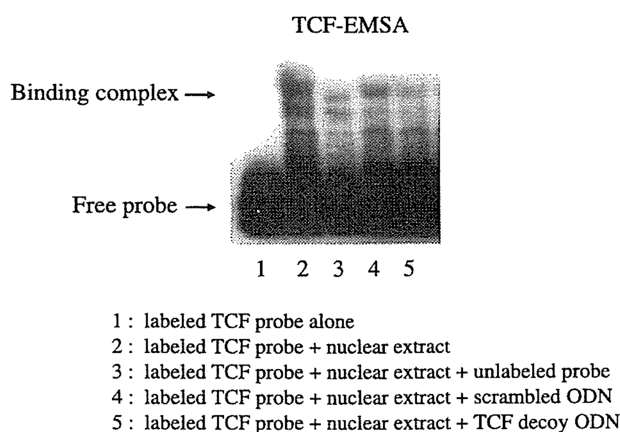


Figure 6. Electrophoretic mobility shift assay. The following reaction mixtures were run on 6% native polyacrylamide gels. Nuclear extracts containing active β-catenin/TCF complex were prepared from HCT116. *Lane 1,* labeled TCF probe (1.5 pmol) alone. *Lane 2,* labeled TCF probe + nuclear extract (5 μg). *Lane 3,* labeled TCF probe + nuclear extract + unlabeled TCF probe (200 pmol). *Lane 4,* labeled TCF probe + nuclear extract + scrambled oligodeoxynucleotide (7 pmol), *Lane 5,* labeled TCF probe + nuclear extract + TCF decoy oligodeoxynucleotide (7 pmol). The TCF decoy decreased the binding of TCF probe and nuclear extract when compared with the scrambled oligodeoxynucleotide. A repeat experiment using 3.5 pmol scrambled oligodeoxynucleotide or TCF decoy oligodeoxynucleotide showed similar results (data not shown).

A TCF decoy strategy is based on competitive binding of β-catenin/TCF complex to the endogenous target sequence in the presence of the exogenously introduced decoy, as schematized in Fig. 2B. If the β-catenin/TCF complex is in large excess, the TCF decoy might be insufficient to block the oncogenic Wnt signaling. Indeed, HCT116 cancer cells showed a strong β-catenin accumulation in the nucleus and cytoplasm and displayed ~16-fold higher endogenous TCF activity than nontumor HEK293 cells. The present study showed that the TCF decoy inhibited TCF activity even in the tumor cells. Such inhibitory effect of TCF activity was also confirmed in DLD1, another colon cancer cell line that displays ~50-fold higher TCF activity than HEK293 cells (data not shown). FITC labeling of decoy revealed that the oligodeoxynucleotide was incorporated in the nucleus in nearly all cells at 6 hours and remained in the cells for up to 72 hours in HCT116. However, we should emphasize that the decoy underwent degradation in a time-dependent manner. Therefore, continuous administration or certain molecular modification to protect nucleolysis should be essential in the next stage.

Nevertheless, the decoy strategy is not only an experimental tool; it has potential as a nontoxic therapeutic agent (39). The E2F decoy has already been used in human patients receiving bypass vein grafts, and no major adverse effects were reported (18). Another advantage is that oligodeoxynucleotide decoys should have little or no immunogenicity, and thus could be repeatedly administered to patients. This is in contrast to gene therapy with adenovirus vectors, in which repeated administration elicits an antivirus antibody response that may lessen its

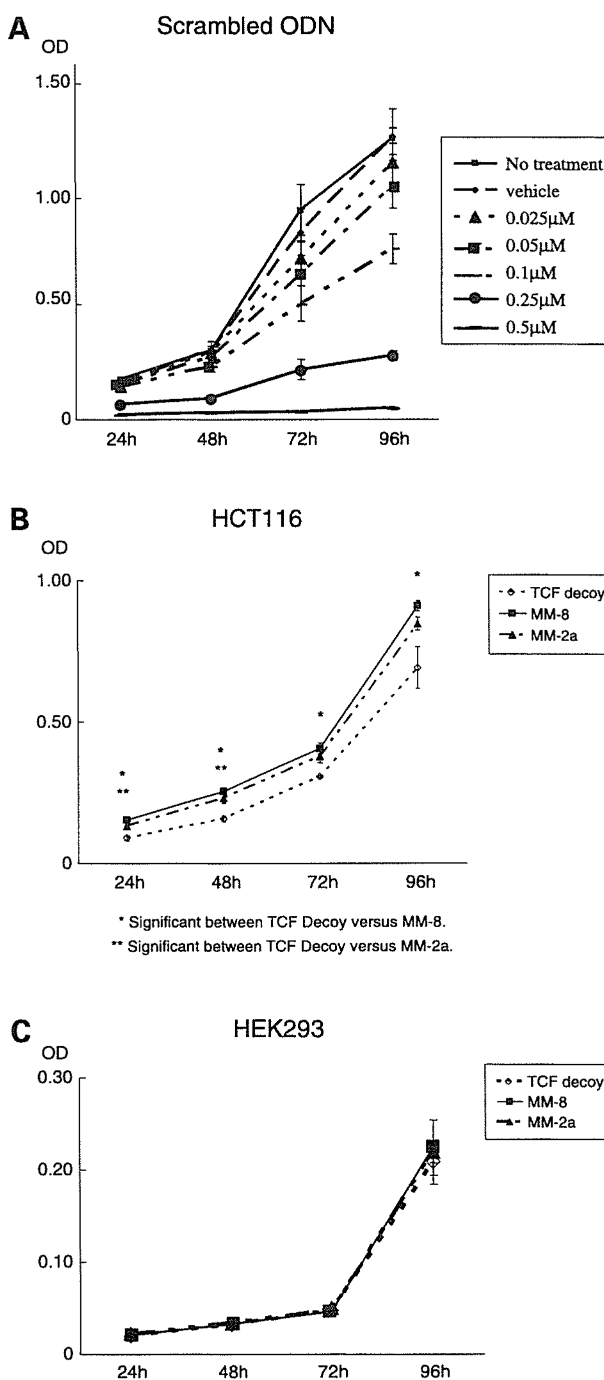


Figure 7. Growth assays. Twelve hours after transfection, cells were harvested and then seeded at 4,000 cells per 100 μL medium into 96-well plates. Cell growth was determined using WST-1 assay kit (Dojindo Laboratories; ref. 27) at indicated time point after transfection. **A,** dose-dependent cytotoxicity of scrambled oligodeoxynucleotide. Compared with nontreatment cultures and vehicle (LipofectAMINE 2000) alone, vehicle plus oligodeoxynucleotide at various concentrations exhibited dose-dependent cytotoxicity. **B,** at 0.05 μmol/L, the TCF decoy significantly inhibited cell growth of HCT116. Differences are significant (*) between TCF decoy and MM-8, and (**) between TCF decoy and MM-2a. **C,** the TCF decoy did not inhibit growth of HEK293.

efficacy and cause side effects. From a clinical point of view, it is noteworthy that growth inhibition of tumor cells was observed by the TCF decoy at only 50 nmol/L, which is considered clinically feasible. In addition, we found a marked growth inhibitory effect by a TCF decoy in three-dimensional collagen cultures (data not shown) and further investigation in combination with chemotherapy is currently under way.

Another notable finding is that TCF decoy was less toxic to nontumor cells than to tumor cells. A similar result was reported for the signal transducers and activators of transcription-3 decoy, which worked selectively in signal transducers and activators of transcription-3–activated tumor cells but not in noncancerous oral cells (17). Thus, there is increasing evidence in the literature that certain transcription targeting strategies, such as response-element decoys, might be especially effective in tumor cells that intrinsically display hypertranscriptional activity, and may be relatively innocuous in nontumor cells. This concept was previously known as "cancer hypersensitivity" or "cancer gene addiction" (40, 41).

In conclusion, our data provide evidence that a TCF decoy not only reduced TCF activity but also efficiently inhibited activation of downstream target genes, with selectivity for tumor cells. Thus, a TCF decoy may be a useful molecular-targeting therapy for controlling the malignant potential of cancer with less toxicity.

Acknowledgments

We thank Dr. Bert Vogelstein for providing the β-catenin expression vector and the c-myc reporter, Dr. Thomas Brabletz for the MMP7 reporter, and Prof. J. Miyazaki for PGK-luc construct.

References

1. Akiyama T. Wnt/β-catenin signaling. Cytokine Growth Factor Rev 2000;11:273 – 82.

2. Morin PJ, Sparks AB, Korinek V, et al. Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. Science 1997; 275:1787 – 90.

