development. In Steel-dickie mutants affecting the Sl gene, TRP2^+ cells are present at E11 (51), showing that EDNRB acts earlier than the c-kit/Sl signaling pathway, a notion also supported by our observations in the avian embryo (ref. 44 and this work).

Previous studies on natural mutants exhibiting an aganglionic bowel indicated that the lethal spotted (ls) mutation is not cell autonomous to the ENS (52–54). As mentioned above, whether the s^1 mutation is cell autonomous for enteric NC-derived cells was less clear. In aggregation chimeras constructed between wild-type and s/s^1 embryos, Kapur et al. (26) showed that s^1 neuroblasts are in certain cases able to colonize the distal intestine. This, however, is not incompatible with our interpretation of the effect of the EDN3/EDNRB pathway on NC development. It is conceivable that in s/s^1 ↔ wild-type chimeras expansion of the wild-type enteric neuronal precursors might be large enough to produce a total population of cells sufficient to invade the whole bowel. This would mean (i) that EDNRB^-/- NC cells have a migratory behavior which is not significantly different from their normal counterpart and (ii) that cell proliferation plays an essential role in colonization of the gut (and skin) by NC cells.

An important result of this work is that EDNRB is expressed strongly in NC derivatives, such as peripheral nervous system ganglia, which are affected neither in s^1 and ls mutants nor in the EDN3 and EDNRB knock-out mice. This suggests that the role of the EDN3 pathway is not crucial for the development of these derivatives, which form in close proximity to their source; the neural primordium.

The next problem to explore concerns the spatiotemporal expression of the endothelins and particularly of EDN3 during the relevant stages of NC cell development.

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