Involvement of gicerin, a cell adhesion molecule, in development and regeneration of chick sciatic nerve

Satoshi Hiroi[a], Yasuhiro Tsukamoto[a,*], Fumihiko Sasaki[a], Naomasa Miki[b], Eiichi Taira[b]

[a] *Department of Veterinary Anatomy, Osaka Prefecture University, Graduate School of Agriculture and Biological Sciences, 1-1 Gakuencho, Sakai, Osaka 599-8531, Japan*

[b] *Department of Pharmacology 1, Osaka University Medical School, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan*

Received 12 August 2003; revised 20 September 2003; accepted 6 October 2003

First published online 20 October 2003

Edited by Michael R. Bubb

Abstract We have examined the role of gicerin, an immunoglobulin superfamily cell adhesion molecule, in chick sciatic nerves during development and regeneration. Gicerin was expressed in the spinal cord, dorsal root ganglion (DRG) and sciatic nerves in embryos, but declined after hatching. Neurite extensions from explant cultures of the DRG were promoted on gicerin's ligands, which were inhibited by an anti-gicerin antibody. Furthermore, gicerin expression was upregulated in the regenerating sciatic nerves, DRG and dorsal horn of the spinal cord after injury to the sciatic nerve. These results indicate that gicerin might participate in the development and regeneration of sciatic nerves.

Key words: Gicerin; Cell adhesion molecule; Sciatic nerve; Development; Regeneration; Neurite outgrowth factor

1. Introduction

Adhesion among cells is an essential property for multicellular organisms in order to organize tissues and organs as well as to establish and maintain cell–cell interactions [1,2]. Cell adhesion molecules (CAMs) are recognized as playing a major role in a variety of physiological and pathological phenomena such as tissue development, regeneration and tumor progression [3]. In the nervous system, several CAMs are necessary for the interaction among neurons, glial cells and target organs, and play important roles such as in neuronal migration and neurite outgrowth, fasciculation and pathfinding. We have identified an integral membrane glycoprotein, *gicerin*, from chicken gizzard smooth muscle as a ligand for the neurite outgrowth factor (NOF), which is a laminin-like embryonic chick muscle protein [4–6]. Gicerin, the same molecule as HEMCAM is a homologous molecule to human MUC18/CD146/Mel-CAM [7–9]. Gicerin exhibits homophilic cell adhesion activity in addition to heterotypic adhesion with respect to NOF [4,5,10]. In the central nervous system of the chicken, gicerin is expressed during the developmental stage when neurons migrate or extend neurites to form a neural network [4,11–13]. Also, gicerin appears transiently in non-neural tissues of the chick embryo and plays an important role in development by its cell adhesion activities [11,14,15]. Furthermore, gicerin re-appears on regenerating epithelial cells of the trachea, oviduct and kidney after injury as well as tumors [14–20]. Although the expression pattern of gicerin in the inner ear development and hair cell regeneration has been reported, the potential role of gicerin in peripheral nervous systems remains unclear [21]. It has been reported that the developmental expression pattern of certain CAMs is recapitulated after peripheral nerve injury, and that this promotes the interaction between axon and axon or axon and Schwann cells in the regenerating nerves [22,23]. Characterization of the development and regeneration of sciatic nerve provides a good model for understanding the function of molecular factors in the formation of peripheral nervous systems [22]. In the present study, we have investigated the expression and the potential function of gicerin in the development and regeneration of the sciatic nerve and dorsal root ganglion (DRG) of the chicken.

2. Materials and methods

2.1. Tissue samples

Embryonic day-8 (E8), newly-hatched and 3-week-old White Leghorn chicks were used in the study. Whole embryos and the sacral spinal cord with DRG and sciatic nerve of the chicks were fixed in Zamboni's solution for immunohistochemistry. They were also frozen for Western blot analysis.

2.2. Sciatic nerve injury model

One-week-old chicks were anesthetized by pentobarbital and their left sciatic nerves were injured (crushed) with a clamp to the femoral region [24]. Their right-side nerves were not injured and used as a non-injured control. On the 14 days post injury, the chicks were killed under deep anesthesia with pentobarbital and their sciatic nerves, DRGs and sacral spinal cords were removed for immunohistochemistry and Western blot analysis.