3. Miyoshi Y, Nagase H, Ando H, et al. Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. Hum Mol Genet 1992;1:229 – 33.

4. Iwao K, Nakamori S, Kameyama M, et al. Activation of the β-catenin gene by interstitial deletions involving exon 3 in primary colorectal carcinomas without adenomatous polyposis coli mutations. Cancer Res 1998;58:1021 – 6.

5. He TC, Sparks AB, Rago C, et al. Identification of c-MYC as a target of the APC pathway. Science 1998;281:1509 – 12.

6. Shtutman M, Zhurinsky J, Simcha I, et al. The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. Proc Natl Acad Sci U S A 1999;96: 5522 – 7.

7. Brabletz T, Jung A, Dag S, Hlubek F, Kirchner T. β-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. Am J Pathol 1999;155:1033 – 8.

8. Takahashi M, Tsunoda T, Seiki M, Nakamura Y, Furukawa Y. Identification of membrane-type matrix metalloproteinase-1 as a target of the β-catenin/Tcf4 complex in human colorectal cancers. Oncogene 2002;21:5861 – 7.

9. Kim PJ, Plescia J, Clevers H, Fearon ER, Altieri DC. Survivin and molecular pathogenesis of colorectal cancer. Lancet 2003;362:205 – 9.

10. Yamada T, Takaoka AS, Naishiro Y, et al. Transactivation of the multidrug resistance 1 gene by T-cell factor 4/β-catenin complex in early colorectal carcinogenesis. Cancer Res 2000;60:4761 – 6.

11. Gupta RA, Tan J, Krause WF, et al. Prostacyclin-mediated activation of peroxisome proliferator-activated receptor δ in colorectal cancer. Proc Natl Acad Sci U S A 2000;97:13275 – 80.

12. Arber N, Doki Y, Han EK, et al. Antisense to cyclin D1 inhibits the growth and tumorigenicity of human colon cancer cells. Cancer Res 1997; 57:1569 – 74.

13. Yamamoto H, Itoh F, Adachi Y, et al. Relation of enhanced secretion of active matrix metalloproteinases with tumor spread in human hepatocellular carcinoma. Gastroenterology 1997;112:1290 – 6.

14. Adachi Y, Yamamoto H, Itoh F, Hinoda Y, Okada Y, Imai K. Contribution of matrilysin (MMP-7) to the metastatic pathway of human colorectal cancers. Gut 1999;45:252 – 8.

15. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 1997;3:917 – 21.

16. Rothenberg ML, Mickley LA, Cole DE, et al. Expression of the mdr-1/ P-170 gene in patients with acute lymphoblastic leukemia. Blood 1989; 74:1388 – 95.

17. Leong PL, Andrews GA, Johnson DE, et al. Targeted inhibition of Stat3 with a decoy oligonucleotide abrogates head and neck cancer cell growth. Proc Natl Acad Sci U S A 2003;100:4138 – 43.

18. Mann MJ, Whittemore AD, Donaldson MC, et al. *Ex vivo* gene therapy of human vascular bypass grafts with E2F decoy: the PREVENT single-centre, randomised, controlled trial. Lancet 1999;354:1493 – 8.

19. Morishita R, Sugimoto T, Aoki M, et al. *In vivo* transfection of *cis* element "decoy" against nuclear factor-κB binding site prevents myocardial infarction. Nat Med 1997;3:894 – 9.

20. Shiratsuchi T, Ishibashi H, Shirasuna K. Inhibition of epidermal growth factor-induced invasion by dexamethasone and AP-1 decoy in human squamous cell carcinoma cell lines. J Cell Physiol 2002;193:340 – 8.

21. Kawamura I, Morishita R, Tsujimoto S, et al. Intravenous injection of oligodeoxynucleotides to the NF-κB binding site inhibits hepatic metastasis of M5076 reticulosarcoma in mice. Gene Ther 2001;8:905 – 12.

22. Faria M, Wood CD, Perrouault L, et al. Targeted inhibition of transcription elongation in cells mediated by triplex-forming oligonucleotides. Proc Natl Acad Sci U S A 2000;97:3862 – 7.

23. Noura S, Yamamoto H, Ohnishi T, et al. Comparative detection of lymph node micrometastases of stage II colorectal cancer by reverse transcriptase polymerase chain reaction and immunohistochemistry. J Clin Oncol 2002;20:4232 – 41.

24. Takemasa I, Yamamoto H, Sekimoto M, et al. Overexpression of CDC25B phosphatase as a novel marker of poor prognosis of human colorectal carcinoma. Cancer Res 2000;60:3043 – 50.

25. Nagel S, Schmidt M, Thiede C, Huhn D, Neubauer A. Quantification of Bcr-Abl transcripts in chronic myelogenous leukemia (CML) using standardized, internally controlled, competitive differential PCR (CD-PCR). Nucleic Acids Res 1996;24:4102 – 3.

26. Yamamoto H, Kondo M, Nakamori S, et al. JTE-522, a cyclooxygenase-2 inhibitor, is an effective chemopreventive agent against rat experimental liver fibrosis. Gastroenterology 2003;125:556 – 71.

27. Yu J, Qiao L, Zimmermann L, et al. Troglitazone inhibits tumor growth in hepatocellular carcinoma *in vitro* and *in vivo*. Hepatology 2005;43: 134 – 43.

28. Darnell JE. Transcription factors as targets for cancer therapy. Nat Rev Cancer 2002;2:740 – 9.

29. Zhang G, Zhou X, Xue L, et al. Accumulation of cytoplasmic β-catenin correlates with reduced expression of E-cadherin, but not with phosphorylated Akt in esophageal squamous cell carcinoma: immunohistochemical study. Pathol Int 2005;55:310 – 7.

30. Zhou YN, Xu CP, Han B, et al. Expression of E-cadherin and β-catenin in gastric carcinoma and its correlation with the clinicopathological features and patient survival. World J Gastroenterol 2002;8:987 – 93.

31. Wong CM, Fan ST, Ng IO. β-Catenin mutation and overexpression in hepatocellular carcinoma: clinicopathologic and prognostic significance. Cancer 2001;92:136 – 45.

32. Nakamura T, Hamada F, Ishidate T, et al. Axin, an inhibitor of the Wnt signalling pathway, interacts with β-catenin, GSK-3β and APC and reduces the β-catenin level. Genes Cells 1998;3:395 – 403.

33. Kishida S, Yamamoto H, Ikeda S, et al. Axin, a negative regulator of the wnt signaling pathway, directly interacts with adenomatous polyposis coli and regulates the stabilization of β-catenin. J Biol Chem 1998;273: 10823 – 6.

34. Tago K, Nakamura T, Nishita M, et al. Inhibition of Wnt signaling by ICAT, a novel β-catenin-interacting protein. Genes Dev 2000;14:1741 – 9.

35. Morin PJ, Vogelstein B, Kinzler KW. Apoptosis and APC in colorectal tumorigenesis. Proc Natl Acad Sci U S A 1996;93:7950 – 4.

36. Shih IM, Yu J, He TC, Vogelstein B, Kinzler KW. The β-catenin binding domain of adenomatous polyposis coli is sufficient for tumor suppression. Cancer Res 2000;60:1671 – 6.

37. Verma UN, Surabhi RM, Schmaltieg A, Becerra C, Gaynor RB. Small interfering RNAs directed against β-catenin inhibit the *in vitro* and *in vivo* growth of colon cancer cells. Clin Cancer Res 2003;9:1291 – 300.

38. Satoh S, Daigo Y, Furukawa Y, et al. AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. Nat Genet 2000;24:245 – 50.

39. Tomita N, Azuma H, Kaneda Y, Ogihara T, Morishita R. Gene therapy with transcription factor decoy oligonucleotides as a potential treatment for cardiovascular diseases. Curr Drug Targets 2003;4:339 – 46.

40. Weinstein IB. Addiction to oncogenes—the Achilles heal of cancer. Science 2002;297:63 – 4.

41. Weinstein IB. Disorders in cell circuitry during multistage carcinogenesis: the role of homeostasis. Carcinogenesis 2000;21:857 – 64.

