Sprouting of sensory neurons in dorsal root ganglia after transection of peripheral nerves*

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Summary. Morphological reaction of sensory neurons of dorsal root ganglia after peripheral nerve transection was investigated by a nerve tracing method using E. coli lacZ (β-galactosidase) gene recombinant adenovirus. The sciatic nerve of the rat was transected and inoculated with the gene recombinant adenovirus from the cutting end of nerve fibers. The fixation was accomplished from one to six weeks after inoculation. A whole mount specimen was observed after the reaction in a X-galactocidase substrate. Newly formed sprouting processes of dorsal root ganglion (DRG) cells appeared, all of them sprouting from the primary segment of DRG cells. Developed branches were morphologically categorized in to two types: one was the "linear type" which showed diverged branches running straightly along the major axis of the DRG; the other was the "winding type" which exhibited a random running pattern to the original axons and wound and extended in all directions in dorsal root ganglia with many branches. Many of this type encircled other cell bodies and formed a ring-like structure. There was no difference in the size of cell bodies in either type or between the ring-like structure forming the cells and those cells encircled by them.

Introduction

For the development of surgical technology for peripheral nerve reconstruction — which aims to accelerate nerve regeneration and functional recovery — it is very important to reveal the various reactions after transection of the peripheral nerve. For such studies, peptides available for the growth of axons sprouting from nerve stumps and the function of glial cells in the elongation of sprouted axons have been determined (Torogoe and Lundborg, 1998; Grados-Munro and Fournier, 2003). Also investigated are the appearances of various molecules and their functions in the process of regeneration after nerve injury (Ma and Bisby, 1999; Tomas Gonzalez-Hernandez, 1999; Kage et al., 2002; Wotherspoon et al., 2005). These studies have gradually revealed the progress of nerve regeneration. Some authors showed that the sprouting of nerve fibers occurred in dorsal root ganglia and formed basket-like structures around other neurons; they suggested that neuropathic pain after peripheral nerve injury would be caused by this phenomenon. It was reported that the origin of sprouting fibers of the basket-like structure was sympathetic fibers (McLachlan et al., 1993; Ramer and Bisby, 1997; Ma and Bisby, 1999) and sensory neurons (McLachlan and

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Hu, 1998; Gonzalez-Hernandez and Rustioni, 1999; Liu et al., 2005). McLachlan and Hu (1998) did a careful identification of fibers based on the peptides in them using an immunohistochemical double labeling technique. They indicated that sensory neurons sprouted and formed the same basket-like structures observed on sympathetic fibers. Liu et al. (2005) also showed that the regeneration bud formed the basket-like structure after the peripheral nerve cutting by NOS immunostaining. However, no original pseudounipolar fiber was shown and the budding position was uncertain in either report. Tandrup (1995) suggested by morphometry that the neurons in the dorsal root ganglia had pseudounipolar axons and no multiple branches in normal conditions. Furthermore, even in the embryonic dorsal root ganglia, no extra-branches were observed except for the central and peripheral process ramified from stem process (Matsuda and Uehara, 1984). These reports indicated that the basket-like structure did not exist in normal ganglia. The reports of McLachlan and Hu (1998) and Liu et al. (2005) did not provide morphological proof; however, it was considered that the basket-like structure observed by them was braided by sprouted fibers of DRG cells. It was noteworthy that, despite the origin of the sympathetic and sensory neurons being the neural crest, their functions and shapes were dissimilar but their morphological reaction was nearly identical. Furthermore, especially in the case of sensory neurons, the sprouting region from pseudounipolar fibers was also of interest.

We investigated morphological reactions of neurons after the transection of peripheral nerves in whole mount specimens by a nerve tracing method using E. coli lacZ (β-galactosidase) gene recombinant adenovirus (Miwa et al., 2001; Zenzai et al., 2004). Here we report on the extra-branches sprouting from pseudounipolar neurons in dorsal root ganglia with obvious bifurcated region.

