DORSAL ROOT REGENERATION INTO TRANSPLANTS OF DORSAL OR VENTRAL HALF OF EMBRYONIC SPINAL CORD*

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Abstract
Adult cut dorsal root axons regenerate into the transplants of embryonic spinal cord (ESC) and form functional synapses within the transplants. It is unknown whether the growth is specific to transplants of dorsal half of ESC, a normal target of most dorsal root axons, or whether it is due to properties shared by transplants of ventral half of ESC. We used calcitonin gene–related peptide (CGRP) immunohistochemistry to label to the subpopulations of regenerated adult dorsal root axons, quantitative analysis to compare the extent of dorsal root regeneration, and also compare neuronal composition within both transplants. Adult Sprague–Dawley rats received intraspinal grafts of dorsal or ventral half ESC (E14), and the L4 or L5 dorsal root was cut and juxtaposed to the transplants. Three months later sagittal sections were prepared for CGRP immunohistochemistry and Nissl–Myelin stain. Dorsal root axons regenerated into both kinds of transplants, but growth into dorsal half of ESC was more robust than that into ventral half of ESC. Histograms of the perikaryal area showed that the transplants of dorsal half ESC consisted of small neurons predominantly, whereas transplants of ventral half ESC consisted neurons of variable sizes with abundant myelination. These results indicate that both kinds of ESC may help to rebuild damaged spinal reflex circuits after spinal cord injury.

Key words : Calcitonin gene–related peptide, Dorsal root regeneration, Embryonic spinal cord tissue, Immunohistochemistry, Intraspinal transplantation

Introduction

The severed central branches of adult mammalian dorsal root ganglion (DRG) neurons are known to regenerate within the dorsal root but the dorsal root entry zone forms a barrier that prevents regrowth into the spinal cord[1-5]. One crucial extrinsic constraint leading to failure of regeneration is the presence of a thickened glial limiting membrane[5] at the transitional zone between dorsal root and spinal cord[1,7]. When transplants of embryonic central nervous system (CNS) tissues[7-12] or Millipore implants coated with embryonic astrocytes[13] are substituted for adult spinal cord, however, cut central processes of adult DRG neurons cross the dorsal root–transplant interface and regrow extensively within the implants. Synaptic terminals of regenerated DRG axons are permanently retained within embryonic spinal cord (ESC) and

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brain transplants[8], and they establish electrophysiologically functional connections with transplant neurons[11]. The dorsal root axon-embryonic CNS transplant system therefore provides an in vivo model for studying whether embryonic CNS transplants can be used to reconstruct injured spinal reflex arcs.

In the present study we used immunohistochemical methods to label regenerated dorsal roots that contain calcitonin gene-related peptide (CGRP). Approximately 50% of DRG neurons, primarily those that are small- and medium-sized, contain CGRP, and these are the source of unmyelinated (C fibers) and myelinated (Ad and Aāb fibers) axons that project to the normal dorsal horn[14,15]. CGRP is also found in motoneurons[14,16], where its appearance is distinctly different from that of the primary afferent fibers. Because these CGRP-containing process derive exclusively from DRG neurons[14,17], CGRP has served as a marker for primary afferent fibers both in the normal dorsal horn (reviewed by Willis and Coggeshall[18]) and in transplants of embryonic CNS tissures[7-12,19,20]. We also used immunohistochemistry for growth-associated protein (GAP)-43 to evaluate the difference of the axonal development in ESC.

Here we report that transplants of dorsal and ventral half of ESC enable cut dorsal roots to regenerate into both transplants, and that these regenerated axons arborize more robustly in the transplants of dorsal half ESC, normal target of most DRG axons, than ventral half ESC. These results encourage expectations that the transplants of dorsal half ESC can be used to recostruct reflex circuitry interrupted by spinal cord injury. Some of these results have appeared in abstract form[21].