2.3. Immunohistochemistry

Tissue samples fixed in Zamboni's solution were frozen and cut into 10-μm sections. DRG explants cultured on a dish (see below) were also fixed in Zamboni's solution. They were incubated with polyclonal antibodies for gicerin or for NOF for 1 h at 37°C [17]. The sections of the injured and non-injured sciatic nerves were double stained with a polyclonal antibody for gicerin and a monoclonal antibody for S-100 protein (Sigma). They were visualized with an fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG and a rhodamine-conjugated anti-mouse IgG.

2.4. Western blot analysis

Tissue samples were homogenized and solubilized by 10 mM Tris/

*Corresponding author. Fax: (81)-722-54 9918.
E-mail address: ytsuka@jyui.vet.osakafu-u.ac.jp (Y. Tsukamoto).

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)01176-1

acetate (pH 8.0), 1 mM ethylenediamine tetraacetic acid (EDTA), 0.5% NP-40 and then centrifuged at 40 000×*g* for 30 min. The resultant supernatants were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to a polyvinylidene difluoride (PVDF) membrane and visualized by an enhanced chemiluminescence (ECL) system using a polyclonal anti-gicerin antibody and a secondary anti-rabbit IgG-horseradish peroxidase (HRP) conjugate [17].

2.5. Assay for neurite extension from DRG

Dishes (3 cm in diameter) were coated with a chimeric protein of gicerin extracellular domain with human IgG Fc fraction (gicerin-Fc) (50 μg/ml) or NOF protein (20 μg/ml) and incubated for 2 h at 37°C [13,15]. DRGs were picked up from E8 chicks and dissociated with 0.05% trypsin and 0.53 mM EDTA for 20 min at room temperature. After centrifugation at 800×*g* for 10 min and gentle suspension in Dulbecco's modified Eagle's medium (DMEM), they were incubated on a dish for 40 min at 37°C and floating cells were re-plated on non-coated dishes, gicerin-Fc- or NOF-coated dishes and incubated for another 12 h at 37°C in DMEM containing 10% fetal calf serum (FCS) with a control IgG or an anti-gicerin antibody (4 mg/ml). Also, an anti-NOF antibody (4 mg/ml) was added in the explants on the gicerin-Fc-coated dishes and incubated for 12–24 h at 37°C.

3. Results

3.1. Expression of gicerin in the spinal cord, DRG and sciatic nerve of chick embryo

In the embryos, gicerin was abundantly observed in the spinal cord, DRG and other tissues, such as myotomes, chondroblasts, blood vessels and notochords. The gray matter of the spinal cord exhibited significantly greater staining for gicerin than the white matter (Fig. 1A). The ventral and dorsal horns and their roots as well as most neurons in the DRG were gicerin-positive (Fig. 1A–C). The dorsal horn and funiculus, especially the region adjacent to the dorsal root were more strongly stained for gicerin than the ventral side (Fig. 1C). Also, gicerin was observed in the sciatic nerve fibers of the embryo (Fig. 1D). Expression of gicerin was dramatically decreased after hatching and only the dorsal horn, dorsal root (Fig. 1E), DRG (data not shown) and sciatic nerve (Fig. 1F) were weakly positive for gicerin. On the other hand, NOF was expressed in the pia mater, neurium, myotome, blood vessels

