RESEARCH ARTICLE

Postischemic administration of Sendai virus vector carrying neurotrophic factor genes prevents delayed neuronal death in gerbils

M Shirakura^{1,2}, M Inoue¹, S Fujikawa¹, K Washizawa¹, S Komaba¹, M Maeda³, K Watabe⁴,
Y Yoshikawa² and M Hasegawa¹

¹DNAVEC Research Inc., Tsukuba, Japan; ²Department of Biomedical Science, Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo, Japan; ³First Department of Anatomy, Osaka City University Medical School, Osaka, Japan; and

⁴Department of Molecular Neuropathology, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan

Sendai virus (SeV) vector-mediated gene delivery of glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) prevented the delayed neuronal death induced by transient global ischemia in gerbils, even when the vector was administered several hours after ischemia. Intraventricular administration of SeV vector directed high-level expression of the vector-encoded neurotrophic factor genes, which are potent candidates for the treatment of neurodegenerative diseases. After occlusion of the bilateral carotid arteries of gerbils, SeV vector carrying GDNF (SeV/GDNF), NGF (SeV/NGF), brain-derived neurotrophic factor (SeV/BDNF), insulin-like growth factor-1 (SeV/IGF-1) or vascular endothelial growth factor (SeV/VEGF) was injected into the

lateral ventricle. Administration of SeV/GDNF, SeV/NGF or SeV/BDNF 30 min after the ischemic insult effectively prevented the delayed neuronal death of the hippocampal CA1 pyramidal neurons. Furthermore, the administration of SeV/GDNF or SeV/NGF as late as 4 or 6 h after the ischemic insult also prevented the death of these neurons. These results indicate that SeV vector-mediated gene transfer of neurotrophic factors has high therapeutic potency for preventing the delayed neuronal death induced by transient global ischemia, and provides an approach for gene therapy of stroke.

Gene Therapy (2004) 11, 784–790. doi:10.1038/sj.gt.3302224
Published online 12 February 2004

Keywords: Sendai virus; cerebral ischemia; delayed neuronal death; GDNF; NGF

Neurons are postmitotic and highly differentiated, and are extremely vulnerable to ischemic injury. Pyramidal cells of the hippocampal CA1 region are well known to be especially vulnerable to cerebral ischemia.^{1,2} Neuronal cell death in the CA1 region itself is not death-dealing but results in severe deficits of memory function.^{3–5} Since the regeneration of neuronal cells remains critically difficult at present, protection against the neuronal loss induced by ischemic injury is vital in cerebrovascular-type dementia.

Glial cell line-derived neurotrophic factor (GDNF) is a potent neurotrophic factor that promotes the cell survival and differentiation of dopaminergic neurons^{6,7} and motoneurons.^{8,9} Nerve growth factor (NGF) also has a potent ability to protect neurons from various injuries and promote the survival of cholinergic neurons.^{10–12} These neurotrophic factors may be valuable as candidates for use in therapy of neurodegenerative diseases. It has been reported that the neuronal cell death induced by ischemic injury was prevented by the administration of GDNF^{13–15} and NGF^{16–18} proteins. However, the usefulness of such protein factors in patients is limited because of their poor bioavailability and short half-lives.

Moreover, these agents might be ineffective without direct injection and continuous infusion into the ventricle, striatum or cerebral cortex. Therefore, virus vector-mediated gene transfer is expected to be an effective approach for the delivery of therapeutic proteins into the central nervous system (CNS). Even in the case of unsustained, but transient, expression by the vectors, it would enable significant cutting down of the number of required administrations. Previous studies demonstrated that gene transfer of neurotrophic factors such as GDNF¹⁹ and NGF^{20,21} rescued neuronal cells from ischemic injury in animal models. However, there have not been any reports in which neurotrophic factors expressed using conventional vectors such as adenovirus, retrovirus or adeno-associated virus were shown to promote the survival of neurons when the vectors were administered after ischemia.