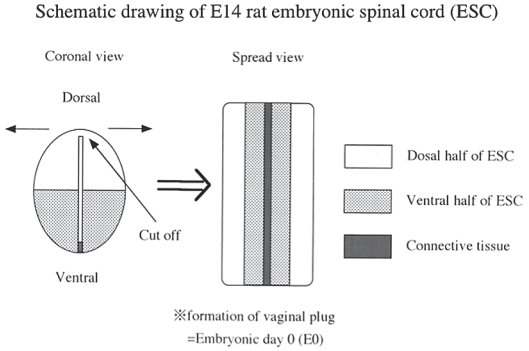

Fig. 1. Schematic drawing of the structure of the embryonic day 14 (E14) spinal cord. Under a dissecting microscope, lateral (=dorsal) and medial (=ventral) longitudinal half portions were dissected from E14 Sprague-Dawley rat pups as dorsal and ventral half of ESC respectively.
The day of vaginal plug formation is the embryonic day 0 (E0).

Materials and methods

Surgical Procedures

Twenty-seven adult female Sprague-Dawley rats (weighing 200-300 g) served as graft recipients.

The rats were anesthetized with an intraperitoneal injection of ketamine hydrochloride (76 mg/kg), xylazine (76 mg/kg), and acepromazine maleate (0.6 mg/kg). Under an operating microscope, a laminectomy of the T13 or L1 vertebra was performed with a speed drill to expose the lumbar enlargement. After sharp transection of the left L4 or L5 dorsal root at the dorsal root entry zone, the distal portion of the root was reflected caudally. A dorsal quadrant cavity approximately 2 mm in length was aspirated from the left side of the lumbar enlargement. Under a dissecting microscope, lateral (N=15) and medial (N=12) longitudinal half portions were dissected from Sprague-Dawley rat pups (embryonic day 14) as dorsal and ventral half of ESC respectively (Fig. 1), and introduced into the cavity. The transected dorsal roots were juxtaposed to the transplants, the dural opening was tightly stitched with interrupted 10-0 nylon sutures, securing the dorsal root in place, and covered with a piece of hydrocephalus shunt film (Durafilm ; Codman Surlef, Inc.). The resected vertebral arch and bone chips were replaced, and the superficial wound was closed in layers. Following surgery, operates were maintained under a heating lamp until fully awake. The procedure have been previously described in detail[3,7-12] (Fig. 2).

Immunohistochemistry

GAP-43. To evaluate the axonal development in embryonic spinal cord transplants removed from embryos (E14) of timed pregnant Sprague-Dawley rat, embryonic spinal cord with surrounding tissues (N=3) were immersed in 4% paraformaldehyde in 0.1 M phosphate buf-

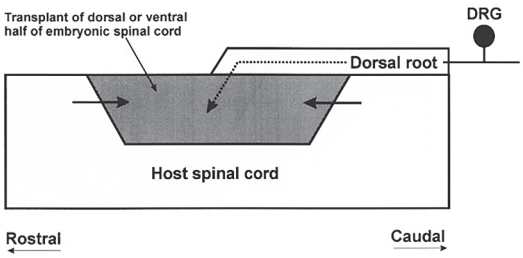

Fig. 2. Schematic drawing of the experimental model showing the relationship of the cut dorsal root, transplant of dorsal or ventral half of embryonic spinal cord, and host spinal cord. In order to distinguish regenerated DRG axons (dotted line) entering directly from the cut dorsal roots from dorsal root collaterals (arrows) that entered the transplant after ascending or descending in the host spinal cord, an important feature is that the dorsal root stump is positioned at the middle of the dorsal surface of the transplant and far from the lesioned dorsal column in this experimental model.

fer pH 7.4 overnight, and transverse vibratome sections (40 μm) were incubated in primary antiserum against growth-associated protein (GAP)-43 (a generous gift from Dr. Schreyer) at 1 : 4,000 dilution in PBS containing 0.5% bovine serum albumin, 2% horse serum, and 0.1% triton X-100 overnight. Sections were then immersed in biotinylaated goat antirabbit IgG and avidin-biotinylated horseradish peroxidase (HRP) complex (Vector Laboratories, Burlingame, CA) and processed for HRP visualization using a solution of 0.05% 3,31-diaminobenzidine tetrahydrochloride (DAB ; Sigma Chemical Co., St. Louis, MO) as the chromagen and 0.01% hydrogen peroxydase in 0.05 M Tris-HCl buffer, pH 7.4.

