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Expression of Deleted in Colorectal Cancer in the Rat Trigeminal Ganglia

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The deleted in colorectal cancer (DCC) protein mediates attractant responses to netrin during axonogenesis. In the rat trigeminal ganglia (TG), axons must extend toward and grow into the trigeminal nerve to innervate target tissues such as dental pulp. Our present study aimed to investigate the expression of DCC in the TG. Four developmental timepoints were assessed in the experiments: postnatal days 0, 7 and 10 and adulthood. RT-PCR and western blotting revealed that the expression of DCC mRNA and protein does not significantly change throughout development. Immunohistochemistry demonstrated that DCC expression in the TG was detectable in the perikarya region of the ganglion cells during development. Nerve injury at 3 and 5 days after the mandibular nerve had been cut did not induce altered expression of DCC mRNA in the TG. Moreover, DCC-positive cell bodies also showed similar immunoreactive patterns after a nerve cut injury. The results of this study suggest that DCC constitutively participates in an axonogenesis attractant in ways other than expression regulation.

Key words: DCC, TG, Nerve

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Introduction

Normal development of the nervous system depends on appropriate cellular migrations and axonal pathfinding to establish connectivity. Observations of developing axonal projections reveal that axons extend to the vicinity of their corresponding target regions in a stereotyped and directed manner. One important question in axonal pathfinding in the developing nervous system is to understand the mechanism allowing growing axons to navigate through the environment to reach their target. A variety of guidance signals have been shown to direct axons along defined pathways to form a complex network of neuronal connections. Several gene families encoding cues for axonal growth cone and cell migrations have been identified. Guidance molecules provide attractive and repulsive signals to these specific receptors present on the growth cone. For example, bifunctional cue UNC-6/netrin in the *Caenorhabditis elegans* guides axon migrations by attractive or repulsive mechanisms depending on the response of its receptor. Cells and axons expressing UNC-5, the UNC-6 receptor are repelled by an UNC-6 gradient [1-3]. In contrast, UNC-6 is predominantly attractive for cells and axons expressing the UNC-40 receptor [4]. In vertebrates, attractive effects of netrin have been shown to require the transmembrane receptors of the vertebrate, unc-40 homolog DCC (deleted in colorectal cancer) [5-7], while another receptor molecule, Roundabout (Robo) is postulated to be required on the axons for repulsion [8]. DCC

encodes a cell surface receptor and was originally found to be altered in colorectal cancers, supporting the notion that DCC might be a tumor suppressor. However, since DCC is known to be involved in axon guidance, much attention has been focused to axonogenesis, rather than tumor suppression. The role of DCC as a guidance receptor responsible for directing axonal projections has been confirmed in mice lacking functional DCC [9]. Thus, the migration route of axons may follow sequential steps, each of which requires a particular set of molecules. The trigeminal nerve conveys information through three main divisions, the ophthalmic, the maxillary, and the mandibular. In general, the ophthalmic division, which is predominantly sensory, serves the skin of the upper parts of the face and parts of the nasal and paranasal mucosa. The maxillary division, also mainly sensory, innervates the teeth, mucosa of the maxilla, the upper lip, the lateral nose, the maxillary sinus and the nasopharynx. The mandibular division is mixed nerve and innervates the teeth and mucosa of the tongue, the mandible, the temporomandibular joint, skin covering the mandible, and the masticatory muscles. A majority of the trigeminal sensory neurons have their cell bodies clustered in the trigeminal ganglia (TG) [10,11]. In mammals, the proportion of unmyelinated fibers to myelinated is much lower in the trigeminal nerve branches than in the spinal nerves. This unique characteristic in the trigeminal nerve may influence its response to injury. Therefore, an in-depth elucidation of trigeminal molecular mechanisms may be required to understand the diverse and complex clinical problems.

The present study investigated the temporospatial presence and function of DCC in the TG using RT-PCR, Western blotting and immunohistochemical analyses. Furthermore, the expression of DCC was examined in the TG after surgical cut of the trigeminal nerve branches as a model of nerve injury.

Materials and Methods

Animals and tissue preparation

Sprague-Dawley rats were brought up in Association for Accreditation and Assessment of Laboratory Animal Care-Approved Facilities and provided with regular food and tap water. Pups at postnatal days 0, 7 and 10 were sacrificed and the TG were isolated immediately. Some of them were fixed in 4% paraformaldehyde for immunohistochemical analysis. The rest of the trigeminal ganglia were rapidly frozen in liquid nitrogen for reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting analysis. Adult rats weighing 180-220 g were anesthetized by intraperitoneal injection of ketamine (50 mg/ml). All of the left mandibular and maxillary teeth were extracted with a forcep and the lingual and mylohyoid nerves were cut under the mylohyoid muscle. Animals were sacrificed by perfusion with 4% paraformaldehyde at day 3 and 5 after the operation, and the TG were isolated. The right TG were used as a 'control'. The isolated tissues were postfixed, embedded in paraffin, and cut into 5 μm thick sections for immunohistochemical analysis. The rest of the TG were rapidly frozen in liquid nitrogen for RT-PCR.

