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Bone morphogenetic proteins regulate enteric gliogenesis by modulating ErbB3 signaling

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ABSTRACT

The neural crest-derived cell population that colonizes the bowel (ENCDC) contains proliferating neural/glial progenitors. We tested the hypothesis that bone morphogenetic proteins (BMPs 2 and 4), which are known to promote enteric neuronal differentiation at the expense of proliferation, function similarly in gliogenesis. Enteric gliogenesis was analyzed in mice that overexpress the BMP antagonist, noggin, or BMP4 in the primordial ENS. Noggin-induced loss-of-function decreased, while BMP4-induced gain-of-function increased the glial density and glia/neuron ratio. When added to immunoisolated ENCDC, BMPs provoked nuclear translocation of phosphorylated SMAD proteins and enhanced both glial differentiation and expression of the neuregulin receptor ErbB3. ErbB3 transcripts were detected in E12 rat gut, before glial markers are expressed; moreover, expression of the ErbB3 ligand, glial growth factor 2 (GGF2) escalated rapidly after its first detection at E14. ErbB3-immunoreactive cells were located in the ENS of fetal and adult mice. GGF2 stimulated gliogenesis and proliferation and inhibited glial cell derived neurotrophic factor (GDNF)-promoted neurogenesis. Enhanced glial apoptosis occurred following GGF2 withdrawal; BMPs intensified this GGF2-dependence and reduced GGF2-stimulated proliferation. These observations support the hypotheses that BMPs are required for enteric gliogenesis and act by promoting responsiveness of ENCDC to ErbB3 ligands such as GGF2.

Introduction

The enteric nervous system (ENS) is the largest and most complex division of the peripheral nervous system (PNS) (Furness, 2006; Gershon and Ratcliffe, 2006). Its component neurons are phenotypically diverse and form microcircuits that allow the ENS to regulate the behavior of the bowel independently of input from the brain and/or spinal cord (Gershon and Tack, 2007). Like the brain, the ENS lacks internal collagen and derives support for its neuronal elements from glia that resemble their counterparts in the central nervous system (CNS)(Gershon and Rothman, 1991). The ENS develops from precursors that migrate to the bowel from vagal, rostral truncal, and sacral levels of the neural crest (Burns and Le Douarin, 1998; Durbec et al., 1996; Heanue and Pachnis, 2007; Kapur, 2000; Le Douarin and Teillet, 1973; Le Douarin and Teillet, 1974; Pomeranz and Gershon, 1990; Pomeranz et al., 1991). Although many individual crest-derived cells are already determined before they reach the bowel (Henion and Weston, 1997; Pham et al., 1991; Reedy et al., 1998a,b), the enteric population of crest-derived cells (ENCDC) is multipotent (Natarajan et al., 1999). This population responds to growth factors and other components of the enteric microenvironment, the actions of which are responsible for the development of the unique properties of the ENS (Gershon and Ratcliffe, 2006; Heanue and Pachnis, 2007).

It is likely that the early ENCDC population contains precursors that are common both to neurons and to glia (Natarajan et al., 1999; Young et al., 2003, 2005). Relatively little is known about signals that act on these uncommitted precursors to cause them to differentiate and give rise to divergent neuronal and glial cell lineages. Early ENCDC express both Phox2b and Sox10; however, ENCDC that commit to a neuronal lineage downregulate Sox10 expression while maintaining Phox2b (Pattyn et al., 1997; Young et al., 1999, 2003). Conversely ENCDC committed to a glial lineage downregulate Phox2b expression while maintaining Sox10 (Bondurand et al., 2006; D'Autreaux et al., 2007; Deal et al., 2006; Kim et al., 2003; Paratore et al., 2001; Stanchina et al., 2006). Sox10, moreover, controls expression of ErbB3 (Britsch et al., 2001) and enteric glia are absent in mice that lack ErbB3 (Riethmacher et al., 1997), suggesting that this receptor is important in enteric gliogenesis. The neuregulin (Nrg) family of ligands signals through ErbB receptors (Birchmeier, 2009; Brinkmann et al., 2008). Nrgs are produced by mesenchymal cells and promote glial lineage commitment by primary neural crest cells *in vitro* at the expense of a neuronal fate (Shah and Anderson, 1997; Shah et al., 1994). Only one