CGRP. After survival periods of 3 months following transplantation, the host rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with 50 ml of physiological saline followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. Spinal segments containing transplants and inserted dorsal roots were removed, sectioned sagittally at 14 μm on a cryostat and mounted onto subbed slides. Every fifth section was then processed for CGRP immunohistochemistry according to the ABC procedure that has been described previously[9,10,12]. Sections were then processed for the ABC procedures and HRP visualization with DAB as the chromagen as described above. Tissues were dehydrated and viewed with an Olympus BX50 microscope (Olympus Optical Co. Ltd., Tokyo, Japan).

To evaluate transplant morphology and the interfaces between dorsal root-host spinal cord and transplant-host spinal cord and to determine the localization of regenerated axons within the transplants, adjacent every fifth section was stained with chromoxane cyanine R for myelin and counterstained with cresyl violet.

Morphometric analysis

The extent to which CGRP-labeled axons regenerated into the transplants was measured for animals with transplants of dorsal half (N=5) and ventral half (N=5) ESC after survival period of 3 months. We have previously reported that dorsal root regeneration into embryonic spinal cord transplants was completed by 3 months after transplantation[7]. Because the transected dorsal root stump was positioned at middle of dorsal surface of the transplants and far from the lesioned dorsal column in this experimental model, regenerated axons entering directly the transplant after ascending or descending in the host spinal cord[20,22]. In addition, for most of the CGRP-containing axons measured in transplants we were able to observe at the transition between host dorsal root and transplant.

To determine the amount of CGRP-labeled axonal regeneration within the transplants, we used a point-counting stereological analysis to measure the area occupied by CGRP-labeled axons. Sagittal sections that contained labeled axons were examined under a light microscope. A micrometer 10 mm×10 mm in size (10^4 μm^2) composed of 1-mm grid squares (Olympus, Tokyo) that was fitted in an ocular lens was used as a sampling lattice, and the number of times that CGRP-labeled axons intersected the corners of the grid squares was counted. The area occupied by labeled axons in each individual sampling lattice was calculated and the area occupied by labeled axons in the whole sagittal section was then calculated by multiplication of the number of sampling lattices contained labeled axons. Five consecutive sections containing the most abundant CGRP immunoreactivity in the

transplant were examined per animal and the results were averaged.

To determine the arborization of CGRP-immunoreactive axons, we measured the distribution of CGRP-immunoreactive axons in 5 sagittal sections by making composite montages that consisted of all the individual sampling lattice examined and the results were averaged.

To compare the neuronal populations in both kinds of transplants, sagittal sections stained with cresyl violet and cyanine R were examined. Perikaryal area of cells contained in a sampling rectangle (7,450 μm^2) was measured using a Bioquant System IV (R&M Biometrics, TN). Three consecutive sections were examined per animal receiving a transplant of dorsal half (N=5) and ventral half (N=5) ESC. A histogram was made from these data to evaluate the differences of cellular composition between both kinds of transplants.

The significance of the differences between both transplants was determined by Mann-Whitney two sample test (P<0.05). All statistical analyses were performed by using NCSS97 Statistical System for Windows (NCSS statistical software, Kaysville, UT).