Gene Transfer of Hepatocyte Growth Factor Gene Improves Learning and Memory in the Chronic Stage of Cerebral Infarction

Munehisa Shimamura, Naoyuki Sato, Satoshi Waguri, Yasuo Uchiyama, Takuya Hayashi, Hidehiro Iida, Toshikazu Nakamura, Toshio Ogihara, Yasufumi Kaneda, Ryuichi Morishita

Abstract—There is no specific treatment to improve the functional recovery in the chronic stage of ischemic stroke. To provide the new therapeutic options, we examined the effect of overexpression of hepatocyte growth factor (HGF) in the chronic stage of cerebral infarction by transferring the HGF gene into the brain using hemagglutinating virus of Japan envelope vector. Sixty rats were exposed to permanent middle cerebral artery occlusion (day 1). Based on the sensorimotor deficits at day 7, the rats were divided equally into control vector or HGF-treated rats. At day 56, rats transfected with the HGF gene showed a significant recovery of learning and memory in Morris water maze tests (control vector 50±4 s; HGF 33±5 s; $P<0.05$) and passive avoidance task (control vector 132.4±37.5 s; HGF 214.8±26.5 s; $P<0.05$). Although the total volume of cerebral infarction was not related to the outcome, immunohistochemical analysis for Cdc42 and synaptophysin in the peri-infarct region revealed that HGF enhanced the neurite extension and increased synapses. Immunohistochemistry for glial fibriary acidic protein revealed that the formation of glial scar was also prevented by HGF gene treatment. Additionally, the number of the arteries was increased in the HGF group at day 56. These data demonstrated that HGF has a pivotal role for the functional recovery after cerebral infarction through neuritogenesis, improved microcirculation, and the prevention of gliosis. Our results also provide evidence for the feasibility of gene therapy in the chronic stage of cerebral infarction. (***Hypertension*. 2006;47:742-751.**)

Key Words: cerebral ischemia ■ genes ■ microcirculation ■ rats

Middle cerebral artery occlusion (MCAo) is one of the most common causes of focal stroke in humans[1] and causes severe sensorimotor deficits and cognitive dysfunction. The ischemic changes closely resemble those produced in a MCAo model in rats,[1] which causes infarction mainly in the dorsolateral and lateral portions of the neocortex and the entire caudoputamen.[2] Several growth factors are upregulated immediately after MCAo, such as fibroblast growth factor (FGF),[3] brain-derived neurotrophic factor,[4] glial cell line-derived neurotrophic factor,[5] vascular endothelial growth factor (VEGF),[6] and hepatocyte growth factor (HGF),[7] and thought to protect neurons or promote angiogenesis after MCAo. In fact, the extension of infarction is prevented by administration of growth factors or gene transfer of growth factors before or immediately after MCAo.[8–11] However, the therapeutic time window of such treatment is too short for clinical use,[12] because they focused on preventing the extension of neuronal death in the penumbra in the acute stage.

Recently, HGF and c-Met/HGF have been reported to be upregulated mainly in the peri-infarct region as long as 28 days after permanent MCAo[13] and up to 14 days in FGF[3] or VEGF.[14] HGF is a well-known potent pleiotropic cytokine that exhibits mitogenic, motogenic, and morphogenic activity in a variety of cells.[15–17] Both HGF and the c-Met/HGF receptor of membranes spanning tyrosine kinase are expressed in various regions of the brain.[17] HGF is also involved in the development and maintenance of cortical neurons during differentiation, motogenesis, neuritogenesis, and neuronal survival during the development of the rat cerebral cortex.[18] Interestingly, HGF promotes proliferation and neuronal differentiation of neural stem cells from mouse embryos.[19] In vivo, it has also been demonstrated that HGF promotes angiogenesis in cerebral ischemia in rodents[20–23] without disrupting the blood–brain barrier.[10]

From these viewpoints, we speculated that HGF might play a pivotal role in the functional recovery in the chronic stage of ischemic insult, and its overproduction could improve the cognitive dysfunction. To clarify this speculation, we transferred the human HGF gene into the brain 7 days after MCAo, using the hemagglutinating virus of Japan (HVJ)-

Received October 23, 2005; first decision November 14, 2005; revision accepted January 13, 2006.

From the Division of Clinical Gene Therapy (M.S., N.S., R.M.), Department of Cell Biology and Neuroscience (S.W., Y.U.), Division of Molecular Regenerative Medicine (T.N.), Department of Geriatric Medicine (T.O.), and Division of Gene Therapy Science (Y.K.), Graduate School of Medicine, Osaka University, Osaka; Department of Advanced Clinical Science and Therapeutics (M.S.), Graduate School of Medicine, Tokyo University, Tokyo; and Department of Investigative Radiology (T.H., H.I.), National Cardiovascular Center, Research Institute, Osaka, Japan.

Correspondence to Ryuichi Morishita, Division of Clinical Gene Therapy, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita 565-0871, Japan. E-mail morishit@cgt.med.osaka-u.ac.jp

© 2006 American Heart Association, Inc.

Hypertension **is available at http://www.hypertensionaha.org** **DOI: 10.1161/01.HYP.0000208598.57687.3e**

envelope vector,[10] and examined behavioral tests, MRI, and histological changes. Here, we demonstrated that gene therapy delayed for as long as 7 days improved outcome from ischemic stroke, and HGF is an important growth factor for the recovery of cognitive function in the chronic stage of MCAo through reconstitution of the neuronal network.

Methods

Preparation of HVJ-Envelope Vector

HVJ-envelope vector was prepared as described previously.[24,25] Briefly, virus suspension (15 000 hemagglutinating units) was inactivated by UV irradiation (99 mJ/cm^2) and mixed with plasmid DNA (400 μg) and 0.3% Triton-X. After centrifugation, it was washed with 1 mL of balanced salt solution (10 mmol/L Tris-Cl (pH 7.5), 137 mmol/L NaCl, and 5.4 mmol/L KCl) to remove the detergent and unincorporated DNA. After centrifugation, the envelope vector was suspended in 100 mL of PBS. The vector was stored at 4°C until use.

Construction of Plasmids

To produce an HGF expression vector, human HGF cDNA (2.2 kb) was inserted into a simple eukaryotic expression plasmid that uses the cytomegalovirus promoter/enhancer.[26] This promoter/enhancer has been used to express reporter genes in a variety of cell types and can be considered constitutive. The control vector had the same structure as the expression vector plasmid, including the promoter but not containing HGF cDNA. Plasmids were purified with a QIAGEN plasmid isolation kit (Qiagen).

Surgical Procedure

Male Wistar rats (270 to 300 g; Charles River Japan, Atsugi, Japan) were used in this study. To generate a permanent MCAo model, the right middle cerebral artery (MCA) was occluded by placement of poly-L-lysine-coated 4-0 nylon around the origin of the MCA, as described previously.[2] In vivo gene transfer was performed by intracisternal injection as described previously.[25] Briefly, rats were anesthetized with ketamine (Sankyo) and xylazine (Bayer Ltd). HVJ-envelope vector (100 μL) containing the human HGF gene was infused at 50 μL/min after removing 100 μL of cerebrospinal fluid (CSF). The protocol was approved by the Committee on the Ethics of Animal Experiments in the Osaka University. To examine transfection of the HGF gene in the CSF, CSF (100 μL) was collected 4, 7, 14, and 21 days after gene transfer. The concentration of HGF was determined by enzyme immunoassay using anti-human HGF antibody (Institute of Immunology, Tokyo, Japan) as described previously.[10]

Protocol for Treatment and Behavioral Tests

Ten rats were only anesthetized (sham operation), and 60 rats were subjected to MCAo (day 1). Based on the neuromuscular function and body weight evaluated on day 7, the rats were divided equally into control vector-treated (n=23) and HGF-treated (n=23) groups. Rats showing no palsy on day 7 or that died before day 7 were excluded from the present study (n=14). On day 55, neuromuscular function and locomotor activity were evaluated in the surviving rats (n=20 for control vector-treated and n=22 for HGF-treated rats). Then, cognitive function was examined by Morris water maze (MWM) and passive avoidance task from day 56 to 90. On day 96, MRI was performed to evaluate the volume of infarction.

Sensorimotor Deficit and Locomotor Activity

Although there are various batteries for testing sensorimotor deficit, we used a simple protocol[27] to evaluate sensorimotor deficit, which used the following categories (maximum score is 4). For forelimb flexion, rats were held by the tail on a flat surface. Paralysis of the forelimbs was evaluated by the degree of left forelimb flexion. For torso twisting, rats were held by the tail on a flat surface. The degree of body rotation was checked. For lateral push, rats were pushed either left or right. Rats with right MCA occlusion showed weak or no resistance against a left push. For hind limb placement, one hind limb was removed from the surface. Spontaneous locomotor activity was also measured via the open field test for 30 minutes using an automated activity box (Muromachi Kikai).

