Neurotoxicity Research, 2003, VOL. 5(7). pp. 539-548

F.P. Graham Publishing Co.

Protective Effects of Adenoviral Cardiotrophin-1 Gene Transfer on Rubrospinal Neurons After Spinal Cord Injury in Adult Rats

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(Received 22 September 2003; Revised 05 November 2003; In final form 05 November 2003)

Cardiotrophin-1 (CT-1), a muscle-derived cytokine, supports the survival of motoneurons *in vivo* and *in vitro*. The present study investigated whether adenoviral huCT-1 gene transfer protected injured neurons from cell death or atrophy and promoted regeneration of rubrospinal tract (RST) after spinal cord injury in adult rats. Administration of the adenoviral CT-1 vector (Adv-CT1) to C3-4 lateral funiculus hemisection cavity, that completely interrupted RST, led to sustained CT-1 expression. Providing Adv-CT1, which rescued 20% of neurons, could prevent the loss of injured rubrospinal neurons 8 weeks post-injury. Retrograde tracing with FluoroGold showed that 1.2% of RST neurons regenerated at least two segments caudal to the injury site. Anterograde tracing with biotinylated dextran amine revealed that the RST axons terminated in white matter and gray matter. Behavioral testing revealed a significant functional recovery in limb usage. This observation indicated that adenoviral CT-1 gene transfer into the injured cord promoted survival and regeneration of rubrospinal neurons in adult rats.

Keywords: Spinal cord injury; Cardiotrophin-1; Adenovirus vector; Red nucleus; Rubrospinal tract; Gene therapy; Axon regeneration; Anterograde and retrograde tracing

INTRODUCTION

Promotion of retrograde degenerated neuronal survival and axonal regeneration by administration of exogenous neurotrophic factor is a promising approach for the treatment of spinal cord injury (SCI). A number of neurotrophic factors, including brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), and glial-derived neurotrophic factor (GDNF), have been identified as being able to promote the survival and regeneration of axotomized neurons from several descending pathways after SCI (Namiki *et al.*, 2000; Novikova *et al.*, 2002). The survival of central neurons and axonal regeneration depend on multiple neurotrophic factors produced by different cell types. It is important to identify effective and specific factors for the protection of specific descending and ascending neuronal pathways after SCI.

Cardiotropin-1 (CT-1), an interleukin-6 (IL-6)-related cytokine, was originally identified to cause hypertrophy of cardiac myocytes (Pennica *et al.*, 1995a). Like LIF, CNTF and oncostatin M (OSM), CT-1 signals via the LIFRß/gp130 receptor complex (Pennica *et al.*, 1995b) and a third specific receptor component (CT-1Ralpha) (Robledo *et al.*, 1997). Besides its similar biological activities to the IL-6 members (review see, Latchman, 2000), more attention has been paid to its effects on neuronal cells like CNTF. CT-1, a muscle-derived cytokine, supports the survival of motoneurons *in vivo* and *in vitro*, including spinal motoneurons (Pennica *et al.*, 1996; Arce *et al.*, 1998; 1999; Oppenheim *et al.*, 2001), brain motoneurons (Oppenheim *et al.*, 2001), embryonic cranial motoneurons (Naeem *et al.*, 2002), and developing oculomotor neurons (Rind and von Bartheld, 2002). CT-1 has also been shown to have protective effects on motoneurons in murine models of inherited progressive neuromuscular degeneration such as progressive motor neuropathy (*pmn*) (Bordet *et al.*, 1999), Wobbler (Mitsumoto *et al.*, 2001) and amyotrophic lateral sclerosis (ALS)

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ISSN 1029 8428 print/ ISSN 1476-3524 online.

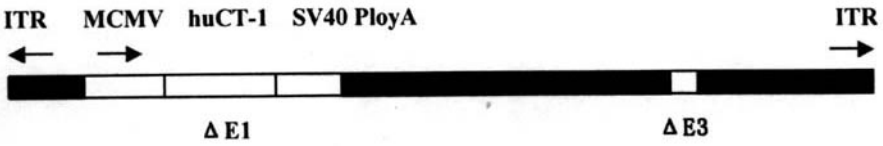


FIGURE 1 The schematic diagram depicting structure of adenoviral human cardiotrophin-1 vector used in the present study.

(Bordet *et al.*, 2001). However, there is little information about neurotrophic roles of CT-1 in descending neuronal groups.

In the present study, we used a gene therapy strategy, the effective way to deliver therapeutic genes with high efficiency in SCI (Lacroix *et al.*, 2000; Tuszynski and Jones, 2001), to observe the effects of CT-1 on the survival and regeneration of the rubrospinal tract (RST). The rubrospinal neurons are known to be sensitive to CNTF (Houle and Ye, 1999), which shares the same LIFRß/gp130 receptor complex with CT-1. In addition, the rubrospinal system is readily identified by retrograde or anterograde tracers with no collateral sprouting which may complicate the interpretation (Waldron and Gwyn, 1969).