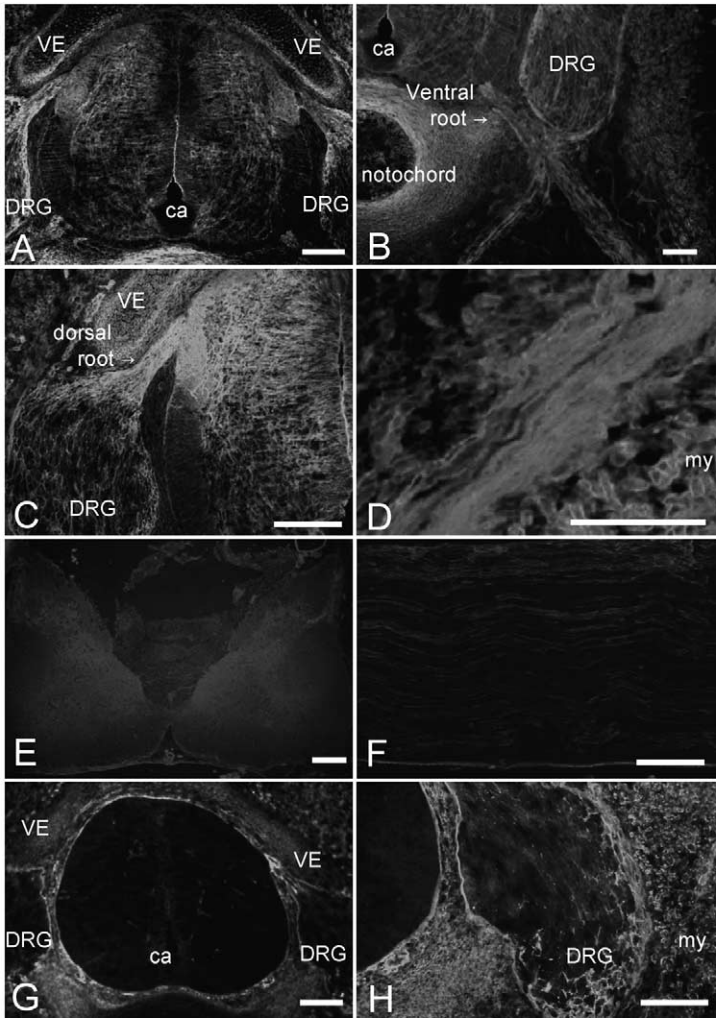

Fig. 1. Expression of gicerin in spinal cord, DRG and sciatic nerve from E8 and newly-hatched chick. Immunofluorescent staining for gicerin (A–F) and NOF (G, H). A: Whole view of spinal cord of E8 (transverse section: dorsal is up and lateral is both sides). B: DRG and ventral root of E8 (transverse section). C: Dorsal horn and dorsal root of E8 (transverse section). D: Sciatic nerve of E8 (longitudinal section). E: Spinal cord of newly-hatched chick (transverse section). F: Sciatic nerve of newly-hatched chick (longitudinal section). G: Whole view of spinal cord of E8 (transverse section: dorsal is up and lateral is both sides). H: DRG of E8 (transverse section). VE, vertebra; ca, central canal; my, myotomes. Bar, 200 μm.

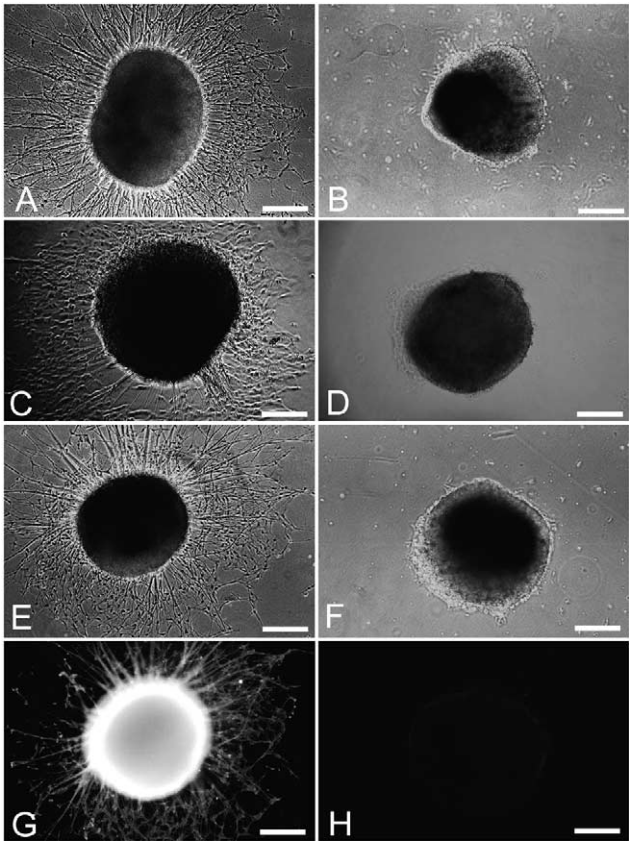

Fig. 2. Neurite extension from explants of DRG. DRGs from E8 were cultured on gicerin-Fc protein (A), on gicerin-Fc protein with anti-gicerin antibody (B), on gicerin-Fc protein with anti-NOF antibody (C), on non-coated dishes (D), on NOF protein (E) and on NOF protein with anti-gicerin antibody (F). Neurite extension is observed on both gicerin- and NOF-coated dishes (A, E), but disturbed with anti-gicerin antibody (B, F). Anti-NOF antibody shows no effect on neurites on gicerin-Fc protein (C). On non-coated dish, a few neurites emerge from DRG (D). Immunocytochemically, gicerin is expressed on DRG and neurites on NOF protein (G), while NOF is not found on the neurites on gicerin-Fc protein (H). Bar, 300 μm.

and notochords in the embryo (Fig. 1G,H), and the expression level decreased after hatching.