MWM Task

A cylindrical tank 1.5 m in diameter was filled with water (25°C), and a transparent platform 15 cm in diameter was placed at a fixed position in the center of 1 of the 4 quadrants (O'Hara & Co, Ltd). In the hidden platform test, the platform was set below the water level, and it was not seen by the rats. The platform was fixed at 1 quadrant, and the starting point was changed in each trial. A previous study showed a difference in the latency of reaching the platform until day 6 of the session between rats exposed to MCAo 12 to 14 weeks before and control rats, if the tests were performed twice a day.[28] Based on the results, we carried out the tests twice a day for 6 days. If the rat could not reach the platform, the latency was set at 60 s. In the visible platform test, a flag was placed on the platform, which could be seen by the rats. The tests were carried out twice a day for 6 days. In this trial, the platform and the starting point were changed in each trial. Throughout the tests, the path of swimming was captured by a charge-coupled device video camera and analyzed by National Institute of Health image.

Passive Avoidance Task

A step-through type of passive avoidance task was used in the present study. The apparatus (Medical Agent) consisted of an illuminated chamber and a dark one. To habituate the rats, they were placed in the illuminated chamber, and the door was opened so that they could enter the dark one. Rats have a habit of entering the dark chamber, because they prefer darkness. In an acquisition trial, the rats were placed in the illuminated chamber and exposed to a 6.0-mA foot shock when they entered the dark chamber. Each trial was continued until the rat learned not to enter the dark chamber for 300 seconds. In retention trials, they were placed in the illuminated room 3 days after the acquisition trial. We evaluated the latency (maximum: 300 s) of their staying in the illuminated room.

Immunohistochemical Study

For histopathologic analysis, other rats (control vector-treated [n=4] or HGF-treated [n=4] rats in each experiment) were treated the same as described above and euthanized on day 11, 14, or 56, followed by transcardial perfusion fixation with normal saline followed by 4% paraformaldehyde. The brain was removed, postfixed, cryoprotected, and cut on a cryostat at 12 μm. After blocking, sections were incubated in 3% normal goat serum and anti-MAP2 (1:1000; mouse monoclonal; Sigma-Aldrich, St Louis, MO), GFAP (1:1000; mouse monoclonal; Sigma-Aldrich), and Cdc42 (1:100; mouse monoclonal; Santa Cruz Biotechnology, Santa Cruz, CA) followed by anti-mouse goat fluorescent antibody (1:1000 for MAP2 and GFAP, 1:250 for Cdc42, Alexa Flour 546, Molecular Probes). For immunostaining of human HGF or synaptophysin, sections were treated with 2% H_2O_2 to block endogenous peroxidase and then incubated with an antibody against human HGF-β (H714; 1:250; rabbit polyclonal; Immuno-Biological Laboratories, Gunma, Japan) or synaptophysin (1:500; mouse monoclonal; Chemicon, Temecula, CA) at 4°C O/N. They were incubated with streptavidin-horseradish peroxidase (Vectastain Elite ABC; Vector Laboratories, Burlingame, CA), and the biotin-streptavidin-peroxidase complex was detected with diaminobenzidine (human HGF) or tetramethylbenzidine (synaptophysin) peroxidase substrate solution (Vector Laboratories). Negative control sections from each animal received identical preparations for immunohistochemical staining, except that primary antibodies were omitted.

Quantitative Histological Analysis

To quantify the immunoreactivity for GFAP and synaptophysin, the acquired image was imported into Adobe Photoshop (version 7.0, Adobe System). The color image was converted into a grayscale image. This was imported into Mac SCOPE (version 2.5, Mitani Corporation). The region of interest was set at the peri-infarct region

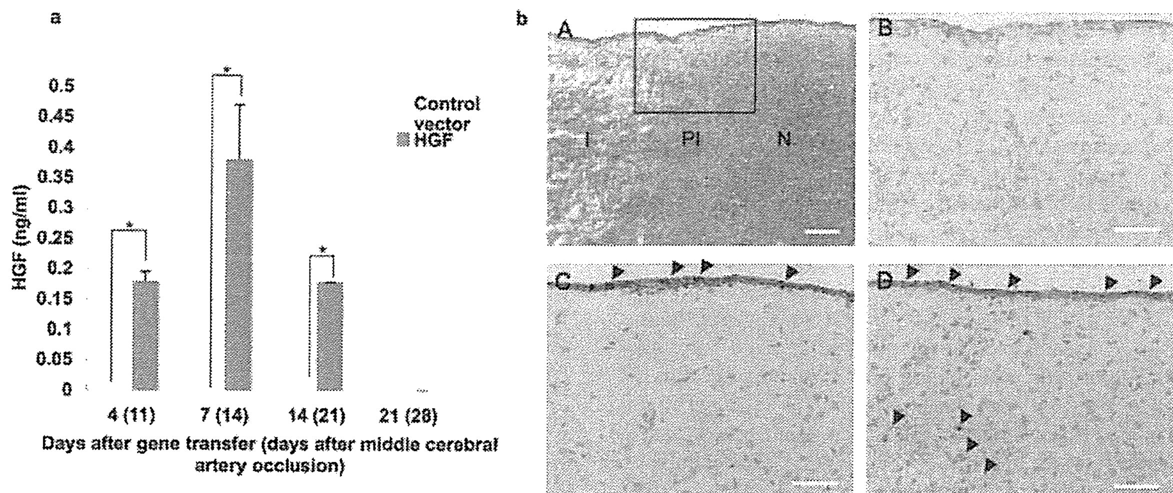


Figure 1. (a) Concentrations of human HGF in cerebrospinal fluid at 4, 7, 14, and 21 days after gene transfer (11, 14, 21, and 28 days after middle cerebral artery occlusion). Control vector indicates rats transfected with control vector (n=4); HGF, rats transfected with HGF vector (n=4). *$P<0.01$ vs Control. (b, part A) HE staining at 4 days after gene transfer (11 days after middle cerebral artery occlusion). I, infarct region; PI, peri-infarct region; N, normal region. Bar=100 μm. (B through D) Representative images of immunohistochemical staining for human HGF. (B) Peri-infarct region in rats transfected with control vector (rectangle area in A). Bar=50 μm. (C) Contralateral intact region in rats transfected with HGF vector. Bar=50 μm. (D) Peri-infarct region in rats transfected with HGF vector. Arrowhead showed immunopositive cells for human HGF. Bar=50 μm.

in the cerebral neocortex. The peri-infarct region is defined as the area surrounding the lesion, which morphologically differs from the surrounding normal tissue (Figure 1b, part A).[29,30] The number of pixels for which the signal was >25 was counted. Immunoreactivity was calculated by the equation: % Area=(Number of high signal pixels)/(Total number of pixels). To quantify the cerebral edema, we calculated the percentage of measured infarct area in the corrected infarct area at 0.7 mm from bregma. The corrected infarct area was calculated as [LT-(RT-RI)], where LT is the area of the left hemisphere, RT is the area of the right hemisphere, and RI is the infarct area.[10] The infarct region is edematous when the percentage is >100%. The infarct brain is atrophic if the percentage is <100%.

Alkaline Phosphatase Staining

For alkaline phosphatase (ALP) staining, sections were washed in Tris-HCl and incubated for 30 minutes in substrate solution (a mixture of naphtol AS-BI phosphate [σ-Aldrich] and fast red violet LB salt [σ-Aldrich]). Five consecutive sections in each rat were observed, and acquired images were imported into Adobe Photoshop. The color image was converted into a grayscale image. Then, the ROI was set as the region in the peri-infarct region. The area or length of vessels was analyzed with an Angiogenesis Image Analyzer (version 1.0, Kurabo).

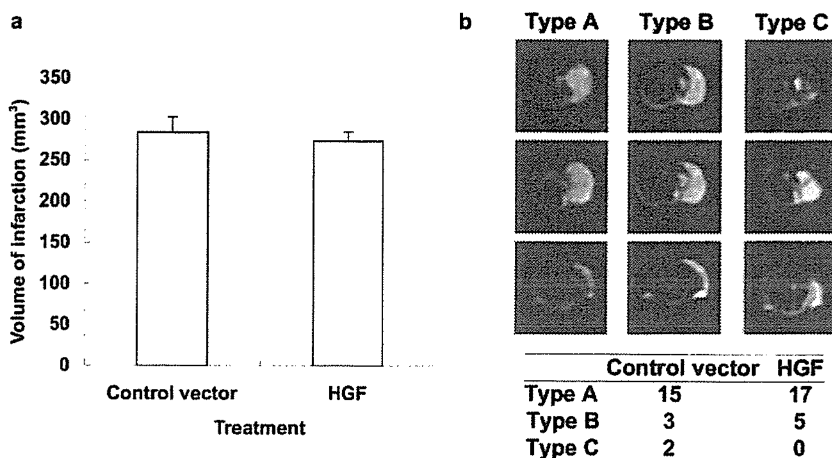
Statistical Analysis

All of the values are expressed as mean±SEM. ANOVA was used to determine the significance of differences in multiple comparisons. $P<0.05$ was considered significant.