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of the 4 types of Nrg1 isoform, glial growth factor 2 (GGF2; a type II isoform) is a secreted molecule (Marchionni et al., 1993); it promotes development of satellite cells, which are in a glial lineage, in developing sensory ganglia (Leimeroth et al., 2002).

During early fetal life, ENCDCs respond to and require stimulation by glial cell derived neurotrophic factor (GDNF), which promotes proliferation, differentiation, survival, and migration of enteric precursors (Asai et al., 2006; Chalazonitis et al., 1998a; Hao and Young, 2009; Hearn et al., 1998; Laranjeira and Pachnis, 2009; Young et al., 2001). The ability of GDNF to stimulate proliferation declines as a function of fetal age (Chalazonitis et al., 1998a) and is limited by the actions of BMP-2 and -4 (Chalazonitis et al., 2004). These BMPs oppose GDNF by enhancing differentiation at the expense of the continued proliferation of precursors. BMPs also induce the expression of certain growth factor receptors, such as TrkC, thereby promoting responsiveness to and dependence on their corresponding ligands, which in the case of TrkC is NT-3. This action of BMP signaling favors the development of some phenotypes of enteric neuron, such as NT-3/TrkC-dependent dopaminergic neurons, at the expense of others (Chalazonitis et al., 2004, 2008).

Although BMP-2 and -4 increase enteric neuronal differentiation, it is not clear whether or not they also enhance development of enteric glia. We therefore tested the hypothesis that BMPs stimulate uncommitted ENCDC to differentiate but do not determine whether they develop as neurons or glia. We postulated that this lineage choice instead depends on other factors, such as GDNF and GGF2, which promote, respectively, neuronal and glial development. Specifically, we proposed that a balance exists between Ret activation by GDNF, which favors the expansion of cells in a neuronal lineage, and ErbB3 activation by a Nrg such as GGF2, which expands the population in a glial lineage. Our observations support these hypotheses and indicate that BMPs enhance the responsiveness of ENCDC both to GDNF-induced neurogenesis and GGF2-induced gliogenesis. Portions of this work have previously been published in abstract form (Chalazonitis et al., 2007, 2009).

Materials and methods

Animals

Pregnant female rats (Sprague–Dawley; Charles River laboratories, Waltham MA) were subjected to CO_2 narcosis and killed by thoracotomy. The Animal Care and Use Committee of Columbia University approved these procedures. Gestational age was counted from the occurrence of a vaginal plug, which was considered E0. The entire gut, from stomach to rectum, was dissected as a single specimen under aseptic conditions. To isolate crest-derived cells, these specimens were pooled from 30 to 32 fetuses at E12, 20–25 fetuses at E14, and 15–20 fetuses at E16. Pregnant female mice (CD-1; Charles River) were euthanized as described above.

Transgenic mice

The neuron specific enolase (NSE) promoter was employed to overexpress noggin or BMP4 in the gut of transgenic animals (Chalazonitis et al., 2004; Gomes et al., 2003; Guha et al., 2004). An IRES–GFP sequence allows expression of the noggin transgene to be monitored by demonstrating the native fluorescence or immunoreactivity of GFP. GFP expression was not detectable in the gut of transgenic mice at E13, although it was observed to be present in the primordia of prevertebral sympathetic ganglia and chromaffin bodies. GFP was abundantly expressed in the gut by E16 and was maintained through P11, not only in the ENS, but also in scattered epithelial cells. By P0, GFP fluorescence was also exhibited by many cells in the subepithelial mesenchyme (Chalazonitis et al., 2008). These observations suggest that the onset of transgenic overexpression of noggin in the gut begins between E13 and E16 and persists into postnatal life. Although the NSE–BMP4 construct did not have an IRES–GFP sequence and thus was not specifically labeled, immunoblots at E16 revealed that the level of BMP4 expression was higher than that of WT animals (Gomes et al., 2003).