MATERIALS AND METHODS

Recombinant Adenoviral Vectors

Adenoviral vectors were constructed according to two-plasmid rescue methods described by Ng *et al.* (Parks *et al.*, 1999; Ng *et al.*, 2000). The human CT-1 cDNA fragment corresponding to nucleotides 1-1018 (GenBank accession no. U43030) containing the coding region was isolated from pBSSSK-CT-1 (gift from Pennica) by digestion with *Hind*III--*Sac*I restriction enzymes and subcloned into shuttle plasmid pDC316, named pDC316-CT1. Recombinant replication-defective adenoviral vectors Ad5CMV-huCT1 (Adv-CT1) were rescued in 293 packaging cells by co-transfection and Cre-mediated recombination of both plasmids pDC316-CT1 and pBHGloxdeltal1'3Cre containing Ad5 genome with the deletion of packaging signal E1 and E3 regions. EGFP cDNA fragment was isolated from pIRES2-eGFP and control recombinant adenoviral vector Ad5CMV-eGFP (Adv-eGFP) was constructed by the same protocol. The vectors contained either CT-1 cDNA or eGFP cDNA in both cases under control of the CMV promoter and SV40 PolyA inserted into E1 of Ad5 genomic DNA with deletion of E3 regions (FIG. 1). Adenoviral vectors were purified by double cesium chloride density gradient and dialysed against phosphate buffer. Concentrations of the viral vectors were expressed as plaque forming units (pfu)/ml. The bioactivity of vectors were tested by PCR analysis of DNA from transfected 293 cell, RT-PCR analysis for RNA from transfected NIH3T3 cells, CT-1 immunocytochemistry assay after Adv-CT1 transfected NIH3T3 cell, and eGFP assay with fluorescent microscope after Adv-eGFP transfected NIH3T3 cell. For injection, the vectors were diluted with virus buffer to the concentration of 2 x 10^7 pfu/ml.

Animals and Surgical Procedure

All the procedures on animals used in this study were approved by the Animal Ethic Committee of Daping Hospital and the animal treatment was under the guide-

Table I Experimental design and the number of animals used in each group

Groups	Expression								Histology		Survival			Regeneration		Function
	RT-PCR				IHC				HE		FG			FG	BDA	Behavior testing
	2d	1W	4 W	8 W	1 W	4 W	6 W	8 W	1 W	4 W	1 W	4 W	8 W	4 W	4 W	2,3,4 W
Normal	2	-	-	-	2	-	-	-	-	-	4	-	-	-	2	4*
GF	2	-	-	-	2	-	-	-	2	2	4	4	4	4	-	4*
Adv-CT1	4	4	4	4	3	3	3	3	2	2	4	4	4	4	4	4*
Adv-eGFP	2	-	2	-	2	-	-	-	2	2	4	4	4	4	4	4*

* The same rats used for assessing the survival of rubrospinal neurons by retrograde FluoroGold labeling 8 weeks after operation. 2d: two days after operation; W: week (s); IHC: immunohistochemistry; HE: hemotoxylin and eosin staining; FG: FluoroGold tracing; BDA: biotin dextran amine tracing.

line of Chinese National Nature and Science Foundation. Every effort was taken to minimize the pain and the number of animals used in this study. Adult female Wistar rats (from The Third Military Medical University), weighing 180-240 g, were anesthetized with a peritoneal injection of acepromazine maleate (0.7mg/kg) and underwent laminectomy at the C3-C4 level to expose the spinal cord segment. After the spinal cord midline and the dorsal root entry zone were identified and the dura matter was removed, a shallow incision was made on the left dorsal spinal cord. Such lesion completely disrupted the lateral funiculus (containing the RST) and partially lesioned the ipsilateral ventral funiculus and gray matter but left the dorsal columns intact. The rostrocaudal extent of the lesion cavity was ~2-3mm (FIG. 2A). A piece of gelfoam soaked with phosphate buffer alone (gelfoam group), Adv-eGFP (Adv-eGFP group) or Adv-CT1 (Adv-CT1 group) was implanted into the cavity. The dura, muscle and skin were closed in layers. After the surgery, the animals were kept on heating pads until wake-up, then returned to their home cages and kept for different periods of time before sacrifice for histological studies. The bladder was expressed 3 times a day after the surgery until the reflex was established. Table I showed the experimental design of animal groups, time points and the number of animals in all procedures.

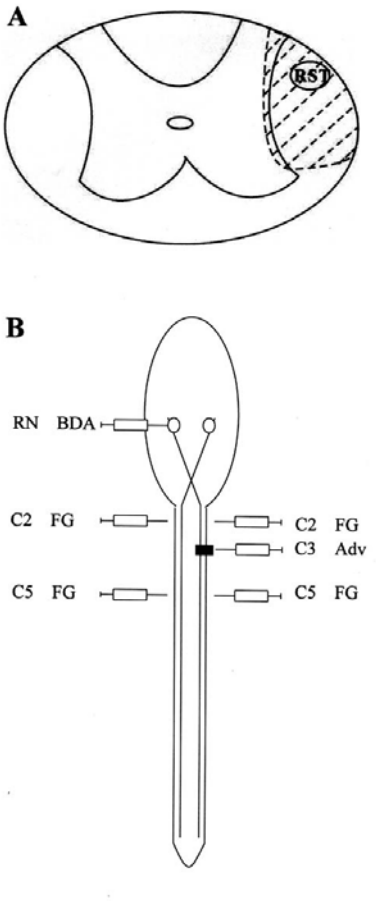


FIGURE 2 The schematic diagram showing the area of spinal cord lesion (**a**) and experimental paradiagm (**b**). RST: rubrospinal tract; RN: red nucleus; BDA: biotin dextran amine; FG: FluoroGold; C: cervical.

Retrograde Tracing with Fluorogold (FG)

The animals were anesthetized as above and received laminectomy at C2-C3 or C4-C5 level 3 days before sacrifice; 1 μl of 2% FG (Fluorochrome, Englewood, CO, USA) was pressure-injected into each side of spinal cord, and animals were killed 3 days later (FIG. 2B).

Anterograde Tracing with Biotin Dextran Amine (BDA)

The animals were anesthetized and positioned on a stereotaxic apparatus 15 days before being killed. A dental drill was used to make a burr hole according to the coordinates described previously (Himes *et al.*, 1981). Then, 0.5 μl of 10% BDA-10,000 (Molecular Probes, Eugene, OR, USA) was slowly injected over 2-3 min with five 200 nl pulses using a 10 μl Hamilton syringe. The needle was left in place for another 20 min and gradually withdrawn over 2-3 min (FIG. 2B).