Results

General histology

Over 90% of the hosts had clearly identifiable transplants. Transplants of dorsal and ventral half ESC survived in the adult host spinal cord and differentiated into patterns that were characteristic for each region. Intraspinal transplants of dorsal half ESC contained regions that resemble substantia gelatinosa based on the presence of numerous small neurons and relative paucity of myelination[3,12,23] (Fig. 3). Ventral half ESC composed of neurons of variable sizes showed abundant myelination. A few large-sized neurons likely corresponded to the motoneurons found in normal ventral cord were present in ventral half ESC, but their morphology and incidence were different from adult normal motoneurons. The number of the large-sized neurons in ventral half ESC was obviously small compared with the host motoneurons, and Nissl bodies characteristic of the perikarya of the normal motoneurons were scant in large-sized neurons in ventral half ESC (Fig. 4).

Fig. 3. Sagittal section of graft of dorsal half ESC 3 months after transplantation. Transplant of dorsal half ESC (TP) contains aggregates of small0sized neurons. Transplant is well integrated with dorsal root (DR). Stained with chromoxane cyanine R and cresyl violet. Calibration bar=500 μm.
Arrowhead ; host spinal cord-transplant interface.

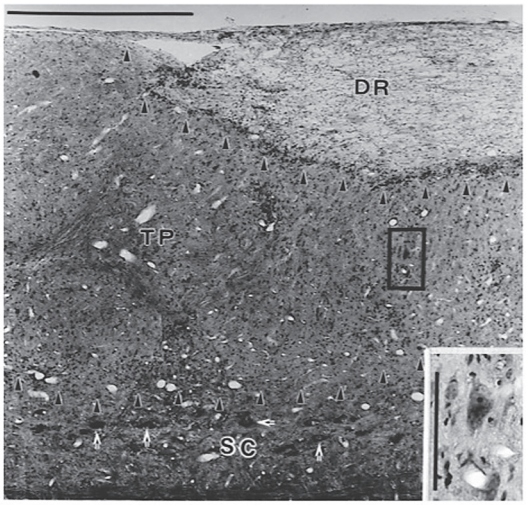

Fig. 4. Sagittal section of graft of ventral half ESC 3 months after transplantation. Transplant of ventral half ESC (TP) consists of various-sized neurons and exhibits extensive myelination. Transplant is well integrated with dorsal root (DR) and host spinal cord (SC). Calibration bar=500 μm.
Inslet : An enlargement of the region outlined by the rectangle shows a large-sized neurons with scant Nissl bodies. Calibration bar=100 μm. Nissl-Myelin stain.
Arrowhead ; borderline between TP and DR and SC.
Arrows ; motoneurons of host spinal cord.

The transplants were generally well-integrated with host spinal cord and dorsal root. The interfaces between the dorsal root and the transplant were, however, readily recognized by the clear contrast in cell density between

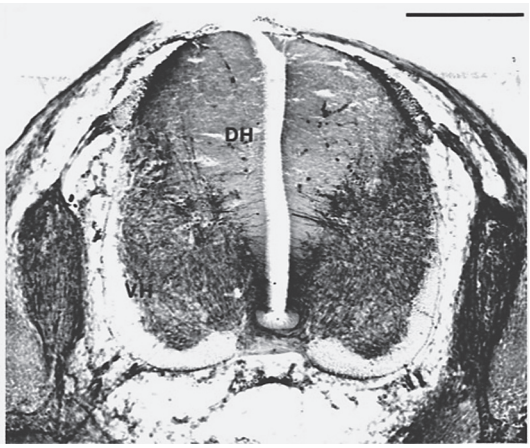

Fig. 5. Transverse section of embryonic spinal cord show that GAP-43 was immunoreactive for ventral cord except the lateral motor column, surrounding area of ventricular zone contained neuronal precursors, dorsal roots, and dorsal root ganglia at the embryonic day 14. Calibration bar=500 μm.