RT-PCR

Pairs of oligonucleotide primers for amplification of cDNAs encoding GGF2, ErbB3, $p75^{NTR}$ and β-Actin1 were designed from published rat cDNA sequences. The Gene bank accession number, the sequences of the primers used for PCR, the conditions of amplification and the expected sizes of the products are shown in Table 1. The identities of all PCR products were confirmed by sequence analysis. For this purpose, PCR products were subcloned into pGEM-T Easy vectors (Promega, Madison, WI) using the TA-cloning kit (Invitrogen Corporation, Carlsbad, CA). Inserts in two individual clones were sequenced by the dideoxynucleotide-chain termination method in the DNA Facility of Columbia University. The sequences of the PCR products obtained from brain and gut with the indicated primers were found to be identical to those of the appropriate regions of the GenBank sequences of the amplified cDNAs (Table 1).

Real time PCR

Real time PCR was used to quantify transcripts encoding GGF2 in the fetal and adult rat gut. Primers for GGF2 were chosen to amplify a PCR product that includes the Kringle domain, which is unique to GGF2 (Buonanno and Fischbach, 2001). The expression of GGF2 was normalized to that of β-actin1. Transcripts encoding β-actin1 and GGF2 were quantified by real time PCR (RT PCR) with a SYBR Green I kit (Roche Diagnostics Corporation, Indianapolis, IN) and Jumpstart *Taq* ReadyMix (Sigma, St. Louis, MO) using a LightCycler™2.0 instrument as previously described (Chalazonitis et al., 2008; D'Autreaux et al., 2007). Amplifications were carried out in a final volume of 20 μl that contained Taq DNA polymerase, reaction buffer, dNTPs in which dTTP is replaced by dUTP, SYBR Green I dye, and $MgCl_2$. The final concentration of primers used for the amplification of cDNA encoding β-actin1 or GGF2 was 0.5 μM. The final concentration of $MgCl_2$ was 4.0 mM. To this mixture 1 μl of either the serially diluted plasmid PGEM-T with the inserted PCR product DNA (standards) or the cDNA prepared from tissue was added. Measurements were obtained by referring to standard curves that were prepared by serially diluting plasmid DNA containing an insert of a PCR product that includes a portion of the sequence of GGF2 or β-actin1. The dilutions of β-actin1 and GGF2 plasmid DNA ranged from 1 ng to 100 fg in 5 series, each of which covered a 10-fold range. The standards and the cDNA from tissues were simultaneously subjected to RT PCR analysis in parallel capillary tubes. A first denaturation step for each round of PCR was carried out at 94°C for 10 min to activate the polymerase. The PCR reactions were carried out according to the programs in Table 1. The appearance of double stranded DNA was quantified by measuring the fluorescence of SYBR Green after each step of elongation. The ramp rate was 20 °C/s during the amplification program. A melting curve analysis with a ramp rate of 0.1 °C/s was carried out to verify that a single moiety had been amplified. Data were analyzed with computer assistance employing the Light-Cycler™ software. Three independent experiments for GGF2 and 2 independent experiments for β-actin1 were carried out. The sequences of the products obtained from gut with the indicated primers (Table 1) were found to be identical to those of the appropriate regions of the GenBank sequence of Nrg1 type 2 amplified cDNAs.

Immunoselection

Crest-derived cells were immunoselected from the fetal rat gut with antibodies to the common neurotrophin receptor, $p75^{NTR}$, at E12, E14 and E16 as described previously (Chalazonitis et al., 1998a,b, 2001, 2004, 2008). Briefly, the bowel was dissociated with collagenase. The resulting