At various time points, the animals were anesthetized and perfused through the heart with 200 ml of phosphate buffered saline followed by 500 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The entire brain and spinal cord were dissected out and immersed in 0.1 M PB at 4ºC overnight followed by cryoprotection in 30% sucrose in 0.1M PB containing 0.5 mM Thimersal for 3-5d. Spinal cord and brain tissue were cut at 20 μm or 40 μm respectively on a cryostat and mounted onto gelatin-coated slide.

Analysis of CT-1 Expression by RT-PCR and Immunocytochemistry

Adenoviral vector transfected NIH3T3 cells and rat cervical spinal cords were fresh-frozen in liquid nitrogen. Total RNA was isolated using Tripure reagent (Roche) and reverse transcribed with the M-MLV RT (GIBCO BRL). RT-PCR analysis of Adv-CT1 transcripts was performed with primers in CT-1 cDNA (P1: 5ACCTCCTCACCAAATAGG3 136-153 of the huCT-1 cDNA sequence, P2: TAGAGGCCGCAAACG3 574-560 of huCT-1 cDNA, GenBank accession no. U43030), and Ad5CMV-eGFP with primer in eGFP

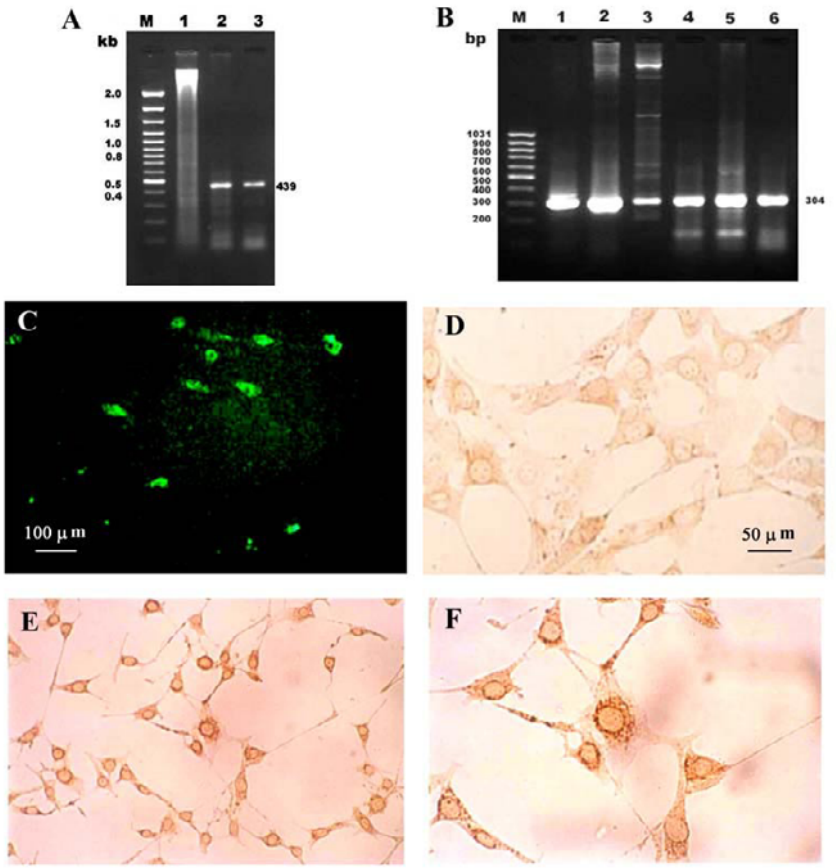
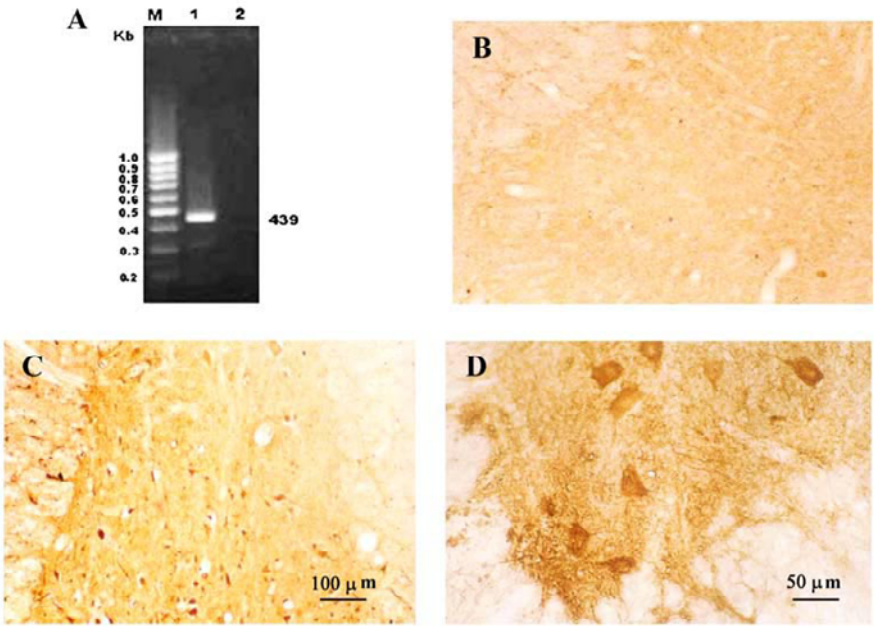