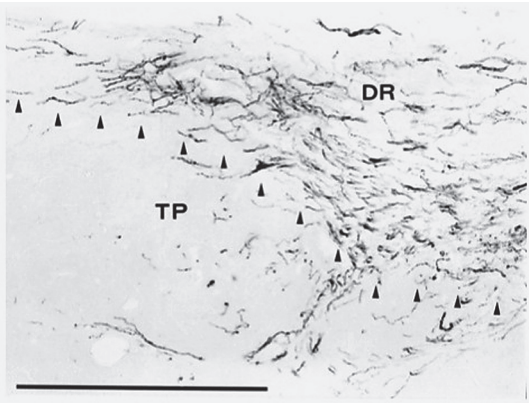

Fig. 6. CGRP-immunoreactive axons in the transplants of dorsal half ESC 3 months after graft. Sagittal section. Regenerated axon cross the interface between host dorsal root (DR) and transplant (TP) and form a dense plexus near the interface (between upward and downward arrowheads). Interface was identified in the adjacent Nissl-stained section. Calibration bar=500 μm.

the numerous closely packed glial cells found in the dorsal roots and the more loosely cellular transplants.

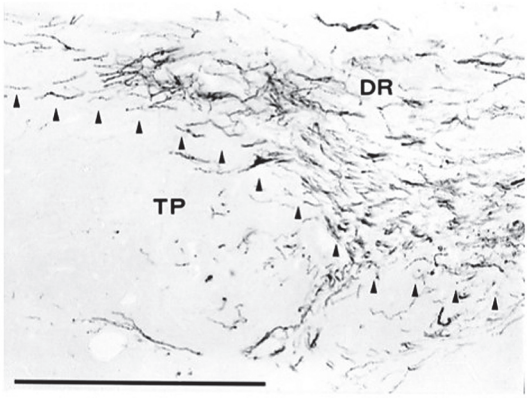

Fig. 7. CGRP-immunoreactive axons in the transplants of ventral half ESC 3 months after graft. Sagittal section. Regenerated axons cross the dorsal root (DR)-transplant (TP) interface (arrowheads) and grow sparsely and extensively close to the interface without the formation of obvious plexuses. Calibration bar=500 μm.

Immunohistochemistry

GAP-43. GAP-43 was immunoreactive for ventral cord except the lateral motor column, surrounding area of ventricular zone contained neuronal precursors, dorsal roots, and dorsal root ganglia at the embryonic day 14 (Fig. 5).

CGRP. Dorsal root axons immunoreactive for CGRP regenerated into every transplant examined. CGRP-labeled axons showed distinctive patterns of distribution within both kinds of transplants. In the transplants of dorsal half ESC, CGRP-labeled axons arborized extensively near the surface of transplants, and in some portions the axons tangled together and formed dense bundles (Fig. 6). In the transplants of ventral half ESC, CGRP-immunoreactive axons extended sparsely but diffusely close to the dorsal root-transplant interface and individual axons but not bundles of axons could be recognized (Fig. 7).

Morphometric analysis

The point-counting stereological analysis showed the area fraction occupied by regenerated CGRP-containing dorsal root axons in transplants of dorsal or ventral half ESC (Fig. 8). These regenerated axons occupied a mean

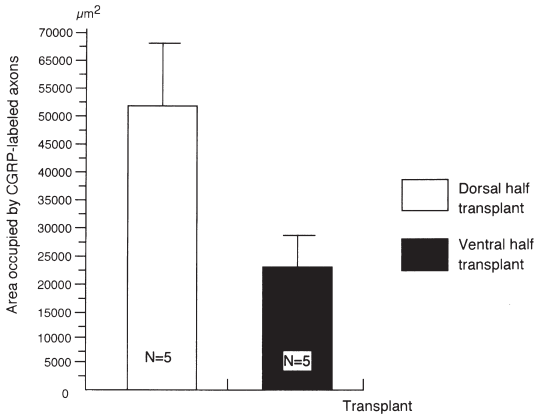

Fig. 8. Comparison of area occupied by CGRP-labeled axons (mean+SE) in the transplants. Regenerated dorsal root axons immunoreactive for CGRP occupy a significantly larger area in the transplants of dorsal half ESC than ventral half ESC. Area occupied by CGRP-labeled axons is calculated by point-counting stereological analysis (see text). Overall significance is determined by Wilcoxon test ($P<0.05$).