Results

Transfer of HGF Gene Improves Learning and Memory After Cerebral Infarction

To test for successful gene transfer via the subarachnoid space, the concentration of human HGF in CSF was measured by ELISA at 4, 7, 14, and 21 days after gene transfer. As expected, human HGF could be detected in the CSF of rats transfected with human HGF vector 4 at 7 days after gene transfer, whereas human HGF protein could not be detected in control rats (Figure 1a). Human HGF protein was detected in the pia mater in the



	Control vector	HGF
Type A	15	17
Type B	3	5
Type C	2	0

Figure 2. Magnetic resonance images of brain. (a) Volume of infarction in all rats calculated in T2-weighted images. Control vector indicates rats transfected with control vector (n=20); HGF, rats transfected with HGF vector (n=22). (b) Typical T2-weighted image of coronal section of rat brain. The images were divided into 3 groups: types A, B, and C (described in text). Most rats showed type A, and fewer showed type B or type C.

normal region (Figure 1b, part C), as well as in the pia mater and parenchyma in the infarct and peri-infarct region 4 days after gene transfer using immunohistochemistry (Figure 1b, part D). Although HE staining at 4 days after gene transfer showed that the infarct brain is atrophic in this timing, as reported previously,[31] there was no significant difference between rats transfected with the human HGF gene and control vector (control vector 87.1±8.1%, HGF 81.0±4.3%; *P* value not significant).

To confirm the severity of cerebral infarction, all of the rats were examined by T2-weighted MRI on day 96. Although the total volume of infarction calculated in T2-weighted images was not different between rats transfected with the human HGF gene and control vector (Figure 2a), the pattern of cerebral infarction was divided into 3 groups: (1) type A, high-intensity area seen in the dorsolateral and lateral portions of neocortex and the entire caudoputamen; (2) type B, high-intensity area seen in the dorsolateral and lateral portions of neocortex and in part of the caudoputamen; and (3) type C, high-intensity area seen in part of the lateral neocortex and caudoputamen (Figure 2b). In type C, most part of lateral neocortex was intact.

In the hidden platform test of MWM, which examined spatial learning and memory, the latency in rats transfected with control vector was markedly longer as compared with sham-operated rats, and the latency in rats transfected with HGF vector was significantly shorter than that of rats transfected with control vector (Figure 3a). There were no differences both in swimming speed and visible platform test, which excluded the possible influences of visual loss, sensorimotor deficit, and motivation on the results,[32] between rats transfected with control and HGF vector (data not shown). Thus, spatial learning and memory partly, but significantly, recovered in rats transfected with HGF vector. In the passive avoidance task, which was used to measure associated learning and memory,[32] the retention of memory was longer in rats transfected with the HGF vector (Figure 3b), which demonstrated a trend toward significance ($P=0.053$). Sensorimotor deficit and locomotor activity were also tested, because they have some influence on tests of cognitive function.[32] Sensorimotor deficit had spontaneously recovered to some extent by day 55 in both groups, and there was no difference between the

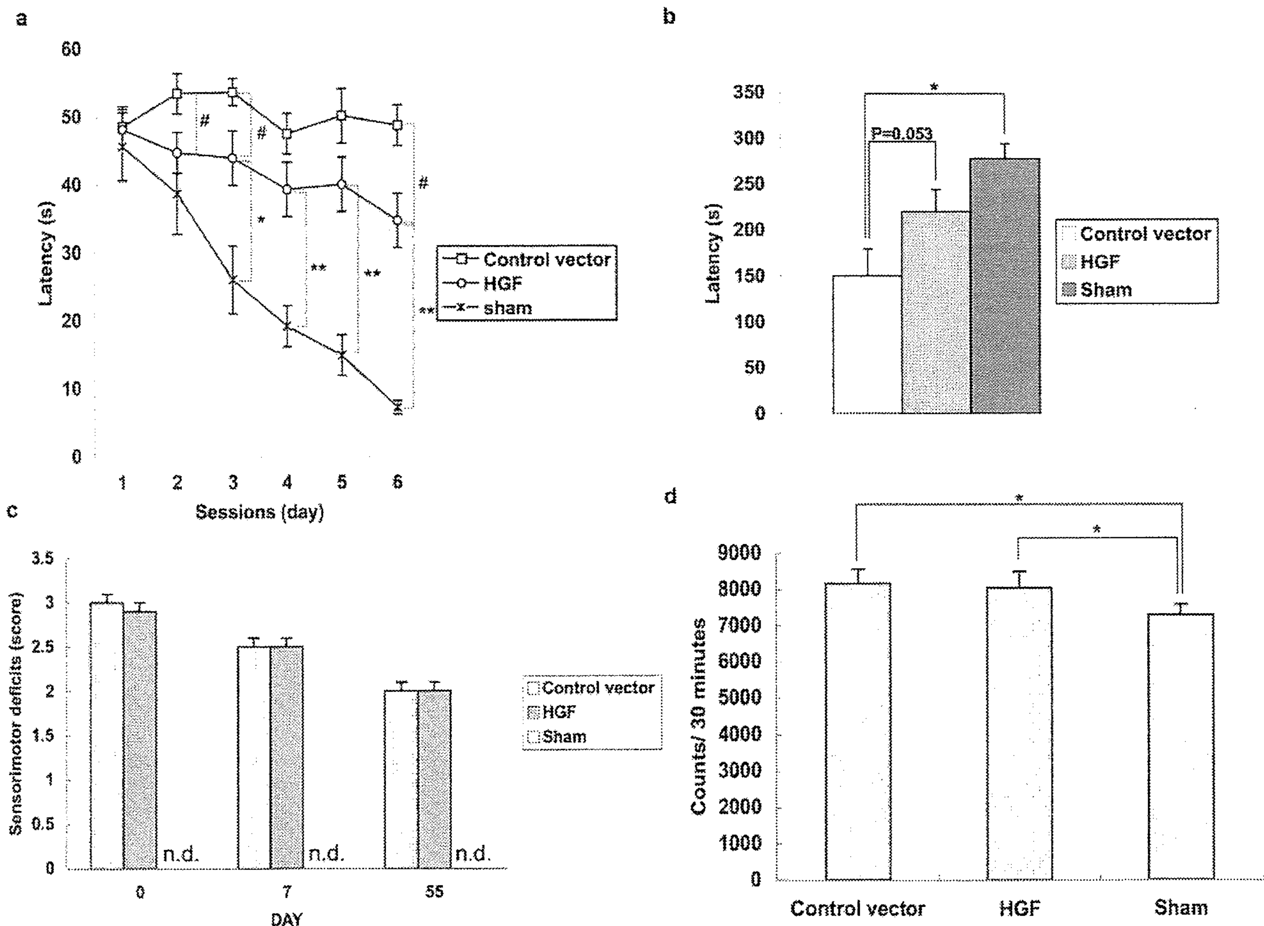


Figure 3. Learning and memory in the chronic stage of cerebral infarction. (a) Hidden platform test in MWM test in all rats. Although rats subjected to middle cerebral artery occlusion hardly reached the hidden platform as compared with sham-operated rats, rats transfected HGF vector could reach faster than that of control vector. (b) Retention trial in passive avoidance task in all rats. The latency of rats staying in the illuminated chamber was calculated. (c) Sensorimotor deficit and (d) spontaneous locomotor activity in all rats. There is no sensorimotor deficit in sham-operation rats in "c" (shown as "n.d."). Control vector indicates rats transfected with control vector (n=20); HGF, rats transfected with HGF vector (n=22); Sham, sham-operated rats (n=10); $^{*}P<0.05$, $^{**}P<0.01$ vs Sham, $^{\#}P<0.05$ vs Control.