We have developed a new type of gene transfer vector using Sendai virus (SeV), which is classified as a type I parainfluenza virus belonging to the family *Paramyxoviridae* with a negative-strand RNA genome.^{22,23} SeV has a strictly cytoplasmic life cycle in mammalian cells, that is, its genomic RNA is restricted to the cytoplasm and has no interaction with the host chromosomes.²² Therefore, SeV vector causes no genotoxicity such as the permanent integration in the target cells sometimes observed with other conventional viral

Correspondence: M Inoue, 1-25-11 Kamondai, Tsukuba-shi, Ibaraki 305-0856, Japan

Received 16 May 2003; accepted 29 November 2003; published online 12 February 2004

vectors. SeV has the ability to infect most mammalian cells such as neuronal and muscular cells and directs high-level gene expression in these cells.^{23–26} Indeed, we observed potent infectivity of SeV vector in ependymal cells after intraventricular administration in the CNS.²⁷ When the SeV vector carrying enhanced green fluorescent protein gene (SeV/GFP) was administered into the left lateral ventricle of gerbils, intense GFP expression was observed around the ependymal layer of the lateral ventricles (Figure 1c) and around the hippocampus (Figure 1b). Immunohistochemical analysis using anti-SeV antibody clearly showed that the cells supporting SeV replication were ependymal cells in the lateral ventricles (Figure 1d), third ventricle (Figure 1e) and around the hippocampus (Figure 1f). We previously showed that SeV vector-mediated gene transfer of GDNF 4 days before transient ischemia prevented the delayed neuronal death induced by transient global ischemia in gerbils.²⁷ However, SeV vector was administered prior to the ischemic insult in that case, too, whereas gene therapy must be applied after the occurrence of a stroke for clinical application. In the present study, we examined the effects of postischemic administration of SeV vectors carrying GDNF, NGF and other neurotrophic factor genes on the delayed neuronal death induced by transient global ischemia. Our results suggest that SeV vector-mediated gene transfer has a therapeutic high potential for cerebral ischemia.

In order to confirm the efficient gene transfer and expression of SeV in the CNS, the proteins derived from the genes harbored in the SeV vector were quantified. SeV vectors such as SeV/GDNF, SeV/NGF and SeV/GFP were administered into the left lateral ventricle of gerbils at 5×10^6 PFU/head, and the amount of GDNF and NGF proteins in the hippocampus was quantified by ELISA assays. High-level expression of GDNF (114 ± 6 pg/mg tissue) and NGF (1130 ± 60 pg/mg tissue) proteins was detected in the hippocampus of gerbils as early as 1 day after injection of SeV/GDNF and SeV/NGF, respectively (Figure 2a, b). In contrast, only a very small amount of GDNF or NGF protein was detected when the gerbils were treated with SeV/GFP. The expression of GDNF (2340 ± 200 pg/mg tissue) and NGF (3360 ± 290 pg/mg tissue) proteins reached peak levels 4 days after injection of SeV/GDNF and SeV/NGF, respectively, and then returned to the original level 14 days after the injection. In another experiment, an increment of GDNF expression in the cerebrospinal fluid was detected 8 h after injection of SeV/GDNF (data not shown). These results indicate that rapid and high-level expression of neurotrophic factors can be achieved by the administration of SeV vectors in the CNS. Also, the expression level achieved using SeV vectors was remarkably high compared with that achieved using adenovirus. For example, the GDNF concentration was reported to be 2.2 ± 0.5 pg/mg tissue 1 day after the

Figure 1 Identification of cell types supporting SeV replication. SeV vector carrying GFP gene (SeV/GFP; 5×10^6 PFU/head) was injected into the left lateral ventricle of gerbils as described previously,²⁷ and the GFP expression 4 days after the injection was observed under a stereoscopic fluorescence microscope (Leica, Germany) from the surface of the top of brain (a) and with coronal sections around the hippocampus (b) and lateral ventricle (c). For the coronal sections, the brain was sliced into 300- μ m-thick slices with a microslicer (DTK-1000; Dosaka, Japan). Representative photographs of immunohistochemical staining for SeV are shown (d–f). The paraffin sections were pretreated with 0.3% H_2O_2 in PBS, followed by washing thrice. After blocking with 10% normal goat serum (NGS) in PBS for 1 h, the sections were incubated overnight at 4°C with a rabbit polyclonal antibody to SeV (anti-SeV)²⁸ in 3% NGS and 0.3% Triton X-100 in PBS. The sections were then washed and incubated for 1 h with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA), followed by incubation for 1 h with the reagents for avidin–biotin complex formation (Vector Laboratories). Immunopositive cells were visualized by reaction with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (WAKO Pure Chemicals, Tokyo, Japan) and counterstained with hematoxylin. Scale bars = 100 μ m. The ependymal cells along the (d) lateral ventricle, (e) third ventricle and (f) hippocampus were SeV positive.