Materials and Methods

Male Wistar rats (200–300g of body weight) were anesthetized by an intraperitoneal injection of ketamine (Sanka Yell Yakuhin, Tokyo)/xylazine (Bayer HealthCare, Leverkusen, Germany). The right sciatic nerve and its branches, that is, the tibial and peroneal nerves, were exposed at the posterior aspect of the thigh. The tau-LacZ gene recombinant adenoviral suspension (5 × 10^3–1 × 10^10 pfu/ml) was dropped on the tip of a scalpel and the exposed sciatic nerve was transected with that scalpel for inoculation of the virus from the cut end of the nerves (details of this method noted by Zenzai et al., 2004). The wound was closed, and the operated rat was returned to a cage for feeding until fixation.

After one to six weeks, each rat was anesthetized in the same way mentioned above. They were perfused transcardially with heparinized 0.01M phosphate buffered saline (PBS, pH 7.4) followed by a mixture of 1% paraformaldehyde and 1.25% glutaraldehyde in the same buffer. After fixation, the sciatic nerve with its related lumbar roots and spinal cord was resected from the body and was taken out as a whole mount material. The specimen was kept immersed in the same fixative for two hours or more. Then it was washed two hours in PBS in 1% NP-40. Redundant soft tissues around the nerve were removed as much as possible during the treatment of NP-40. The specimen was incubated two or more hours in X-gal substrate [5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 2 mM MgCl2, 0.1% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)] at room temperature or overnight in the refrigerator at 4°C. The specimen was observed repeatedly and removed from incubation when sufficient staining was obtained. Following this, the material was immersed in 50% glycerol and sequentially concentrated to 100% glycerol to be transparentized. The blue stained cell body, axons, and extension form of branches were observed using a stereoscopic microscope.

Results

Observations in eighteen dorsal root ganglia taken out from approximately fifty rats showed abnormal branches that appeared to be sprouting. In other dorsal root ganglia, the crowded stained cell bodies and their fibers made it difficult to achieve a fine observation of branches in dorsal root ganglia, or only normal pseudounipolar axons were observed without extra-branches, or no labeled soma nor axon was observed at all. In the same ganglion many labeled neurons showed a typical pseudounipolar shape, that is, round-shaped soma and one stem process which bifurcated and extended as the central and peripheral process, though some other labeled cells developed ectopic processes. As the blue stained normal pseudounipolar neurons were observed with neurons extending ectopic branches in the same ganglion, obviously not all the cells sprouted.

Only in the material of the short period from labeling to fixation were sprouted short processes observed (Fig. 1), although, extended developing ectopic processes were also seen already in a week (Fig. 2c, d). In the present method, the staining reaction gradually declined after four weeks of labeling; therefore, it was very hard to obtain materials after a long time. Consequently, the fate of sprouted branches was not investigated. All the sprouted
Fig. 1. Short sprouted branches observed in DRG after two weeks of operation. a: A simple short budding (arrowhead) is observed near the cell body (arrow) which has typical pseudounipolar processes (white arrowheads). b: A short sprout having three branches (arrow).

Fig. 2. Linear type of sprouted branches. a and b: Sprouted branches show a simple figure. c and d-1: Branches appear relatively complicated. a: A neuron indicated by an arrow projects a single branch proximal of the DRG (black arrowheads). b: A DRG neuron having an ectopic fiber (black arrowheads) similar to the original pseudounipolar fiber (white arrowheads) is seen. The tip of the proximal and distal processes is in the DRG (data not shown). c: Developed sprouted branches that have grown from near the cell body elongate in the distal or proximal direction of the DRG. d-1: Ectopic branches show a complex distribution; however, all branches eventually lead toward the proximal or distal direction of the DRG. Figure d-2 is traced d-1 to show the details of branching. It is obvious that the sprout occurs near the root of stem process. A large black arrowhead in d-1 and d-2 indicates the sprouting position. A soma is indicated with red. White arrowheads in each figure indicate the original pseudounipolar fiber. a: 4 weeks, b: 5 weeks, c, d: 1 week after operation.

branches were bifurcated from the stem process (Fig. 1, 2), and no sprouting was seen from central or peripheral process. Furthermore, the sprouting position on the stem process was close to soma. Some sprouted processes diverged repeatedly and showed an intricate distribution with multiple branches. Others extended linearly to locations distal or proximal of the dorsal root ganglia like a normal axon of the sensory neuron. Based on these morphological aspects, these developed processes were broadly sorted into two groups as follows.