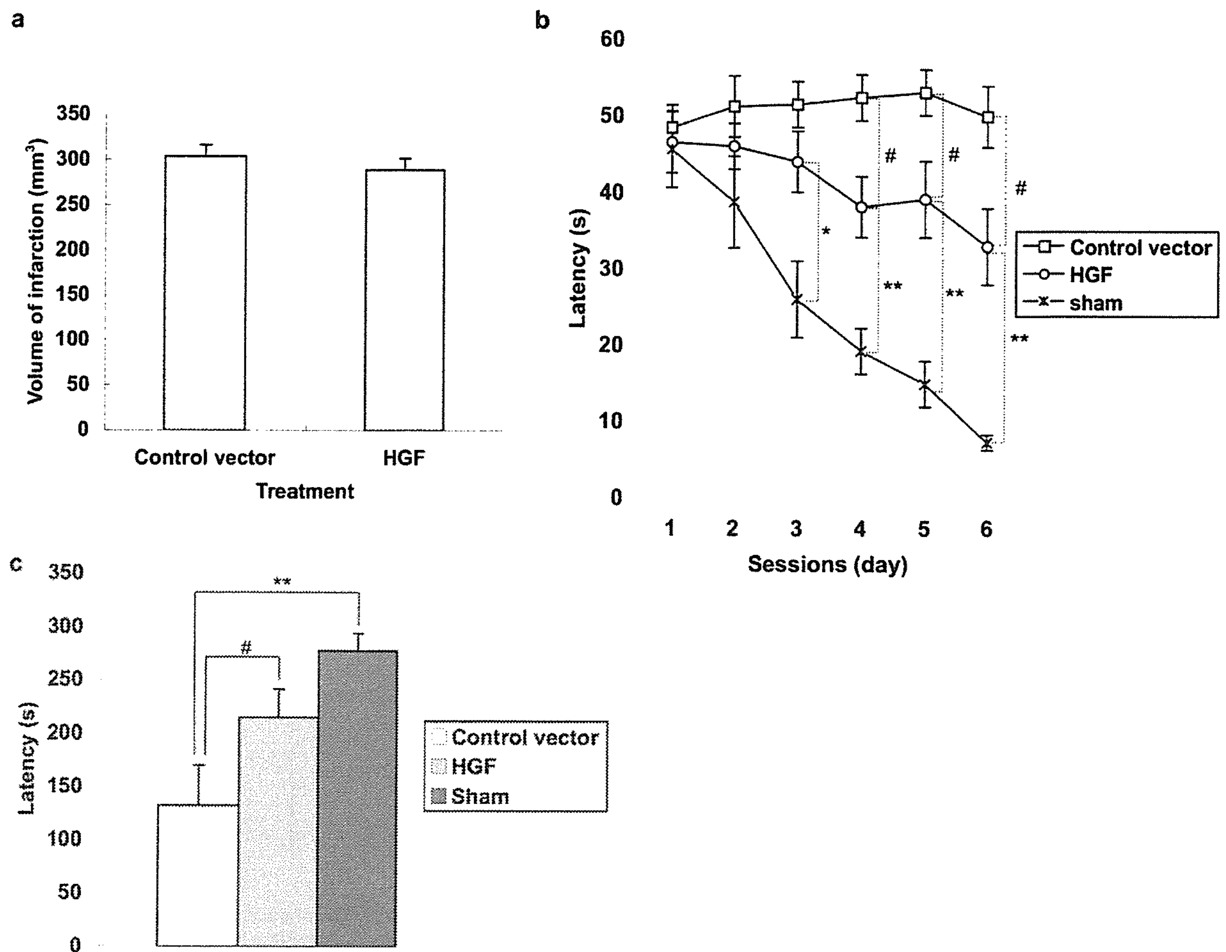


Figure 4. (a) Volume of infarction in type A rats. (b) Hidden platform test in MWM test in type A rats. (c) Retention trial in passive avoidance task in type A rats. Control vector indicates rats transfected with control vector (n=15); HGF, rats transfected with HGF vector (n=17); Sham, sham-operated rats (n=10). $^{*}P<0.05$, $^{**}P<0.01$ vs Sham, $^{\#}P<0.05$ vs Control.

2 groups (Figure 3c). Locomotor activity of rats subjected to MCAo was increased as compared with sham-operated rats, as described before,[33] but there was no difference in rats transfected with control and HGF vector (Figure 3d).

To exclude the influence of the pattern of cerebral infarction on the cognitive function, we additionally focused on type A rats. The volume of cerebral infarction in type A rats was not different between rats transfected with human HGF gene and control vector (Figure 4a). Even type A rats transfected with HGF vector showed the improvement in the learning and memory in MWM test (Figure 4b). Also, rats transfected HGF vector showed the significantly longer retention of memory in the passive avoidance task (Figure 4c). Type A rats showed no significant difference in sensorimotor deficit and locomotor activity (data not shown).

These data supported the results that in the MWM and passive avoidance task were not influenced by the sensorimotor and locomotor activity and the volume and pattern of cerebral infarction. Overall, these data suggest that rats transfected with HGF vector maintain their memory longer as compared with those transfected with control vector.

HGF Enhances Neuritogenesis and Synaptogenesis

To examine whether HGF induced neuritogenesis and/or synaptogenesis, we focused on Cdc42, which belongs to the Rho family of GTPases and has positive effects on neuronal process extension,[34] and synaptophysin, which is used as presynaptic markers and synaptogenesis.[35,36] According to previous reports that the neuronal process extension occurred until 14 days after focal cerebral ischemia[36] and synaptogenesis in the chronic stage of the insult,[36] we measured the immunopositive cells against Cdc42 at day 14 and synaptophysin at day 56. Although the number of Cdc42-positive neurons was the same in the contralateral neocortex in both groups, the peri-infarct region in the neocortex of rats transfected with the HGF vector showed a significant increase in the number of Cdc42-immunoreactive cells (Figure 5a and 5b). Also, the immunoreactivity of synaptophysin was significantly increased at day 56 in rats transfected with the HGF gene, especially in the peri-infarct region (Figure 5c and 5d).

HGF Prevents Glial Scar Formation

Then, we investigated whether HGF had influences on astrocytes, because the neuron–glia interaction is also impor-