FIGURE 3 Characterization of recombinant adenoviral vectors. **A**: gel analysis of Adv-CT1 vector and PCR products. M: molecular markers; l: Total DNA extracted from Adv-CT1 vector prepared as described in the Materials and Methods; 2: CT1 PCR product using vector DNA as a template and 3: CT1 PCR product using reversed transcribed cDNA as a template and the mRNA was extracted from Adv-CT1 vector infected 293 cells. **B**: gel analysis of Adv-eGFP vector and PCR products. M: molecular marker; 1: eGFP PCR product using pIRES2-eGFP plasmid as a template; 2: eGFP PCR product using pMD18T-eGFP plasmid as a template; 3: eGFP PCR product using pDC316-eGFP plasmid as a template; 4: PCR product using eGFP DNA as a template; 5: PCR product using DNA extracted from adv-eGFP transfected 293 cells; 6: RT-PCR product of RNA extracted from adv-eGFP transfected 293 cells. **C**: A micrograph of 293 cells transfected with adv-eGFP vector for 24 h, scale bar also applies for **E**; **E** and **F**: lower and higher magnification micrographs of 293 cells stained for CT1 with polyclonal antibodies to CT1 after Adv-CT1 vector treatment for 24 h. Granular staining was present in cytoplasm of most transfected cells. **D**: Adv-CT1 vector treated cells stained with normal rabbit serum as a negative control.



cDNA (P1: 5GCCACAAGTTCAGCGTGTCC3, P2: AGCTCGATGCGGTTCACCAG3).

NIH3T3 cells seeded on Lab-Tek slides were cultured in the presence of Adv-eGFP or Adv-CT1 for 48 h and fixed in 4% paraformaldehyde. The spinal cords from rats that received gelfoam soaked with Adv-CT1 for 1, 4, 6 and 8 weeks were cut at 20 μm. The transfected cells and spinal cord sections were incubated with an anti-huCT1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:1000 dilution followed by incubations with biotinylated secondary antibody and the ABC kit (Vector Labs, Burlingame, CA, USA), respectively. The specificity of the immunostaining was verified in sister cultures or adjacent sections by omitting primary or secondary antibodies.

Detection of BDA-labeled Fiber and FG-labeled Cells

BDA-labeled fibers were detected by the incubation of sections with the ABC kit (Molecular Probes) and visualized with diaminobenzadine (DAB) as a chromagen. The procedures were performed according to protocol of BDA neuronal tracer kit (Molecular Probes, Eugene, OR, USA). The numbers of BDA-labeled axons at 3 adjacent cross sections in C2-C3, C3-C4 and C4-C5 were counted.

The number of FG-labeled cells was counted in every section throughout the rostrocaudal extent of the RN spanning 1000 μm from the caudal pole with the fluorescent microscope at ˜100 magnification. The criteria for cells counting were described previously (Agrawal and Fehlings, 1997; Liu *et al.*, 1999). Briefly,to avoid overestimating, neurons were counted in every other section, and the number was multiplied by 2 to calculate the total number of neurons in each RN. Only those cells with identifiable nuclei, nucleoli, and characteristic neuronal morphology were counted.

Behavioral Testing

Forelimb usage and behavioral scores were examined as described previously (Liu *et al.*, 1999). Rats were examined for forelimb use during spontaneous vertical exploration, a test that is highly sensitive for the asymmetrical use of forelimbs. Briefly, animals were placed in a transparent Plexiglass cylinder (20 cm in diameter and 20 cm high) for 5 min. The cylinder was used to encourage the use of forelimbs for vertical exploration. The testing session was videotaped, and forelimb usage was analyzed blindly later based on the videotape. The frequency of forelimb contact with cylinder wall was scored respectively: the left forelimb, the right forelimb or both forelimbs. The data were presented as the percentage use of the contralateral forelimb, the ipsilateral forelimb, and both forelimbs, respectively, against the total number of contacts.

Statistical Analysis

The data for FG cell count, the number of BDA labeled fibers, and the percentage of forelimbs usage were expressed as mean ± SEM and compared by two-way ANC (treatment X preferred limb) or one-way ANOVA between groups. Statistically significant differences were considered at $p < 0.05$.

RESULTS

Transgene Expression *in vitro* and *in vivo*

The expression of the transgene delivered by the adenoviral vector was tested by PCR, RT-PCR, immunocytochemistry or fluorescent microscopy *in vitro*. PCR analysis of DNA from adenovirus transfected 293 cells cytopathic effect (cpe) and RT-PCR analysis for RNA from transfected NIH3T3 cells showed specific bands at 304bp for Adv-CT1 and 438bp for Adv-eGFP (FIG. 3A, 3B). CT-1 antibody specifically stains nearly all of the Adv-CT1 transfected 3T3 cells (FIG. 3C) but none of the unmodified primary NIH3T3 cells (FIG. 3D). Adv-eGFP transfected NIH3T3 cells were shown green fluorescence under fluorescent microscope (FIG. 3E).

Between 1 and 8 weeks after injection of Adv-CT1 into the injured spinal cord, motor neurons around the injection sites clearly expressed the transgene as shown by RT-PCR and immunocytochemistry (FIG. 4). The expression level of Adv-CT1 reached maximum between 1 and 2 weeks after injection and had declined but was still significant at 6 weeks (data not shown).

The Effects of CT-1 Gene Delivery on RN Survival

Counting the number of retrogradely FG labeled neurons in the rubral nucleus (RN) is a quantitative measure of the persistence of survived neurons after SCI when the dye was injected at the rostral stump. Labeled cells were seen in the bilateral RN and there were 2822

FIGURE 4 Expression of CT1 transgene in the spinal cord after Adv-CT1 vector. **A**: RT-PCR analysis of CT1 mRNA extracted from spinal cord 1 week after Adv-CT1 vector treatment; M: molecular markers; 1: RT-PCR product from Adv-CT1 vector transfected spinal cord; 2: RT-PCR from Adv-eGFP vector treated cord. **C**, **D**: Lower and higher magnification micrographs of the spinal cord stained for huCT1 with a polyclonal antibody to CT1 after treatment of Adv-CT1 vector for one week, specific staining was found mainly in neurons; **B**: CT1 immunostaining in the spinal cord treated with control Adv-eGFP vector for 1 week.