RT-PCR

For preparation of RNA, total RNA was extracted from the TG using Trizol® reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. Reverse transcription reactions were performed as follows. After the RNAs were treated in DNase I, they were mixed with 25 μg/ml oligo$(dT)_{12-18}$ and heated at 70℃ for 10 min. For the generation of 1st strand cDNA, the RNAs mix was then added to solution composed of 0.5 mM dNTP each, 10 mM dithiothreitol and 1st strand buffer, followed by incubation at 42℃ for 60 min and subsequent incubation for 15 min at 70℃. RT controls were carried out using the same RT reaction mixures except substituting cDNA for DEPC-treated H_2O. PCR cycles were performed in a Perkin-Elmer Gene Amp PCR system 2400. Preliminary studies were performed to determine the optimum number of cycles for quantitation. PCR products were resolved on a 1.2% agarose gel and visualized using ethidium bromide. The size was confirmed using 1 kb DNA ladder (Gibco BRL, MD, USA).

The PCR primers used in the present study and corresponding GenBank accession numbers were listed in Table 1.

Western blot analysis

The TG were resuspended in a lysis buffer [50 mm Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM thenylmethylsulfonyfluroide, 1% aprotinin, 1% leupeptin, 1 mM NaF], incubated on ice for 10 min, and then lysed using a homogenizer. The lysates were centrifuged to remove cell debris, and the supernatant was collected and frozen at -70℃ until furt

Table 1. The sequence of PCR primers

	Forward (5'→ 3') Reverse (5'→ 3')	Annealing Temp (℃)	Product Size (bp)	Genbank accession number
GAPDH	CCA TGG AGA AGG CTG GGG CAA AGT TGT CAT GGA TGA CC	65	195	AF106860.2
DCC	CGG AAT TCC GGA ACA CGC CAG TGA GG GCG GAT CCA CAG GAG TGG AGG CAG ACG	57	153	NM_012783.1

her use. Protein lysates (50 μg) were boiled for 5 min in a denaturing sample buffer and loaded onto 10% continuous gradient SDS-polyacrylamide gel for the transfer to nitrocellulose membrane (Amersham Pharmacia Biotech, IL, USA). The membrane was blocked with TBST buffer [10 mM Tris-buffered isotonic saline (pH 7.0), 0.1% merthiolate, 0.1% Tween-20] containing 5% nonfat dry milk for 30 min at room temperature with shaking and incubated with goat anti-DCC primary antibody at a dilution of 1:200 (Santa Cruz, CA, USA) in TBST buffer containing 5% nonfat dry milk for 24 h at 4℃ with gentle shaking. The membrane was washed twice with TBST for 10 min and reacted with horseradish peroxidase-conjugated secondary antibody at a dilution of 1:3000 (Cell Signaling Technolog, MA, USA). Bound antibodies were visualized using ECL (Amersham Pharmacia Biotech, IL, USA) according to the manufacturer's protocol.

Immunohistochemistry

For the immunohistochemical staining, the streptavidin-biotin-peroxidase method was used. Endogenous peroxidase in tissue sections was inactivated using 0.3% hydrogen peroxide. Sections were then reacted with goat anti-DCC primary antibodies at a dilution of 1:200 (Santa Cruze, CA, USA) diluted in primary antibody diluent (Invitrogen, CA, USA), and incubated overnight at 4℃. They were washed then in phosphate-buffered saline (pH 7.4) for 10 min and incubated with biotinylated anti-goat immunoglobulin for 1 h, followed by incubation with streptavidin-labelled peroxidase solution for 1 h. AEC was used for visualization. Normal goat serum instead of the primary antibody was used for the negative control. All controls yielded negative results.

Results

DCC expression in the TG in development

RT-PCR was performed to examine DCC expression level in TG at postnatal days 0, 7 and 10 (P0, P7, P10) and adult. DCC mRNA were not changed during developmental time points (Fig. 1).

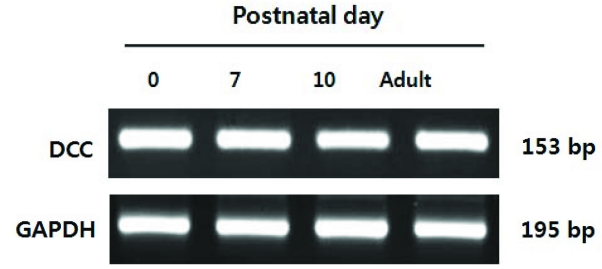

Fig. 1. Ethidium bromide-stained 1% agarose gel image of RT-PCR products for DCC in the trigeminal ganglion at four time points: postnatal days 0, 7 and 10 and adult. DCC expression was unaltered during these developmental periods.