injection into the cortex of adenovirus (1×10^8 PFU/head) carrying the GDNF gene.²⁹

We next examined the effects of the postischemic administration of SeV vectors on the delayed neuronal death of the hippocampal CA1 pyramidal cells induced by transient ischemia. It has been reported that the direct administration of the bcl-2 gene mediated by adeno-associated virus (AAV) into pyramidal neurons within 1 h after ischemic insult prevents the delayed neuronal death in gerbils.³⁰ However, direct injection of virus vectors into the cerebral parenchyma cells, especially in

Figure 2 Kinetics of the expression of GDNF and NGF proteins in the hippocampus. Gerbils were injected with SeV vectors carrying GDNF (SeV/GDNF), NGF (SeV/NGF) or GFP (SeV/GFP) genes (5×10^6 PFU/head, $n=20$ animals per group) into the left lateral ventricle as described.²⁷ At 1, 2, 4, 7 or 14 days after the injection, the concentrations of GDNF and NGF in the hippocampus were measured using ELISA kits (Promega, WI, USA) as previously described.²⁷ The hippocampus was harvested from four gerbils at each time point. SeV/GDNF and SeV/GFP were constructed as previously described.²⁷ SeV/NGF was constructed as described.^{27,28} Briefly, mouse NGF (accession number: M14805) cDNA was amplified with a pair of NotI-tagged (underlined) primers containing SeV-specific transcriptional regulatory signal sequences, 5'-ACTTG CGGCCGCGCCAAAGTTCAGTAATGTCCATGTTGTTCTACACTCG-3' and 5'-ATCCGCGCGCCGCGATGAACCTTACCCCTAAGTTTTCTTA CTACGGTCAGCCTCTCTTGTAGCCTTCCTGC-3'. The amplified fragment was introduced into the NotI site of the parental pSeV18+ b(+), +), which was constructed to produce the exact SeV full-length antigenomic RNA, to generate pSeV/NGF. pSeV/NGF was transfected into LLC-MK₂ cells after infection of the cells with vaccinia virus vTF7-3, which expresses T7 polymerase. The T7-driven full-length recombinant SeV/NGF RNA genomes were encapsulated by NP, P and L proteins, which were derived from the respective cotransfected plasmids. After incubation for 40 h, cell lysates of transfected cells were injected into embryonated chicken eggs to amplify the recovered viruses. The virus titers were determined using a hemagglutination units (HAU) assay. Values are expressed as the mean $\pm$ s.d.