1) The linear type (Fig. 2): A type having sprouted branches running parallel to the original central or peripheral process in dorsal root ganglia. In only one case of this type, sprouted processes ran out of the dorsal
Fig. 3. Winding type of sprouted branches with no orientation. **a-1** and **b-1**: A neuron indicated by a large arrow in **a-1** has very complicatedly diverging branches which meander intricately on the surface of the dorsal root ganglion. Many branches overget the ridge and reach the opposite side of the DRG (**b-1**). Many ring-like structures are found, particularly on **b-1** (white arrowheads). The small arrow and arrowhead in **a-1** and **b-1** indicate the turning point of each corresponding branch from one side to the opposite. Figures **a-2** and **b-2** are traced figures that show the details of the branching in the area limited by the broken red line in **a-1** and **b-1**, respectively. A red neuron in **a-2** corresponds to it, as indicated by a large arrow in **a-1**. A small arrow in **a-2** indicates the sprouting position. **a, b**: 4 weeks after operation.

Fig. 4. Examples of a ring-like structure. Each arrow indicates a ring-like structure. Whole figures clearly show that they are not a basket made by nets but a coiled ring made by a single fiber.
Fig. 5. Distributions of the diameters of LacZ-labeled neurons in DRG. The horizontal axis indicates the diameters of labeled neurons and the vertical axis indicates the number of labeled neurons. Arrows indicate the distributions of diameters of sprouting neurons and ring-like structures. The range of the sprouting neurons is between 45 µm and 63 µm. The diameters of the rings are between 46 µm and 68 µm. No difference in cell size can be observed between the sprouting neurons and neurons encircled by them. As rings are formed on the same sized neurons, the diameter of rings is larger than that of neurons.

root ganglia and reached into the proximal spinal root. Other processes of this type stayed in the dorsal root ganglia. Two cases indicated in Figure 2 had branches that diverged several times, but eventually they extended along the major axis of the dorsal root ganglia. Their distributions differed from the winding type mentioned below. No ring-like structure could be observed in this type.

2) The winding type (Fig. 3): A type having diverging branches which ran randomly to the original central or peripheral process and meandered intricately in dorsal root ganglia. No processes ran out to the dorsal root ganglia. Many of the terminals of this type formed ring-like structures. As this structure was not mesh of a net but encircled other non-labeled neurons (Fig. 4), “ring” was the more appropriate description than “basket”. These rings seemed to be made by a single process that wound around the surface of other neurons. Some of them did not terminate at one soma but further extended to other soma. In order to measure the size of the sprouted cell bodies, five dorsal root ganglia were inspected as a random sampling (Fig. 5). The diameter of all labeled neurons was measured at a minimum 28 µm and maximum 70 µm. The diameter of sprouted neurons was within a range of 45 µm to 63 µm. The diameter of the ring-like structure was also measured, and it was within a range of 46 µm to 68 µm.

Because the labeling method was the same, in all specimens used in the present study, the phenomenon that both types could appear simultaneously in one ganglion was sufficiently considered. However, no such case was detected because the fibers made a complicated meshwork in a ganglion having several labeled neurons.