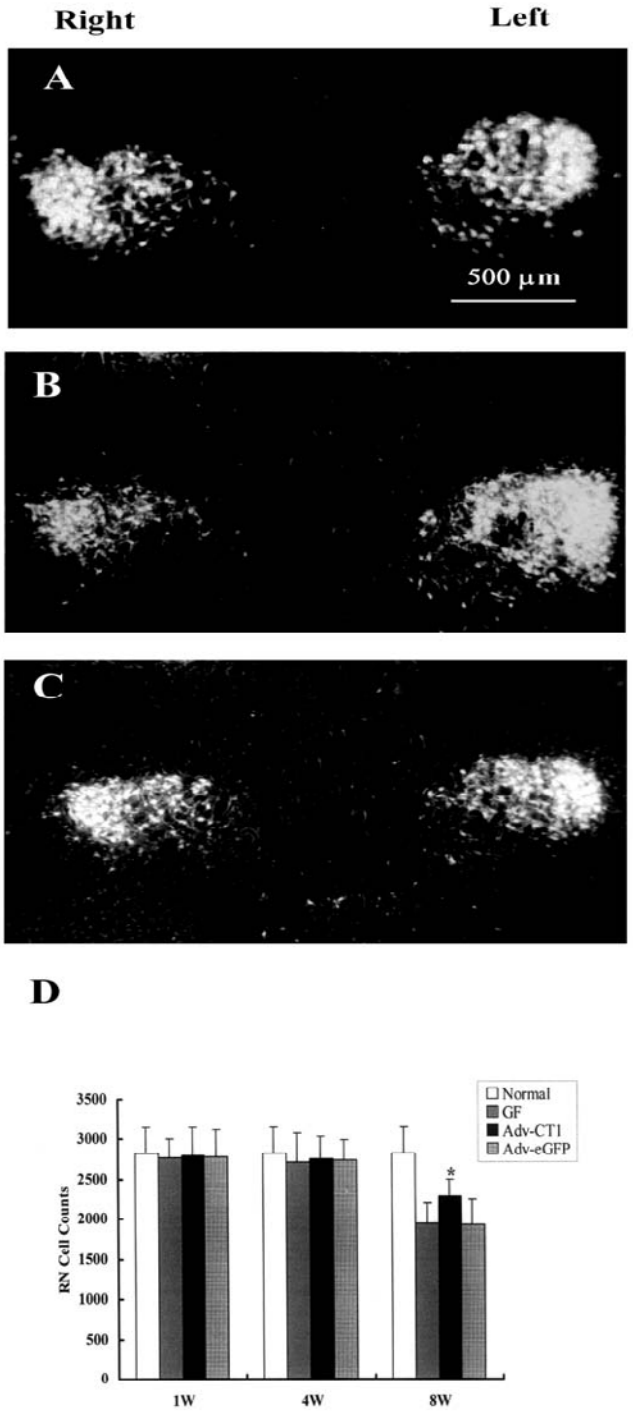


FIGURE 5 FluoroGold Retrograde labeling of total neurons in the red nuclei after FluoroGold was injected in the rostral stumps of injured cord. **A**: A micrograph of Fluorogold labeled neurons in red nuclei from a normal control rat; **B**: a micrograph of Fluorogold labeled neurons in red nuclei from a rat treated with gel form; **C**: A micrograph of Fluorogold labeled neurons in red nuclei from a rat treated with Adv-CT1 vector for 4 weeks; **D**: Histograms show total number of neurons in the red nuclei from different groups. GF: gel foam group; **p* <0.05 compared with gel-foam group or Adv-eGFP group. 1w, 4w and 8w indicate 1, 4 or 8 weeks after injury and treatment. Left: intact; right: injured.