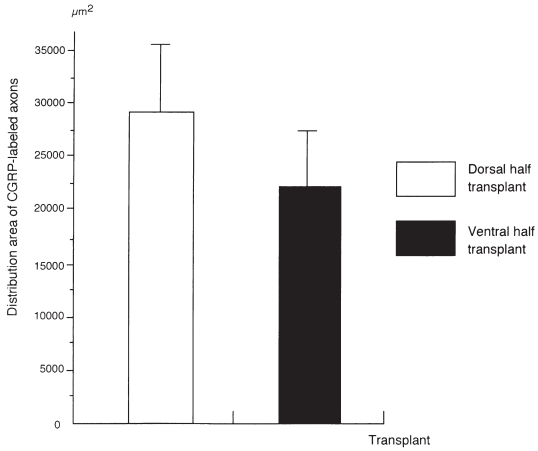

Fig. 9. Comparison of the distribution of CGRP-immunoreactive axons in the transplants. There is not significant difference between dorsal half and ventral half ESC. The distribution of CGRP-labeled axons in the sagittal plane is determined by making montages which consist of all the individual sampling lattices examine (see text). Overall significance is determined by Wilcoxon test ($P<0.05$).

area of $5.42\times10^4\,\mu m^2$ in transplants of dorsal half ESC. The area occupied in animals that had received transplants of ventral half ESC was approximately 40% of that in the rats grafted dorsal half ESC. Regenerated CGRP-immunoreactive axons therefore occupied a significantly larger area in the transplants of dorsal half ESC than in transplants of ventral half ESC ($P<0.05$).

Fig. 9 shows the areas over which regenerated CGRP-labeled axons arborized in the transplants of rats receiving dorsal or ventral half of ESC. The mean area of CGRP-innervated regions within the transplants of dorsal half ESC was not significantly different from ventral half ESC. The regenerated dorsal root axons therefore distributed over a similar area within the both kinds of transplants. These results confirmed our quantitative observations that the regenerated CGRP-labeled axons showed different patterns of growth in the transplants of dorsal and ventral half ESC.

Based on the perikaryal area of neurons within the both kinds of transplants, we classified the neurons into 3 groups : small-sized neurons (50-$100\,\mu m^2$) ; medium-sized neurons (150-$300\,\mu m^2$) ; large-sized neurons (more than $300\,\mu m^2$) (Fig. 10). Transplants of dorsal half ESC included small-sized neurons significantly greater than those of ventral half ESC, whereas ventral half ESC contained medium- and large-sized neurons greater than dorsal half ESC. These results confirmed our qualitative observations. Cells whose area is less than $50\,\mu m^2$ appeared to be glia and there was no significant difference between dorsal and ventral half ESC.

Discussion

The principal findings of the present study are that cut dorsal roots immunoreactive for CGRP regenerate into the transplants of dorsal and ventral half ESC adjacent to the interface between dorsal root and transplants, and that transplants in rats receiving a dorsal half ESC support more robust regeneration than ventral half ESC. The results suggest that transplants of dorsal and ventral half ESC provide factors that permit or enhance dorsal root regrowth and that transplants of dorsal half ESC, a normal target of primary afferent fibers, provide additional more specific cues for dorsal root regeneration than