the hippocampus, is more invasive than intraventricular administration. Therefore, we selected a single intraventricular administration and utilized SeV-transduced ependymal cells to produce proteins from the genes carried by the vectors.²⁷ Accordingly, SeV/GDNF and SeV/NGF (5×10^6 PFU/head) were injected into the lateral ventricles of ischemic gerbils after 30 min of occlusion of the bilateral carotid arteries, and histopathological analysis was conducted 6 days after the injection. The effects of the above vectors were compared with those of SeV vectors carrying brain-derived neurotrophic factor (SeV/BDNF), insulin-like growth factor-1 (SeV/IGF-1) and vascular endothelial growth factor (SeV/VEGF). All the genes carried by the vectors have been reported to prevent neuronal degeneration after transient ischemia,^{31–33} and for each SeV vector, vector-derived expression in infected cells was confirmed *in vitro* (data not shown). In sham-operated gerbils, surviving wheel-like nuclei were observed in the pyramidal cells in CA1 (Figure 3a). However, in gerbils treated with SeV/GFP, almost all of the pyramidal cells in the hippocampal CA1 region showed pyknotic degenerative nuclei in the pyramidal cells (Figure 3g). In contrast, treatment of gerbils with SeV/GDNF or SeV/NGF ameliorated the delayed neuronal death in the hippocampal CA1 pyramidal cells (Figure 3b, c). Treatment with SeV/BDNF also showed ameliorative effects (Figure 3d), but treatment with SeV/VEGF did not (Figure 3f). Treatment with SeV/IGF-1 showed ameliorative effects (Figure 3e) in only two gerbils among eight tested. For quantitative analysis, the number of surviving neurons/1-mm length in the hippocampal CA1 region was counted (Figure 4). Treatment with SeV/GDNF (180.8 ± 11.7 cells/mm) or SeV/NGF (142.4 ± 24.3 cells/mm) significantly prevented neuronal death as compared to treatment with SeV/GFP (10.7 ± 1.9 cells/mm) ($P < 0.01$). Treatment with SeV/BDNF (139.3 ± 29.7 cells/mm) also reduced the cell death of the neurons by about 70%. SeV/GDNF and SeV/NGF (and SeV/BDNF) proved to be better for the treatment of transient global ischemia than SeV vectors carrying genes for the other factors investigated here. As a way to confirm that the vector-derived growth factors actually increased and acted to prevent the neuronal death of the hippocampal CA1 pyramidal neurons, we measured the concentrations of both NGF and GDNF proteins in the hippocampus of 'ischemic' gerbils 4 days after the injection of SeV/GDNF or SeV/NGF into the lateral ventricle. When the SeV/GDNF was injected at 4 h after ischemia, the concentration of GDNF (71.9 ± 12.0 pg/mg tissue) was increased compared to that of SeV/GFP-injected (0.057 ± 0.022 g/mg tissue) or untreated (0.038 ± 0.042 pg/mg tissue) gerbils. However, the concentration of NGF (15.8 ± 0.9 pg/mg tissue) remained at the original level of SeV/GFP-injected (15.4 ± 4.6 pg/mg tissue) or untreated (18.2 ± 6.2 pg/mg tissue) gerbils. When the SeV/NGF was injected at 4 h after ischemia, the concentration of NGF (1570 ± 210 pg/mg tissue) but not that of GDNF (0.074 ± 0.047 pg/mg tissue) increased, and this NGF could show a neuroprotective effect. These results indicate that the vector-derived growth factors rather than the intrinsic ones increase and function to prevent the neuronal death.

To examine the effect of extending the time until the administration of SeV vectors after ischemic insult, which is important for practical use in clinical applications,

SeV/GDNF and SeV/NGF were injected 4 h after ischemia. Surviving neurons in the hippocampal CA1 region were observed in gerbils treated with either SeV/GDNF or SeV/NGF, although a few degenerated nuclei of neurons were observed in these cases (Figure 5b, c). The number of surviving neurons in the hippocampal CA1 region was also counted (Figure 6). Administration of SeV/GDNF (114.7 ± 9.7 cells/mm) or SeV/NGF (127.6 ± 13.8 cells/mm) prevented the neuronal death as compared to administration of SeV/GFP (14.3 ± 5.6 cells/mm) ($P < 0.01$), even if the vectors were administered 4 h after ischemia. Moreover, treatment with SeV/GDNF (74.2 ± 9.6 cells/mm) or SeV/NGF (76.4 ± 10.8 cells/mm) 6 h after ischemic insult was also effective for prevention of the delayed neuronal death as compared to treatment with SeV/GFP (11.7 ± 2.3 cells/mm) ($P < 0.01$) (Figure 5e, f and Figure 6). Equal neuroprotective effects were observed on the ipsilateral and contralateral sides of the hippocampal CA1 region (data not shown), indicating that the proteins expressed by SeV vectors were extensively dispersed. These results indicate that administration of SeV/GDNF or SeV/NGF even several hours after ischemia effectively prevents the delayed neuronal death induced by transient global ischemia. There have been no previous reports showing that the administration of virus vector-mediated genes at 4 h or even 6 h after an ischemic insult was effective for preventing the delayed neuronal death induced by transient ischemia in animal models. Our results indicate that the time window for the treatment of cerebral ischemia could be substantially extended. It was reported that more than 8 h was required for the expression of genes carried by adenovirus vectors in the rat brain.³⁴ Thus, the time needed for expression of genes carried by SeV vector

may be shorter than that needed for genes carried by adenovirus vector. The rapid gene expression achieved using SeV vectors may be due to the rapid replication, high level of mRNA transcription and effective translation of the mRNA of the vectors, that is, the typical features of RNA viruses. This may have led to the protective effect observed even with 6 h postischemic administration of SeV vectors against the delayed

Figure 4 Quantitative analysis of the effect of SeV vector on ischemic injury when administered 30 min after ischemia. SeV/GDNF, SeV/NGF, SeV/BDNF, SeV/IGF-1, SeV/VEGF or SeV/GFP was injected intravenicularly 30 min after ischemic insult. The number of surviving neurons/1-mm length in the hippocampal CA1 region was calculated. Values are expressed as the mean $\pm$ s.d. ($n = 8$ animals per group). Asterisks indicate a significant difference as compared with the SeV/GFP-treated group. ($P < 0.01$, Student's *t* test).