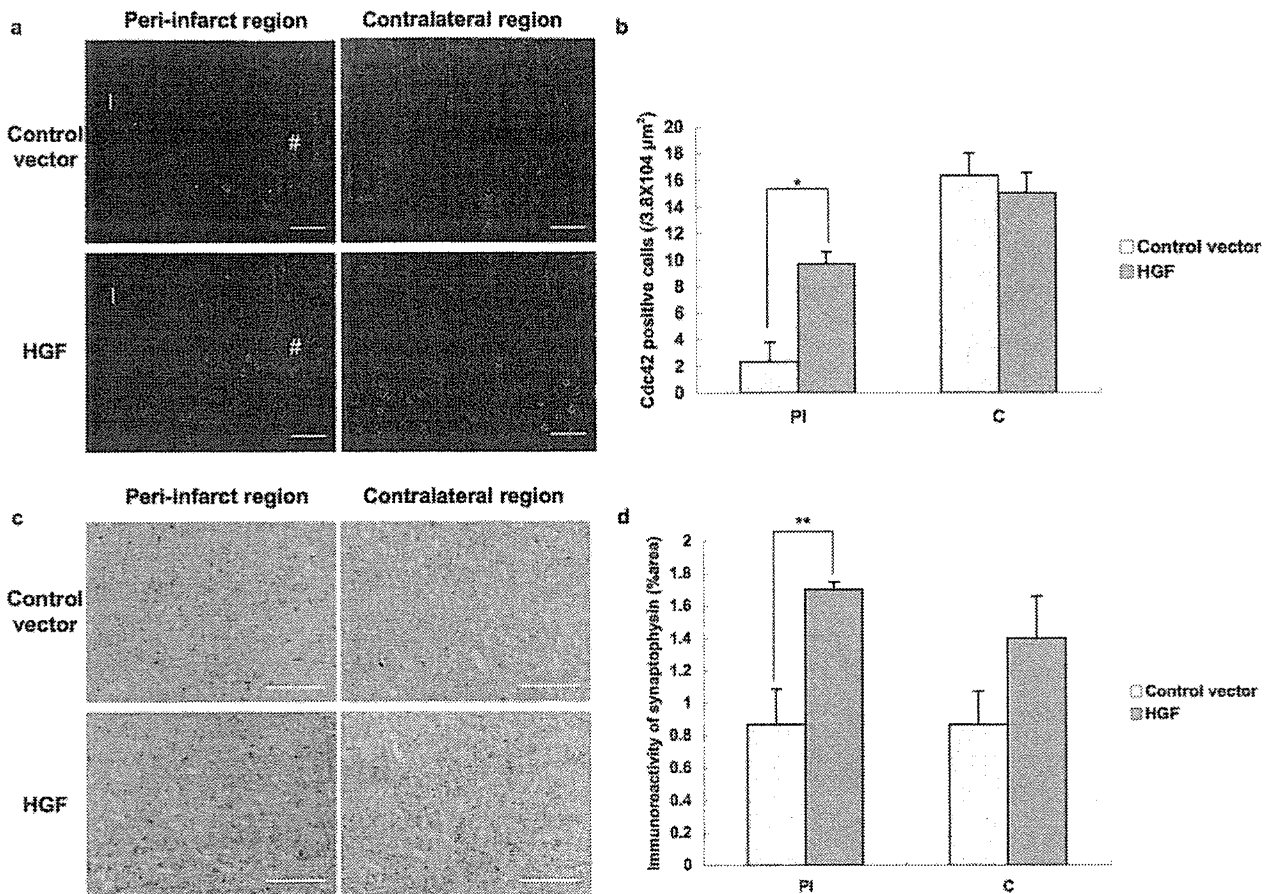


Figure 5. (a) Representative images of immunohistochemical staining for Cdc42 on day 14 in rats transfected with control and HGF vector. The number of cells immunoreactive for Cdc42 was significantly increased in the pyramidal neurons in the peri-infarct region (#) of rats transfected with HGF vector. I, infarct region. (b) Quantitative analysis for Cdc42-immunoreactive cells in peri-infarct region (#). (c) Typical images of immunohistochemical staining for synaptophysin on day 56 (●). (d) Quantitative analysis for the immunoreactivity of synaptophysin. In the peri-infarct region, the immunoreactivity was significantly increased in rats treated with HGF gene. Control vector indicates rats transfected with control vector (n=4); HGF, rats transfected with HGF vector (n=4). $^{*}P<0.05$, $^{**}P<0.01$ vs Control. Bar=100 μm. PI, peri-infarct region in neocortex; C, contralateral region in neocortex.

tant for neuroprotection or neuritogenesis.[37] The immunoreactivity of GFAP was increased on days 14 and 56 in the peri-infarct region in both groups, and the immunoreactivity on day 14 was significantly higher in rats transfected with HGF vector (Figure 6). In contrast, the fewer immunopositive cells against GFAP could be detected in rats transfected with the HGF vector on day 56 as compared with the control vector (Figure 6).

Because some viral vectors, such as adenoviral vector, cause diffuse encephaloventriculitis and substantial leukoencephalopathy,[38] we also performed hematoxylin/eosin staining to examine the inflammation. As expected, there was no inflammatory lymphocyte infiltration in HGF and control vector–transfected rats compared with sham-operated rats (data not shown).

HGF Increases Microvessels in the Peri-Infarct Region

Finally, the arteries in the peri-infarct and contralateral region were also examined using ALP staining on days 14 and 56. In the peri-infarct region, on day 56, arteries were significantly increased in rats transfected with HGF vector as compared with control vector (Figure 7a). Consistently, quantitative analysis showed an increase in the area and length of arteries on day 56 in the peri-infarct region in rats transfected with HGF vector (Figure 7b and 7c). Of importance, in the contralateral region, there was no difference between the groups on days 14 and 56 (Figure 7b and 7c).

Discussion

Disruption of blood flow to the brain initiates a cascade of events that produces neuronal death and leads to neurological dysfunction. From this viewpoint, we and others have reported that pretreatment with neurotrophic factors, such as FGF and HGF, has beneficial effects to prevent brain injury. However, considering their clinical application, pretreatment with neurotrophic factors might not be feasible. Unfortunately, few reports have revealed beneficial effects of treatment after infarction. To develop new therapeutic strategies to treat brain infarction, in this study, we examined the effects of overexpression of HGF after infarction, because HGF has unique actions in the central nervous system, as (1) a survival

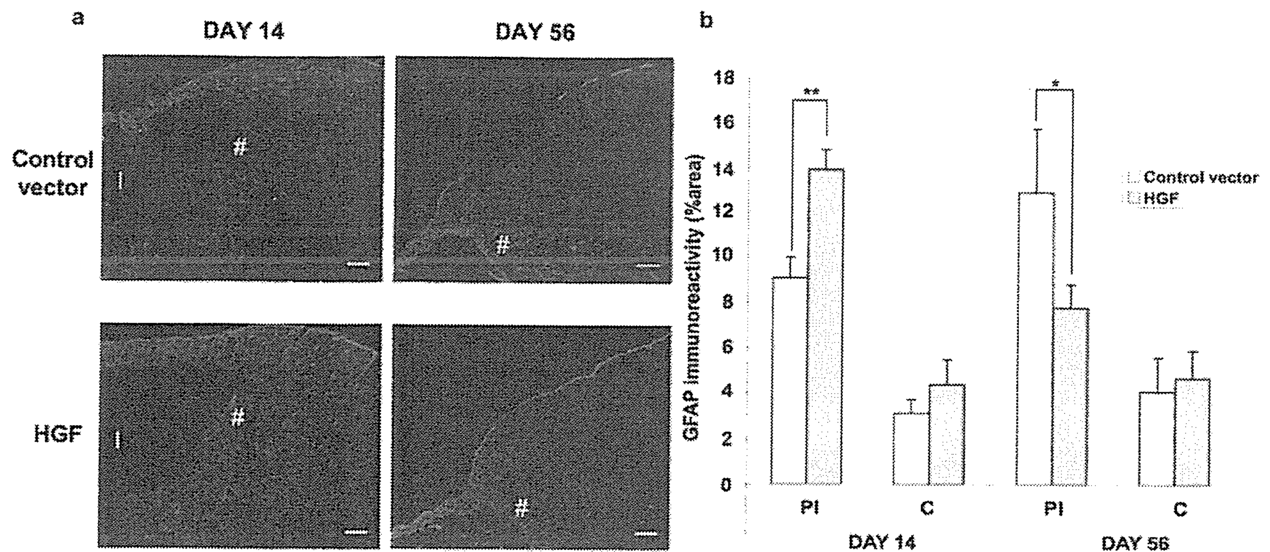


Figure 6. (a) Representative images of immunohistochemical staining for GFAP in ipsilateral neocortex on day 14 and 56 in rats transfected with control and HGF vector. Positive staining for GFAP was increased on day 14 and decreased on day 56 in the peri-infarct region (#) in rats transfected with HGF vector. I indicates infarct region (n=4 in each group; bar=100 μm.) (b) Quantitative analysis of immunoreactivity for GFAP in neocortex. PI, peri-infarct region in neocortex; C, contralateral region in neocortex. Control vector, rats transfected with control vector (n=4); HGF, rats transfected with HGF vector (n=4). **P*<0.05, ***P*<0.01 vs Control.

factor for embryonic motor neurons; (2) a stimulatory factor for the differentiation, survival, and axonal outgrowth of sensory and sympathetic neurons; (3) a neurotrophic factor;[39] and (4) a potent angiogenic growth factor.[10,21] The present study demonstrated that overexpression of HGF resulted in significant improvement of the results in MWM and the passive avoidance task on day 56, without any difference in infarct size and pattern. This study demonstrated that treatment with HGF postinfarction improved learning and memory.

Interestingly, the overexpression of HGF did not act on the disability of sensorimotor function and locomotor activity. The discrepancy of the recovery between the sensorimotor and cognitive functions has also been reported recently.[40] The authors reported that the functional recovery was observed not in the cognitive function but in the sensorimotor deficits when MHP36 stem cells were grafted into the cerebral parenchyma, whereas only spatial learning was improved in rats with intraventricular grafts.[40] Although the reason why the discrepancy was caused was unclear in the present study, we speculate that the functional recovery might be dependent on the kind of growth factor or the route of administration because of the different mechanisms in recovery from sensorimotor and cognitive deficits. Because the improvement of the sensorimotor deficits is also important, additional study is necessary to achieve the improvement of sensorimotor deficits.

The region where the significant histological difference was observed was the peri-infarct region, which was the border region between the frontal and parietal cortex. Because the neocortex was also an important site for learning and memory,[41] we speculate that the functional recovery enhanced by HGF is dependent on that region in the present study. In fact, both the immunohistochemical analysis for Cdc42, synaptophysin, and GFAP and the ALP staining revealed significant differences in that region. Cdc42 belongs to the Ras superfamily of small GTPases and is expressed in hippocampus, cerebellum, thalamus, and neocortex in the rats.[34,42] In general, Rac and Cdc42 have positive effects on process extension, whereas Rho has a positive effect on process retraction. HGF activated Cdc42, concomitant with the formation of filopodia and lammelipodia, in epithelial cells,[43] although it was not still demonstrated in neurons. Considering that the immunoreactivity for Cdc42 in pyramidal neurons, which possess a high density of cholinergic terminals,[41] was enhanced at day 14, the reconstitution of the neural network through neurite extension, so-called "neuritogenesis," might be in progress at the early stage of HGF gene treatment. Also, the immunoreactivity of the presynaptic marker synaptophysin was increased at day 56 in rats treated with the HGF gene, which implies that the neuritogenesis resulted in the formation of new synapses.[35,36] These results suggested that HGF enhanced neuritogenesis and synaptogenesis, which might contribute to the recovery of cognitive dysfunction.