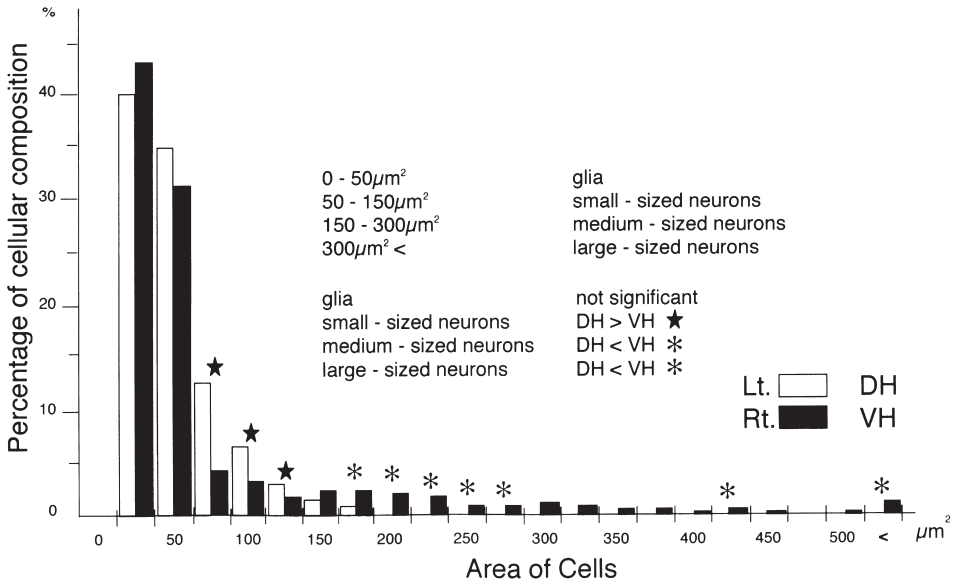

Fig. 10. Distribution histogram showing perikaryal area of neuron in the two types of transplants. Based on the perikaryal area of neurons into 3 groups: small-sized neurons (50-150 μm^3); medium-sized neurons (150-300 μm^3); large-sized neurons (more than 300 μm^3). Transplants of dorsal half ESC include small-sized neurons significantly greater than those of ventral half ESC, whereas ventral half ESC contains medium- and large-sized neurons greater than dorsal half ESC. The significance of the differences between dorsal (N=5) and ventral (N=5) half ESC transplants was determined by Mann-Whitney two sample test (P<0.05).

transplants of ventral half ESC.

Difference of Morphology of Dorsal and Ventral Half ESC

The neuronal composition differed between transplants of dorsal and ventral half ESC. Dorsal half WSC contained small-sized neurons significantly greater than those in ventral half WSC, whereas ventral half ESC contained medium- and large-sized neurons greater than those in dorsal half ESC. Besides the immixture of a few large-sized neurons in transplants of dorsal half ESC during dissection of graft tissues, the number of large-sized neurons likely corresponded to the motoneurons in normal ventral horn was very small even in ventral half ESC and their biological activity also appeared to decrease because of poor Nissl bodies. These results suggested that most motoneurons in ventral half ESC would have died presumably by isolation of a transplant from surrounding host spinal cord except some neural circuits or otherwise would not have prevented perikaryal atrophy. Intraspinal transplants of embryonic spinal cord have been reported to consist predominantly of the small and medium-sized neurons, such as those seen in the intermediate gray and superficial dorsal horn of the normal adult spinal cord[3,23].

Transplants of Dorsal and Ventral Half ESC Effect Different Patterns of Regeneration