Figure 3 Representative photomicrographs of pyramidal neurons in the hippocampal CA1 region after treatment (30 min after ischemia) with SeV vector following ischemic injury. (a) Sham-operated gerbils; (b) SeV/GDNF-, (c) SeV/NGF-, (d) SeV/BDNF-, (e) SeV/IGF-1-, (f) SeV/VEGF- and (g) SeV/GFP-treated gerbils 30 min after ischemic insult. These sections were stained with hematoxylin and eosin. Arrows indicate the pyknotic nuclei of neurons. Scale bar = 50 μ m. SeV vectors carrying the BDNF (SeV/BDNF), IGF-1 (SeV/IGF-1) and VEGF (SeV/VEGF) genes were constructed as described in Figure 1. In this experiment, human BDNF (accession number: XM_006027), human IGF-1 (accession number: X56773) and human VEGF (accession number: AY047581) cDNAs were amplified with a pair of NotI-tagged (underlined) primers containing SeV-specific transcriptional regulatory signal sequences, 5'-ACTTGCGGCCGCCAAAGTTCAGTGATGACCATCTTTCTTAC-3' and 5'-ATCCGCGGCCGCGATGAACCTTACCCTAAGTTTTCTTACTACCGGTATCTTCCCTTTTAATGGTCAATGTAC-3' for BDNF, 5'-ACTTGCGGCCGCCAAAGTTCAGCAATGGGAAAAATCAGCAGTCTTC-3' and 5'-ATCCGCGGCCGCGATGAACCTTACCCTAAGTTTTCTTACTACCGGTATCTTCTGTAGTTCTTGTTCCTGC-3' for IGF-1 and 5'-ACTTGCGGCCGCCAAAGTTCAGTGAACCTTACCCTAAGTTTTCTTACTACCGGTATCTTCTGTAGTTCTTGTTCCTGC-3' and 5'-ATCCGCGGCCGCGATGAACCTTACCCTAAGTTTTCTTACTACCGGTATCTTCTGTAGTTCTTGTTCCTGC-3' for VEGF. Adult male Mongolian gerbils (60–80 g) were used in this experiment. Occlusion of the bilateral common carotid arteries was performed as previously described.^{19,27} Briefly, gerbils were anesthetized by an intraperitoneal injection of chloral hydrate (300 mg/kg)¹⁹ and both common carotid arteries were clamped for 5 min with surgical clips to produce transient forebrain ischemia. The body temperature was measured using a thermocouple probe inserted into the anus and maintained at 37.5°C using a heating pad. Sham-operated animals were treated in the same manner except for occlusion of the bilateral common carotid arteries. At 30 min after the ischemic insults, SeV/GDNF, SeV/NGF, SeV/BDNF, SeV/IGF-1, SeV/VEGF or SeV/GFP (5×10^6 PFU/head, $n = 8$ animals per group) was injected intravenicularly into gerbils. At 6 days after injection, the gerbils were anesthetized with ether and transcardially perfused with normal saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The brains were removed and fixed in the same fixative overnight and then processed into paraffin blocks. Coronal brain sections were cut at 5- μ m thickness and stained with hematoxylin and eosin. Arrows indicate the pyknotic nuclei of neurons. Scale bar = 50 μ m.

Figure 5 Representative photographs of pyramidal neurons in the hippocampal CA1 regions after treatment (4 and 6 h after ischemia) with SeV vector following ischemic injury. SeV/GDNF (b, e), SeV/NGF (c, f) or SeV/GFP (d, g) was administered intraventricularly 4 h (b, c, d) or 6 h (e, f, g) after ischemic insult. The sections were stained with hematoxylin and eosin. Scale bar = 50 μ m. Occlusion of the bilateral common carotid arteries was performed as described in Figure 2.

neuronal death of the hippocampal CA1 pyramidal neurons induced by transient ischemia. Moreover, these attractive features of SeV vectors may make them useful for the treatment of acute diseases such as cerebral ischemia.