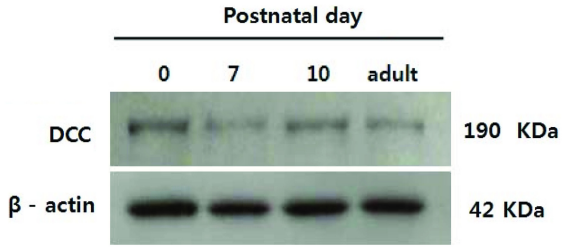

Fig. 2. Total protein from trigeminal ganglia was analyzed by 10% SDS-PAGE and immunoblotted with goat anti-DCC primary antibody. DCC was constantly expressed during the TG development up to postnatal day 10.

Western blot analyses were undertaken with an antibody against DCC in TG at four postnatal developmental time points (P0, P7, P10 and adult). The level of DCC expression during the developmental process was somewhat constant (Fig. 2). These may imply that DCC in developing TG may work as a constant guidance receptor responsible for pathfinding axonal projections.

To localize DCC in nerve cells of the TG, sections of the TG were immunohistochemically stained using an antibody against DCC. Its distribution was determined at four postnatal time points; postnatal day 0, 7, 10 and adult. The exp

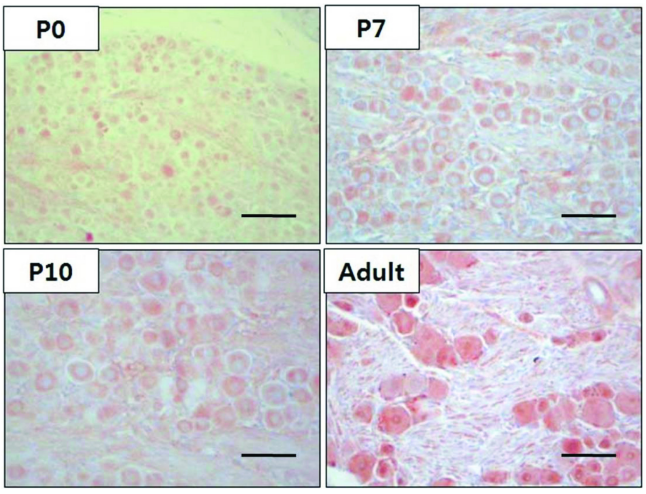

Fig. 3. Immunohistochemical localization of DCC in the TG during the developmental time points (P0, P7, P10 and adult). The immunoreactivities were demonstrated in axons as well as ganglion cell bodies. Each scale bar denotes 50 μm in length.

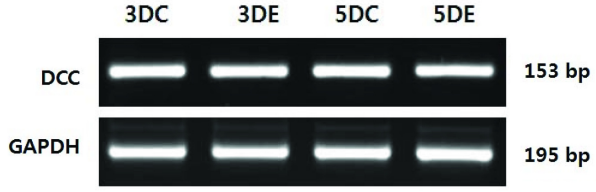

Fig. 4. Ethidium bromide-stained 1% agarose gel image of RT-PCR products for DCC at 3 and 5 days after the trigeminal nerve injury. Unaltered level of the mRNAs was seen after the nerve cut. DC : control, DE : experiment.

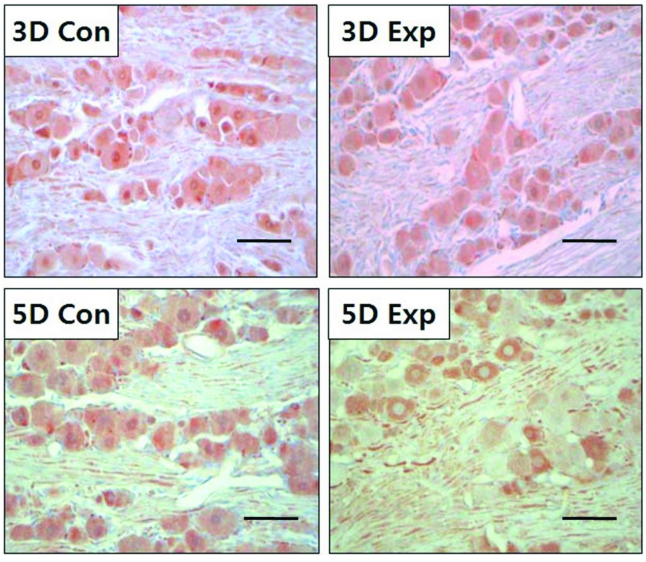

Fig. 5. Immunohistochemical localization of DCC in the control and experimental parts of the TG at 3 and 5 days after the nerve injuries. Compared with the control, the cut side displayed the unaltered immunoreactivities at both 3 and 5 days. Each scale bar denotes 50 μm in length. Con: Control, Exp: Experiment (denervation of the trigeminal nerve)

ression patterns were constantly detected in all the development time points. The immunoreactivities were mainly seen at axons as well as cell bodies of both small and large nerve cells (Fig. 3).