The pattern and extent of dorsal root regrowth differed between transplants of dorsal and ventral half ESC. In the transplants of dorsal half ESC regenerated axons stayed relatively close to the host dorsal root-transplant interface, then arborized extensively. In some portions they often tangled together in plexuses. In contrast, regenerated axons were distributed widely and sparsely near the interface between dorsal root and transplant, and grew as individual axons rather than in bundles or plexuses. These qualitative morphological observations were confirmed by our quantitative studies. Although cut dorsal roots immunoreactive for CGRP regenerate into both kinds of transplants, the area occupied by re-

generated CGRP-labeled axons within dorsal half ESC is significantly greater than ventral half ESC. Both transplants therefore provide an environment conductive to dorsal root regeneration, but dorsal half ESC, a normal target of most primary afferent fibers, provide additional more specific cues for growth. We have previously reported differences in the pattern of dorsal root regeneration into transplants of brain and spinal cord that resemble those that we now find when the target is dorsal half ESC[10]. It is unlikely that inappropriate targets taken from ventral half ESC reproduce precisely the conditions found in dorsal half ESC. Growth into inappropriate targets is therefore consistent with the concept that the early stages of axon extension depend on molecules that are expressed generally throughout the developing nervous system.

Our observations that regenerated DRG axons grew more densely within dorsal half ESC than ventral half ESC suggests the presence of target-specific cues for pathfinding and target recognition that are not provided by ventral half ESC. These results are similar to those of in vitro studies showing that neuritis of explanted embryonic DRG axons grew and arborized more abundantly within co-cultured explants of spinal cord than of tectum[24]. Surface macromolecules likely to mediate the formation of specific pathways include glycoproteins that are expressed transiently by discrete population of neurons[25].

Dorsal Half ESC Enhances Dorsal Root Regeneration in Preference to Ventral Half ESC

Axotomized dorsal roots show limited regeneration in adult mammals, and most terminate as dilated ends within a thickened glial limiting membrane at the interface between the dorsal root and spinal cord[1,6]. Astrocytes at the dorsal root entry zone are thought to impose a mechanical or chemical barrier that repels regrowth into the spinal cord[4]. Some DRG neurons, including those that contain CGRP, are capable of regenerating into intraspinal transplants of embryonic spinal cord or brain[7-12]. At least three mechanisms may account for the ability of embryonic CNS transplants to allow or encourage regenerations. The first is the absence of a glial limiting membrane. The second is the absence of a mature CNS glial environment that express substances inhibitory for regeneration, particularly peptides associated with CNS myelin[26,27]. The third is the expression of growth promoting molecules, among them the family of neurotrophic factors related to nerve growth factor, which includes brain-derived neurotrophic factor, neurotrophin-3 and neurotrophin-4/5[28]. NGF has been reported to enhance regeneration of central primary afferent axons in several different models of injury[19,29,30].

One of the mechanisms account for the preference of dorsal root regeneration in dorsal half ESC is likely to be the present of the ventral-to-dorsal gradient of proliferation and migration of neuronal precursors during normal development of the spinal cord[31,32]. The ventral motor system develops earlier than does the dorsal sensory system. As neural development proceeds, proliferation diminishes in the ventral cord, and by E15 only the most dorsal portion of the ventricular zone remains active[32]. It is well-known that GAP-43 is found in large amounts specifically in outgrowing axons during normal developnent[33]. The present results of GAP-43 immunoreactivity in ESC (E14) confirmed the ventral-to-dorsal gradient of neural development. Since adult dorsal roots begin to regrow into the embryonic spinal cord transplants soon after grafting[8], at the early stage of the axon elongation regeneration of adult rat dorsal roots is likely to be additionally enhanced by the precursor neurons remained in the dorsal cord of ESC.

Our results suggest that dorsal half ESC as well as ventral half ESC provide the conditions that allow or encourage cut dorsal root axons to regenerate and survive. The conditions that constitute a permissive environment for regenerating axons are therefore relatively nonspecific, but dorsal half ESC nevertheless differ in the extent to which they satisfy the requirement for growth of dorsal roots. The results of the present study are therefore an important step toward establishing that the transplants of dorsal half ESC can be used to reconstruct segmental reflex arcs interrupted by spinal cord injury. However, whether neural circuits between this restored afferent limb and the remaining efferent limb of a spinal reflex arc is reconstructed by bridging effect of embryonic spinal cord transplants remains to be determined by electrophysiological methods.

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