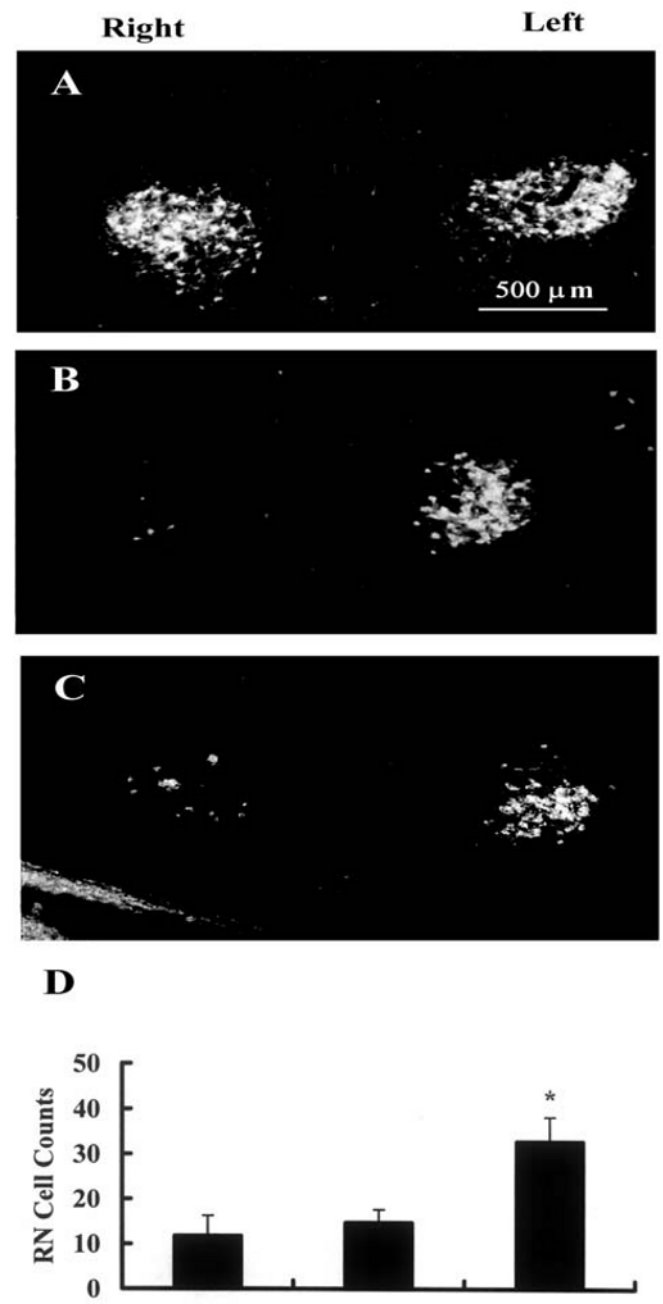


FIGURE 6 Fluoro-Gold retrogradely labeled regenerating neurons after fluorogold was injected into caudal stumps of injured cord. **A**: A micrograph of Fluorogold labeled neurons in red nuclei from a normal control rat; **B**: a micrograph of Fluorogold labeled neurons in red nuclei from a rat treated with control Adv-eGFP vector for 8 weeks; **C**: A micrograph of Fluorogold labeled neurons in red nuclei from a rat treated with Adv-CT1 vector for 8 weeks. **D**: Total numbers of retrogradely labeled regenerating neurons in red nuclei of rats from different groups. **p* <0.05 compared with the gel foam group (GF) or Adv-eGFP group.

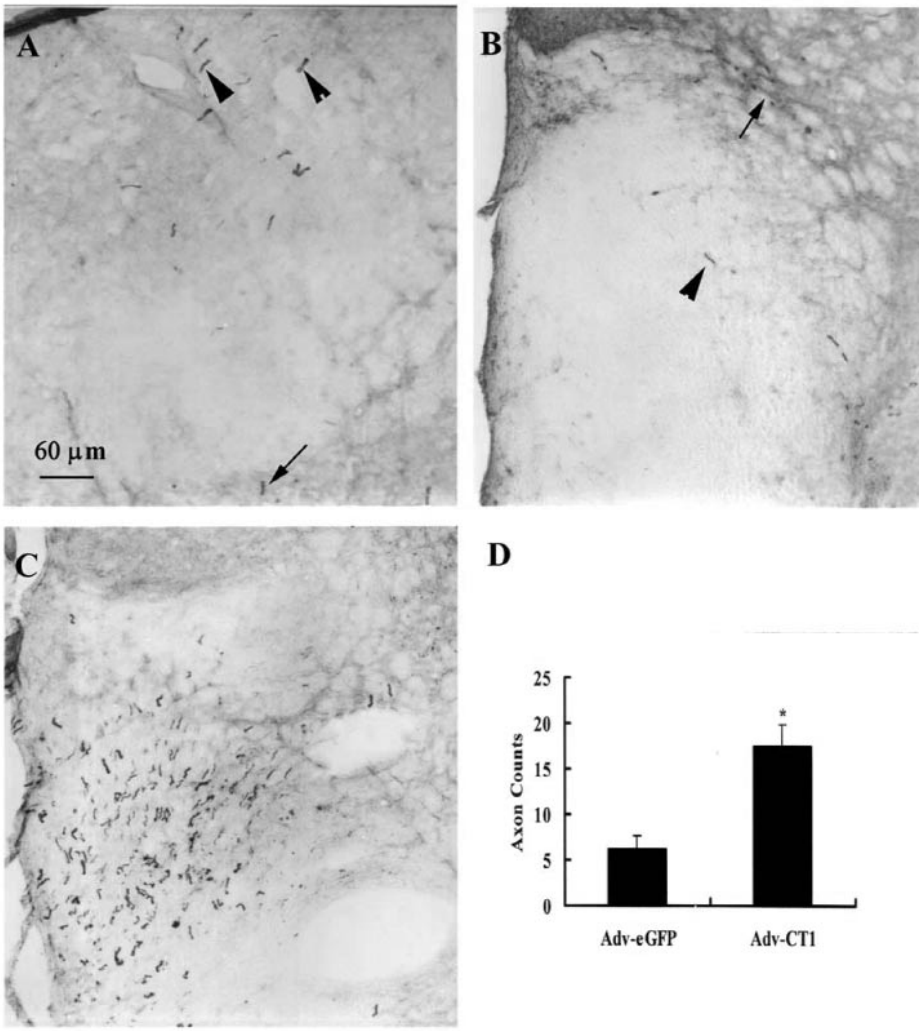


FIGURE 7 BDA anterogradely labeled regenerating axons after injection of BDA into red nuclei. **A**: a micrograph shows BDA labeled axons caudal to the injury site from a rat treated with Adv-CT1 vector. Arrow heads indicate DBA-labeled axons; scale bar also applies to B and C. **B**: a micrograph shows BDA labeled axons caudal to the injury site from a rat treated with Adv-eGFP vector; **C**: DBA labeled axons from a section of rostral stumps of the cord. **D**: Total number of regenerating axons counted from sections 2 segments caudal to the lesion site. *p <0.05 compared with Adv-eGFP treated group.