3.2. Neurite extension from DRG explants on gicerin and NOF proteins

On gicerin-Fc chimeric protein, many neurites were extended from DRG explants, which were blocked by the anti-gicerin antibody but not by the anti-NOF antibody (Fig. 2A–C). On the other hand, only a few neurites were extended from the explants when they were cultured on the non-coated dish (Fig. 2D). Immunocytochemically, NOF was not found on the neurites on gicerin-Fc protein (Fig. 2H). These results suggested that NOF was not involved in the neurite extension on gicerin-Fc chimeric protein. Neurite extension was also prominent on the NOF-coated dish, which was blocked by the anti-gicerin antibody (Fig. 2E,F). Both the DRG and extending neurites grown on NOF-coated dish were positive for gicerin (Fig. 2G). This suggested the involvement of gicerin in the extension of neuritis on NOF.

3.3. Expression of gicerin in the spinal cord and sciatic nerve after injury

After 14 days of injury, the injured site of the sciatic nerve was swollen and whitish, as described by Galloway et al. [24]. Gicerin was strongly re-expressed in the sciatic nerve fibers of the injured side and the distribution of gicerin did not co-localize with S-100 proteins (Fig. 3B–D), indicating that gicerin appeared in axons, not in Schwann cells. And most of the ganglion cells in the DRG of the injured side strongly re-expressed gicerin on their surface (Fig. 3F). On the other hand, gicerin was slightly observed in the sciatic nerve and in the ganglion cells of the DRG of the uninjured side (Fig. 3A,E). The S-100 proteins were expressed in the uninjured sciatic nerves (data not shown). In the spinal cord, expression of gicerin apparently increased in the dorsal horn of the injured side (Fig. 3G). NOF expression increased slightly in the neurium of the injured sciatic nerve (data not shown).

The increase of gicerin expression was also confirmed by Western blot analysis. Gicerin was recognized as a doublet band around 90 kDa and its expression increased only in the sciatic nerve and DRG of the injured side (Fig. 4).