It has been reported that NGF administration prevented the delayed neuronal death when neurons were observed 7 days after ischemic insult, but not when they were observed 28 days after the insult.¹⁸ The long-term effects of the vectors studied here therefore had to be examined to better clarify their potential benefits in clinical use. Therefore, we also examined the long-term effects of SeV/GDNF and SeV/NGF on delayed neuronal death. The effect of SeV vectors for preventing neuronal death could be observed even when analyzed 28 days after the insult, although it was not seen in the case of topical application of NGF protein.¹⁸ However, the number of surviving neurons at 28 days after the ischemia was reduced to almost half of that at 6 days after ischemia in the cases of gerbils treated with SeV/GDNF and SeV/NGF (Figure 7). High-level expression of the vector-encoded protein was detected after the administration of the SeV vector, but this expression reached a peak 4 days after the administration and then decreased to the basal level by 14 days. It is probable that immune responses induced by the virus particles or genes derived from SeV may cause the rapid decline of the gene expression of SeV. However, this limitation may be circumvented by the development of a new generation of SeV vectors that elicit weaker immune responses, which should extend the duration of gene expression. We have developed a series of an attenuated type of SeV vectors that are F gene-deleted,²⁴ F gene-deleted with preferable mutations,³⁵ M gene-deleted,³⁶ or have combinations of deletions of these genes (Kitazato K, unpublished; Inoue M, unpublished). We plan the first clinical application of SeV vector carrying human fibroblast growth factor-2 for the treatment of peripheral arterial disease using F gene-deleted SeV vector. As the F gene-deleted SeV is nontransmissible and shows less cytopathic effect than the wild-type SeV, we should utilize this type of SeV (or further advanced types of SeV vector) for applications to brain ischemia. Improvement of the vector modifications will ultimately provide better protection against ischemic injury. Moreover, alternative administration routes, such as lumbar puncture of the vectors, should be developed for the purposes of human

Figure 6 Quantitative analysis of the effect of SeV vector on ischemic injury when administered 4 and 6 h after ischemia. SeV/GDNF, SeV/NGF or SeV/GFP was injected intraventricularly 4 or 6 h after ischemic insult. The number of surviving neurons/1-mm length in the hippocampal CA1 region was calculated. Values are expressed as the mean $\pm$ s.d. ($n=8$ animals per group). Asterisks indicate a significant difference as compared with the SeV/GFP-treated group. ($P<0.01$, Student's t test).

gene therapy for cerebrovascular diseases. Hayashi and co-workers³⁷ reported that liposome-mediated hepatocyte growth factor (HGF) gene transfer into the subarachnoid space prevented delayed neuronal death in gerbils. Intrathecal injection into the cisterna magna involves no systematic anesthesia, no burr hole and no pain for patients. For use in clinical application, these methods should be tested in future studies. More importantly, we have already confirmed the efficient replication of SeV vectors in primates. In fact, high-level expression of GDNF (more than 100 ng/ml) was observed in cerebrospinal fluid after the injection of SeV vector carrying the GDNF gene (SeV/GDNF) into the lateral ventricle of primates (data not shown). Thus, we are going to evaluate the SeV/GDNF vector for its effects on the recovery from brain ischemia using primates. In such experiments, we hope to show a correlation between CA1 neuroprotection and functional recovery.

In conclusion, the present study demonstrated that postischemic administration of SeV vectors carrying genes for GDNF and NGF effectively prevented the delayed neuronal death of the hippocampal CA1

Figure 7 Long-term effect of the SeV vectors on ischemic injury. SeV/GDNF, SeV/NGF or SeV/GFP was injected intraventricularly 30 min after ischemic insult. At 6 days or 28 days after injection, gerbils were anesthetized, and the number of surviving neurons/1-mm length in the hippocampal CA1 region was calculated. Values are expressed as the mean $\pm$ s.d. ($n=8$ animals per group). Asterisks indicate a significant difference as compared with the SeV/GFP-treated group. ($P<0.01$, Student's *t* test).

pyramidal cells induced by occlusion of the bilateral carotid arteries. It is noteworthy that the neuroprotective effect was obtained even 4 and 6 h after ischemic insults. These results indicate that gene therapy using SeV vectors is of great potential usefulness for the treatment of cerebral ischemia.

Acknowledgements

We thank T Yamamoto for technical assistance, and A Iida, M Okayama and M Fukumura for helpful discussions.

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