± 326 cells in normal rats. One , 4 and 8 weeks after SCI, the numbers of labeled cells in the gelfoam group were 2764 ± 238, 2714 ± 368 and 1954 ± 246, respectively; the numbers in the Adv-eGFP group were 2786 ± 334, 2743 ± 242, 1928 ± 286, respectively; and the numbers in the Adv-CT1 group were 2795 ± 353, 2751 ± 285 and 2287 ± 213, respectively (FIG. 5A, B). There was no significant change in the number of labeled cells in all groups at the time points of 1 and 4 weeks. However, the numbers of labeled RN cells were significantly reduced in all groups 8 weeks after SCI. The numbers reached 69%, 68% and 81% of the normal controls in the gelfoam group, Adv-eGFP group, and Adv-CT1 group, respectively. Compared with the gelfoam and Adv-eGFP groups, the number of FG labeled cells in the Adv-CT1 group was significantly increased (p <0.05) (FIG. 5C).

The Effects of CT-1 Gene Delivery on Regeneration of Rubrospinal Neurons

To examine the regeneration of rubrospinal neurons, the FG dye was injected bilaterally at two segments caudal to the lesion site 4 weeks after the lesion. In animals with a lesion on the left-side and treated only with gelfoam or Adv-eGFP , only few labeled cells were present in the right RN, whereas the number of labeled neurons in the left RN was similar to that of normal animals. The statistical data showed that in the normal animals, up to 3000 neurons were labeled in each RN (FIG. 6A). In animals receiving only gelfoam or Adv-eGFP, less than 10 cells (0.4%) were labeled in the contralateral RN (FIG. 6B). In contrast, up to 30 cells (1.2%) were retrogradely labeled in the contralateral RN in the Adv-CT1 group (FIG. 6C). The number of FG labeled cells in the contralateral RN in the Adv-

Table II Percentage of time used for forelimb contact to the cylinder wall during a full rear($\bar{x} \pm s$, $n = 4$)

Group	2W			3W			4W		
	L	R	B	L	R	B	L	R	B
Normal	27.4±3.5	17.7±7.4	55.3±6.3	-	-	-	-	-	-
GF	0	94.8±1.9	5.2±1.2	1.3±0.5	86.3±4.3	12.4±2.7	3.6±1.4	80.8±6.9	17.0±4.5
Adv-eGFP	0	93.3±4.7	6.7±2.6	1.5±0.3	84.4±5.1	14.1±3.2	4.1±2.6	77.6±9.3	18.4±6.2
Adv-CT1	0	86.5±6.5	13.5±4.7	4.4±1.3*	76.2±8.4	19.4±5.7	9.3±3.3*	66.0±8.7	24.7±5.7

L=left, R=right, B=both, W=week(s), GF: gel foam; *: $P < 0.05$ vs. GF and Adv-eGFP groups.

CT1 group was significantly increased, comparing with those in the control groups (FIG. 6D, $p < 0.05$).

The effect of adenovirus CT-1 vector on the regeneration of axotomized RN neurons was also determined by the anterograde tracing technique with BDA. Figure 7C shows the BDA-labeled RST axons occupy a wedge-shaped area in the superficial dorsolateral white matter with a few scattering to the dorsal horn and the ventral horn. Cross sections were used to visualize the presence of BDA-labeled fibers in the lesion region (C3-C4), caudal segments (C4-C5) and rostral segments (C2-C3) at 4 weeks after the lesion. Numerous BDA-labeled RST axons were seen in C2-C3 in normal rats, Adv-CT1 group and control groups. But the spinal cord lesion completely disrupted the anterograde labeling of RST in the lesion region in all groups, indicating our lesion procedure did not spare any rubrospinal axons. Only a few fibers were present in the gray matter (data not shown). In the normal animals, up to 150 axons were labeled in each section (FIG. 7C). At the caudal segment, 17 ± 2 regenerating axons were present in ipsilateral lateral funiculus and gray matter in Adv-CT1 group (FIG. 7A), whereas only 6 ± 1 axons scattered in the gray matter mainly in the Adv-eGFP group (FIG. 7B). There is a statistically significant difference between these two groups (Fig. 7D, $p < 0.05$).

Behavioral Analysis

When placed in a cylinder, normal rats spontaneously reared and explored the wall of the cylinder using a single forepaw alone (nearly 50%) or both forepaws together (nearly 50%). The hemisection at the upper cervical level produced asymmetry in the usage of forelimbs and the rats with hemisected spinal cord rarely used the forelimb ipsilateral to the injury. By 2 weeks after surgery, animals in all groups did not use the ipsilateral forelimb independently. The rats in the control groups used both forelimbs simultaneously in 5% of the total, as compared with 10% in the Adv-CT1 group. By 3 weeks, approximately 5% of the total number was occupied by the forelimb alone and 20% by both forelimbs in the Adv-CT1 group, whereas the animals in the gelfoam and Adv-eGFP groups used ipsilateral forelimb independently by only 1% and both forelimbs by 12% of the total. By 4 weeks after surgery, the animals in the adv-CT1 group used the ipsilateral forelimb alone in 10% of the total number, as compared with only 4% in the control groups. This result clearly demonstrates the partial functional recovery of injured limb usage in the adv-CT1 group. There is statistically significant difference in the usage of forelimbs among these groups ($p < 0.05$) (Table II).