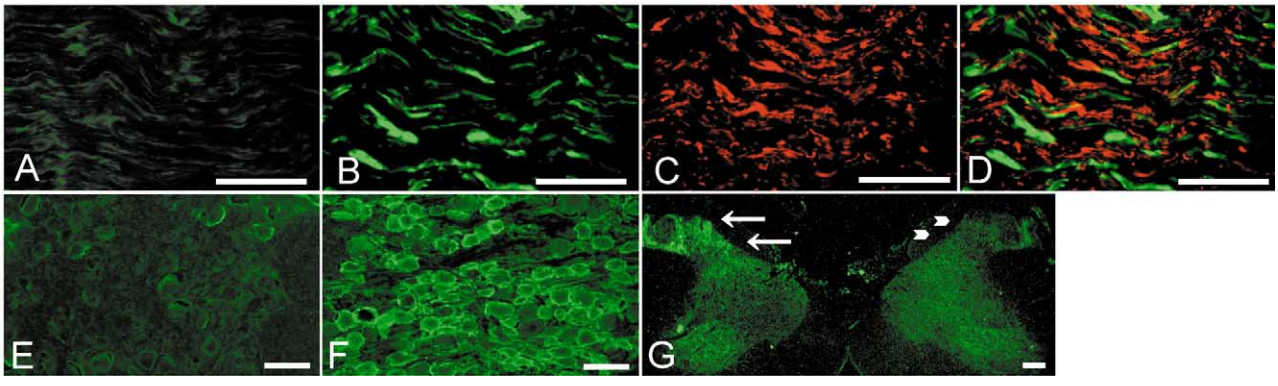

Fig. 3. Expression of gicerin in sciatic nerve and spinal cord of 3-week-old chicks at 14 days post sciatic nerve injury. Gicerin is not expressed on nerve fibers in normal sciatic nerve of leg which was not operated on (A, gicerin only). In contrast, gicerin was visible in sciatic nerve fibers as bundle-like appearances in injured leg, but did not overlap with distribution of S-100 protein in double staining (B: gicerin; C: S-100 protein; D: merge). In DRG, gicerin is slightly found on the cell surface of ganglion cells in the side which was not operated on (E), whereas it is strongly expressed in most ganglion cells in the injury side (F). In spinal cord, the expression level of gicerin is increased in dorsal horn of the injured side (arrows) compared with the side which was not operated on (arrowheads) (G: dorsal is top and left is injured side). A–D: Longitudinal sections. E–G: Transverse sections. Bar, 100 μm.

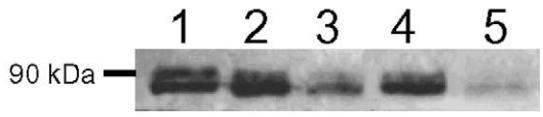

Fig. 4. Western blot analysis for gicerin protein in DRG and sciatic nerves from 3-week-old chicks at 14 days post sciatic nerve injury. Each lane is loaded with 20 μg of membrane fractions. Lane 1, DRG from E8. Lane 2, DRG of the injured side from 3-week-old chick. Lane 3, DRG of the side which was not operated on from 3-week-old chick. Lane 4, sciatic nerve of the injured side from 3-week-old chick. Lane 5, sciatic nerve of the leg which was not operated on from 3-week-old chick.

4. Discussion

In the present study, we first characterized the expression pattern of gicerin and its heterophilic ligand, NOF in the spinal cord, DRG and sciatic nerve during the development. The sciatic nerve consists of fibers from sensory neurons in the DRG and fibers from the motor neurons in the ventral horn, and both of them were gicerin-positive in the embryonic stage. On the other hand, NOF was strongly positive in the neurium. We speculated that gicerin might participate in the formation of the sciatic nerve by its adhesive activities. Homophilic adhesion of gicerin may promote axonal extension, while its heterophilic adhesion with NOF may involve in the axonal guidance on the neurium. To support this paradigm, an in vitro embryonic DRG culture was performed. We found that the neurite extension from the DRG explants was strongly promoted on both gicerin-Fc and NOF proteins, and these extensions were obviously inhibited by the anti-gicerin antibody. These suggested that gicerin participated in the neurite extension system. In case of homophilic interaction, we denied the possibility that NOF was secreted from extending neurons and involved in the neurite extension process. Neurons were negative for NOF and anti-NOF antibody did not inhibit neurite extension on gicerin-Fc. But we cannot exclude the possibility that there is an unknown heterophilic ligand on the surface of DRG neurons and it works on the neurite extension on gicerin-Fc chimeric protein since human homolog MCAM/CD146/MUC18 is supposed to have a heterophilic ligand [25–27]. As for the heterophilic binding activity to NOF, DRG neurons extended neurites in respond to NOF and the activity was also blocked by anti-gicerin antibody, suggesting the involvement of gicerin–NOF interaction in the neurite extension.

One particularly interesting and potentially important finding in the present study was the observation that gicerin reappeared on the regenerating axons in the sciatic nerve. This finding suggests a potential function of gicerin in peripheral nerve regeneration. Gicerin might promote regrowth and fasciculation of regenerating axons by its adhesive activity. Sensory neurons have a potential for central growth when the peripheral axons regenerate and reorganization occurs both in the DRG and the spinal cord during the regeneration of sciatic nerves [22,28]. In the present study, we demonstrated that gicerin increased in the dorsal horn and DRG after sciatic nerve injury. Accordingly, we speculate that gicerin may be involved in the reorganization of sensory neurons in the spinal cord during the regeneration of sciatic nerves. The functional role of gicerin on motor neurons was not elucidated in this study, for which further investigation is needed. Also, it is strongly expected that elucidating the mechanism how gicerin participates in the nerve repair might help treatment such as nerve regeneration.

Acknowledgements: This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan (No. 12680753), a Grant from the Senri Life Science Foundation (No. S-1208) and a Grant-in-Aid for Advanced Scientific Research from Osaka Prefecture University.

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