DCC expression in axotomy

To assess whether expression of DCC was altered as a result of axotomy, tooth extraction and lingual and mylohyoid nerve cut were performed at 3 and 5 days prior to the isolation of the ganglia. The time points were determined to detect the modulation of mRNA levels during the initiation of transcriptional changes associated with the regenerative response. A detailed analysis of the mRNA levels for DCC whose expression was unchanged compared with the control side during the nerve growth in the TG was shown in Fig. 4.

To determine whether DCC protein level was changed in the TG neurons after the nerve injury, the TG neurons isolated at postoperation days 3 and 5 were processed for immunohistochemical staining of DCC (Fig. 5). As shown in Fig. 5, the expression of DCC in the ganglia from the experimental side of the same rat seemed unchanged compared to the control side both at 3 and 5 days .

Discussion

DCC, one of the netrin receptors, is known as an attractant which is expressed on various projecting populations of axons during neural development [5,6,12,13]. Based on these studies, the present study firstly investigated the presence of DCC in the TG using RT-PCR, Western blotting and immunohistochemical analyses. In these ganglia, DCC was detected as signals in cytoplasmic regions at gangion cell bodies and in axon fibers. These findings suggested that DCC might play a role in axonogenesis in TG as in other neural tissues [14]. However, this result was different from the report that DCC is expressed in Schwann cells which are important in nerve regeneration by secreting neurotrophic molecules [15].

The designated postnatal day 10 in the present study for elucidating axonogenesis of the trigeminal nerve was based on the fact that this axonogenesis is active at least until this day. The very first nerve fibers which approach to developing tooth sacs, which further develop into periodontal tissues, can be seen from the bud to cap stage, ramifying and forming plexuses around the sacs. However, the penetration of the nerve fibers into the dental papilla which later constitu-

tes the dental pulp, takes place after the dentinogenesis, which can be firstly seen at the late bell stage, begins. In rats at postnatal day 10, the 3rd molars in rats are in cap or early bell stage. Thus, axonogenesis of the trigeminal nerve may be active before at least postnatal day 10 in rats.

In relations to axonogenesis, several molecules in the rat retinal ganglion which are important for neuronal development such as neurolin [16], extracellular matrix proteins fibronectin [17] and transcription factors such as c- JUN [18] are down-regulated after axons have developed, but reexpressed during regeneration. In a search for a possible role of DCC in controlling adult TG regeneration, this study had examined changes in expression of this molecule during regeneration followed by the trigeminal nerve injury in adult rats. At day 3 and 5 after the axotomy of the trigeminal nerve, TG exhibited unchanged levels of DCC transcripts compared to contralateral uninjured controls. This result was also confirmed by immunohistochemical analysis. For another approach to elucidate a possible role of DCC, the present study investigated DCC changes during axon development. As a result, DCC mRNA was constitutively expressed during developmental stages and its protein level was also unchanged as assessed by both Western blotting and immunohistochemical analysis.

In fact, DCC needs netrin-mediated signaling pathway on the reformed growth cones of injured axons. This mediated attraction was positively regulated by a protein tyrosine kinase, whereas negatively regulated by CLR-1. Such antagonistic effects might reflect opposite effects on the tyrosine phosphorylation state of DCC or a DCC effector, UNC-34/Ena [19]. Another way of enhancement of DCC−mediated guidance is to downregulate RPTP-LAR (Receptor protein tyrosine phosphatases - Leukocyte common-Antigen Related), suggesting that effects of RPTP-LAR in axon guidance might result from regulation of key axon guidance receptors [20]. Expression of RPTP-LAR decreased as axonogenesis in the trigeminal nerve decreased during development in rats. Also, this increased after axotomy, followed by nerve regeneration [21,22]. In the present study, no altered expression of DCC by either the induced nerve regeneration followed by the surgical denervation or sprouting of axon during development was detected. This no alteration reflected that DCC might need phosphorylation for attraction of axonogenesis and dephosphorylation for stop of axonogenesis rather than the regulation of DCC expression.

All together, the present finding that DCC was constantly expressed during axon development and regeneration in the rat TG suggests that DCC constitutively participate in axonogenesis as an attractant in other ways than regulating its expression. Also, tyrosine phosphorylation and the involvement of RPTP-LAR might be the key factors to regulate DCC function for the trigeminal nerve axonogenesis. DCC regulates polarized axon initiation and asymmetric outgrowth of neurons in vertebrates [23]. Trigeminal nerve cannot be the exception. Further studies are needed for roles of DCC in axonogenes as well as its pathways in action [24,25].

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