Discussion

In this study, the LacZ adenovirus was introduced to the sciatic nerve located at the posterior aspect of thigh. Thus, labeled peripheral nerves were axons of the sensory, motor, postganglionic sympathetic, and parasympathetic neurons. However, sympathetic fibers in sensory ganglia were not labeled in the present experiment because these postganglionic fibers were derived from the sympathetic trunk along the posterior roots and were not in the sciatic nerve at the level of the thigh. The motor and parasympathetic neurons had somata in the spinal cord. Therefore, the visualized fibers with cell bodies in dorsal root ganglia in the present method were obviously only sensory fibers and their sprouted branches that came into existence were the results of a direct reaction of the sensory neurons after transaction of their peripheral processes. The previous research
based on the identification of sympathetic fibers by tyrosine hydroxylase or catecholamine already revealed the sprouting of sympathetic fibers in dorsal root ganglia after transection of the peripheral nerve (McLachlan and Hu, 1998; Ma and Bisby, 1999). These fibers, however, were not injured by peripheral transection because they were considered to be the postganglionic fibers sent from the sympathetic trunk to ganglia along blood vessels of the posterior roots. Crutcher (1982) showed that the sprouting of sympathetic fibers occurred even in uninjured neurons. This phenomenon was supposed to be triggered by a chemotactic substance (McLachlan et al., 1993). The sprouting of DRG cells in the present study was a direct reaction induced by nerve injury. Further, the sensory neurons formed a ring-like structure on the surface of the other sensory neurons after nerve transection. These observations indicate that injured sensory neurons may convey the information from them to another sensory neuron and give rise to extremely complicated neuropathic pain. Thus, we suppose that the ring-like structure formed on the other sensory neurons observed in the present study is intimately related to the onset of the mechanism of complex regional pain syndrome (CRPS) type II developing after the peripheral nerve injury.

Shapes of sprouting processes were sorted into two groups. Processes of the winding type formed a ring-like structure, and such a structure was not found in the linear type. It was possible that each type might have a respectively different role or character but it was impossible to certify the difference between them by the present labeling method.

Liu et al. (2005) reported that the axonal sprout from small neurons formed a basket-like structure around the cell body of large neurons in rat dorsal root ganglia after sciatic nerve ligation. In the present study, no sprout was found from the small neurons. Further, the diameter of the ring-like structure was also similar to that of the cell body of sprouted neurons. The diameter of cell bodies of sprouted neurons and that of cell bodies surrounded by rings was within almost same range. Differences in the size of cell bodies between sprouted neurons and the surrounded neurons mentioned by Liu et al. (2005) were not confirmed in our study.

Ramer and Bisby (1997) investigated the sprouting of sympathetic axons of rat dorsal root ganglia after chronic constriction injury and showed that this was already seen only four days after nerve injury. In our tracing method, in order to investigate the whole figure of DRG neurons, we had to wait several days until all parts of the neurons, that is, the cell bodies, axons and terminal branches, were filled with β-galactosidase produced by the LacZ gene recombinant adenovirus. In the present study, the minimum period of observation after labeling was a week. Even then, not only the soma and stem process but also bifurcated central and peripheral processes were already labeled without interruption (see Fig. 2c, d). It was considered that β-galactosidase filled the whole cell body and processes in a week after labeling, that is to say, the distribution of sprouted branches observed in our specimen was thought to show whole shape of the branches. Because the developed branches were already seen only a week after labeling in the present experiment (see Fig. 2c, d), it was possible to say that the sprouting of sensory neurons also starts as early as that of sympathetic neurons. It could be supposed that the short processes observed in the two weeks (Fig. 1) were redundant fibers after the pruning phenomena. If pruning occurs on every branch that did not satisfy the conditions to survive at any time, it could be expected that such short branches would be seen in every week. However, they were not observed after three weeks of operation. Therefore, it is natural to consider that they were not the result of pruning but rather young branches just after sprouting and that new sprouting would occur within almost two weeks after nerve transection. Additionally, pruning would not occur within six weeks in our experiment because well-developed branches were observed in the dorsal root ganglia in six weeks.

In conclusion, it is evident from the present study that the spinal ganglion cells sprout regenerating fibers from the vicinity of the cell body after transection of the peripheral nerve, as shown by stereoscopic observation on whole mount specimens. The extension pattern of the regenerating fiber is classified into 2 types: linear or winding. The possibility of the functional reconstruction can be considered on the linear type by the similar extension pattern of their sprouting fiber to the original pseudounipolar sensory fiber. On the other hand, functional recovery can not be assumed by the winding type showing no organized pattern of its fibers, although we showed the possibility that this type causes CRPS type II.

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References