DISCUSSION

In recent years, there has been dramatic progress in research on spinal cord injury. Injured spinal cord in adult mammals can regenerate in certain conditions. Introduction of permissive factors into the injured cord is one of most effective ways to treat the spinal cord. To improve delivery efficiency of these permissive factors, the gene therapy approach has been used by many researchers. The main advantage of the gene therapy approach is that it only requires the application once to reach therapeutic efficacy. In the present study, we chose to use adenoviral vectors for the following reasons. This vector has high efficiency in the delivery of genes. The delivery efficiency *in vitro* by this vector can reach as high as 100% and is also very high *in vivo*. Our current study showed that almost all adenoviral treated cells express the transgenes of CT1 and eGFP *in vitro*. We also found that CT1 expression maintained for several weeks with high levels. Secondly, the expression of transgene is fast and lasts only for several weeks to several months and the time frame is suitable for the repairing of the injured spinal cord and protection of injured neurons. Thirdly, recombinant replication-deficient adenoviral vector is relatively safe and widely used by researchers and in clinical trials. In the present study, we did not find significant side effects from rats treated with the viral vector compared with

the gelfoam treated rats. Thus, it can be a potential gene delivery vector for clinical trials.

Spinal cord injury causes disruption of descending pathways from the brainstem and cortex and initiates a neuronal response which can lead to atrophy or death of the injured cells (Tetzlaff *et al.*, 1994; Theriault and Tator, 1994). Neurons in RN in neonatal animals appear to be very sensitive to SCI, as over 55% of the neurons die after a mid-thoracic SCI (Bregman and Reier, 1986). After cervical axotomy in adult animals, the number of RN neurons remains stable for four weeks, and then declines significantly. Approximately 25-40% of the neurons die within 4 to 8 weeks (Mori *et al.*, 1997; Houle and Ye, 1999). However, there are many atrophied neurons which are mistaken as dead neurons (Tetzlaff *et al.*, 1994; Houle and Ye, 1999; Kwon *et al.*, 2002). Our data are consistent with these reports. The number of FG labeled RN neurons appeared stable for four weeks, but declined by approximately 30% when examined eight weeks after SCI.

Neurotrophic factor may be partially effective in preventing RN cell loss. For example, neurotrophins such as BDNF/NT4 and NT3, binding respectively to TrkB and TrkC receptors which are expressed by RN cells, prevented some loss of rubrospinal neurons when applied to the spinal cord lesion site after SCI (Xu *et al.*, 1995; Kobayashi *et al.*, 1997). In addition, CNTF prolonged the survival of rubrospinal neurons when applied to the refreshed spinal cord lesion site 4 and 8 weeks after injury (Houle and Ye, 1999). Application of BDNF to the RN cell body reversed the massive atrophy of neurons and promoted the regeneration of rubrospinal axons 1 year after SCI (Kwon *et al.*, 2002). After gene transfer of an adenoviral vector encoding CT-1 into the injured spinal cord, we found that high amounts of mRNA and protein of CT1 were expressed in the injured spinal cord within 2 months of the gene delivery. Like CNTF and GDNF, the AdCT-1 treatment protected 89% of RN neurons from cell death or atrophy and significantly increased the survival of these neurons, compared with the Adv-eGFP treatment. Our data indicate that CT-1 is a neurotrophic factor for RN neurons and that adenoviral vector is effective in the delivery of therapeutic genes after SCI.

Injured neurons in the central nervous system in adult mammals cannot spontaneously regenerate due to the suppression of myelin associated inhibitory factors. However, these neurons have an intrinsic potential for their axonal regrowth after injury, provided that they are supplied with a suitable environment (e.g., fetal neural tissue, peripheral nerve graft, Schwann cell graft, neurotrophic factors). Many investigations have demonstrated that injured neurons in the RN can respond to specific trophic factors or growth factors, such as BDNF, CNTF or FGF2, leading to an enhanced axonal regrowth (Xu *et al.*, 1995; Kobayashi *et al.*, 1997; Liu *et al.*, 1999; Kwon *et al.*, 2002). In our experiment, the regenerating axons did not spread widely in the gray matter away from the injection sites and were only present in the ipsilateral lateral funiculus and a few in the gray matter caudally in the Adv-CT1 group, but none in the Adv-eGFP group.

We have used behavioral tests to examine the functional recovery of forelimbs after cervical SCI. The behavioral results are consistent with those of histological data that Adv-CT1 is effective in the treatment of rubrospinal tract injury. Animals receiving Adv-CT1 injection showed significant improvement in the usage of the forelimb ipsilateral to the lesion. In contrast, those receiving gelfoam alone relied almost entirely on the contralateral limb and failed to recover their independent use of the impaired forelimb. By 1 week rats did not independently use the forelimb ipsilateral to the lesion in this task, but by 3-4 weeks they used the limb alone for 5-10% of the time and for 50% of the time together with the contralateral limb. The progression of the functional recovery may be due to the regeneration of RST axons promoted by CT1 expressed in the spinal cord. However, since only about 1% of neurons in RN could regenerate in response to the treatment, this dramatic functional recovery cannot be fully explained by the regeneration of descending pathways. The treatment with Adv-CT1 may also promote sprouting and regeneration of other pathways, such as the ascending sensory pathway, and enhance the plasticity of local neuronal circuits. Whether CT1 also promotes the survival and regeneration of other descending and ascending neuronal pathways remains to be determined.

In summary, we have constructed an adenoviral expression vector for the CT1 gene which is highly expressed *in vitro* and *in vivo* in mammalian cells. It has been demonstrated that adenoviral CT-1 gene transfer promoted the survival and regeneration of rubrospinal neurons and enhanced the partial functional recovery of forelimb usage after cervical spinal cord injury in adult rats. Our study suggests that CT1 may be used as a drug target for the treatment of the acute spinal cord injury with the adenoviral vector as the gene delivery vehicle.

Acknowledgements

This study was supported by Chongqing Commission of Science grant (1999-003) and NHMRC of Australia

(160052). The plasmid pBSSK-huCT1 was a generous gift of Dr. Dinna Pennica at Genentech Incorporated.

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