The association of neurogenesis is also the center of interest, because HGF is involved in the development and maintenance of cortical neurons during differentiation and motogenesis in the neocortex.[18] In general, adult neurogenesis in the neocortex is still controversial.[44,45] It is also unclear whether adult neurogenesis occurs in the neocortex in rats after focal cerebral ischemia, because Jiang et al[46] showed the existence of neurogenesis, but Zhang et al[47] failed to detect neuronal nuclei and 5-bromodeoxyuridine double-labeling cells in the neocortex. In the present study, the fact that the volume of infarction was not decreased by transfection of the HGF gene and the density of matured neurons assessed by immunohistochemistry for MAP2 was not different (data not shown) implied that neurogenesis was not related to the functional recovery.

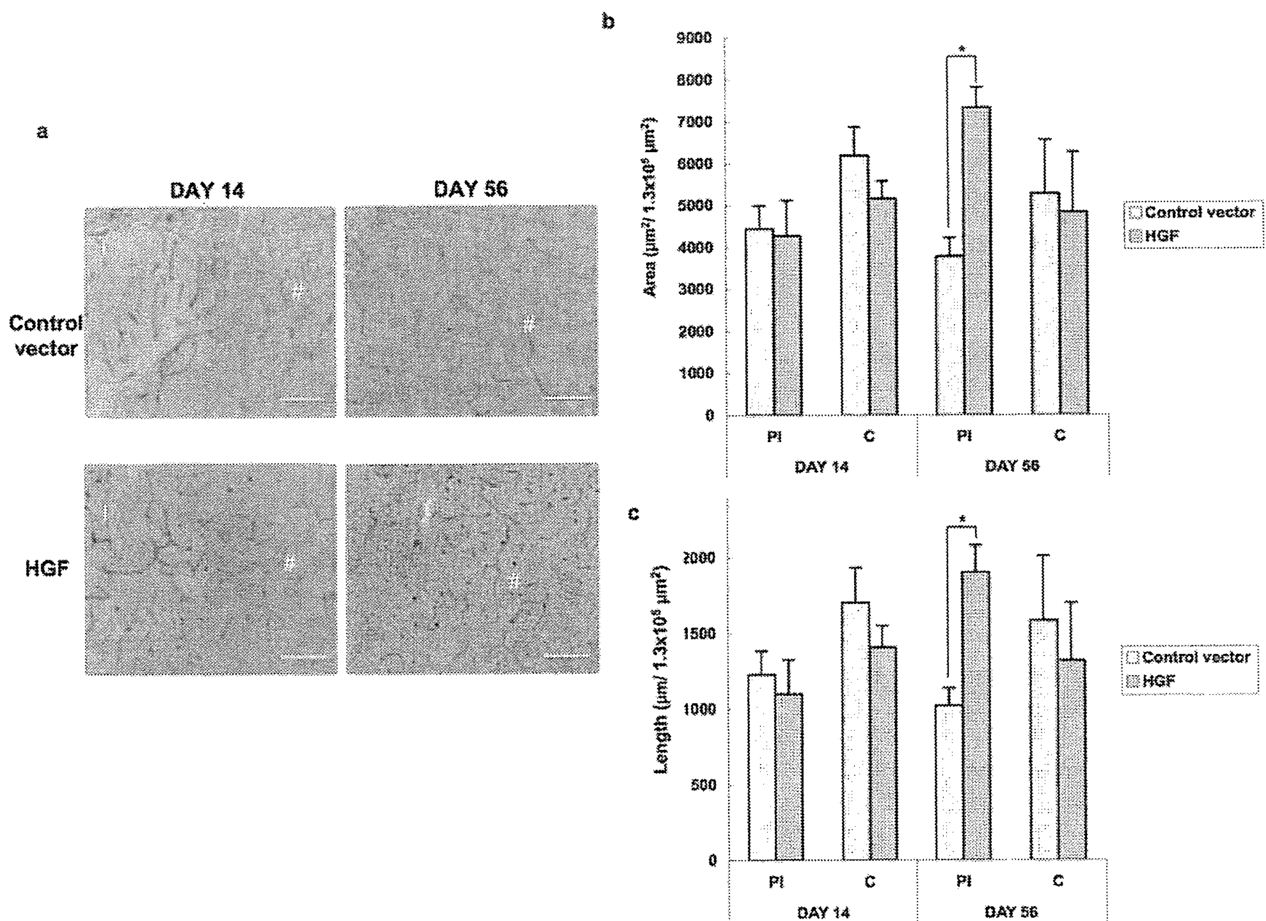


Figure 7. (a) Typical images of ALP staining in ipsilateral neocortex on day 14 in rats transfected with control and HGF vector. Coronal sections of ipsilateral neocortex stained for ALP. The structure of arteries in the peri-infarct region (#) was not different on day 14. However, the arteries in rats transfected with HGF gene showed a more complex pattern on day 56. Bar=100 μm; I indicates infarct region. (b and c) Quantitative analysis of area (b) and length (c) of blood vessels. PI, peri-infarct region; C, contralateral region; Control vector, rats transfected with control vector (n=4); HGF, rats transfected with HGF vector (n=4). *$P<0.05$ vs Control.

Another possible mechanism is that exogenously added HGF would transiently activate astrocytes and induce the production of other neurotrophic factors, resulting in the promotion of neuritogenesis. In fact, immunoreactivity for GFAP was increased on day 14 but decreased on day 56 to the contrary. Similar results were also observed in the recent report showing the effectiveness of forced arm use and brain-derived neurotrophic factor in MCAo.[35] A recent study showed that the activated astrocytes possess qualities that will promote neuronal survival and regeneration, and they do not, by themselves, produce inhibitory extracellular matrix, whereas reactivated astrocytes stimulated by cytokines, including interleukin 1b, interferon γ, tumor necrosis factor α, and transforming growth factors, contribute to the glial scar formation, which inhibit neuronal survival or regeneration.[37] It was also demonstrated that exogenous HGF regulated c-Met expression in cultured astrocytes and might induce other neurotrophic factors from activated astrocytes.[48] Thus, it is likely that the effect of HGF was direct action and/or indirect action via neuron–glia interactions on neuritogenesis.

This study also revealed an increase in microvessels only in the peri-infarct region but not in normal regions. Although the relationship between the improved microcirculation and behavior is still unclear, a recent report demonstrated that restoration of perfusion by collateral growth and new capillaries in the ischemic border zone around a cortical infarct supported long-term functional recovery in rats.[49] Additionally, others reported that some patients who received tissue plasminogen activator therapy with no immediate clinical improvement in spite of early recanalization showed delayed clinical improvement.[50] From the viewpoints, it is likely that the improvement of microcirculation is an important factor for the functional recovery. Although additional study is necessary, the improvement of microcirculation by HGF might be an alternative mechanism to improve learning and memory.

The influence of HGF on cerebral edema is another important issue. In general, the peak of cerebral edema is 3 days, and a significant decrease is 7 days after permanent MCAo in rats.[31] Afterward, the infarct brain becomes atrophic.[31] In the present study, the infarct region was atrophic in rats transfected with the HGF gene, as well as the control vector, and there was no significant difference in the volume. Thus, HGF gene transfer did not exacerbate the cerebral edema. Considering that VEGF

exacerbated cerebral edema,[51] HGF might be safer than VEGF. Additional study is necessary to compare the effectiveness of HGF to other growth factors.

The amount of HGF produced by this method (0.1 to 0.4 ng/mL) is relatively low because of the limited transfected cells in the surface brain and ischemic region, as compared with that of previous reports showing the effectiveness of recombinant human HGF protein for the cerebral ischemia.[11,23] Nevertheless, this low concentration might be enough to have the beneficial effects, because HGF elicited surviving neurotrophic effect at 0.5 to 1 ng/mL in primary cultured hippocampal neurons[17] and enhanced neurite extension at 0.1 to 100 nM (0.1 to 100 ng/mL) in neocortical explant.[52] Indeed, several previous articles demonstrated that the similar amount of HGF produced by gene transfer showed the neurotrophic and/or angiogenic property in several experimental rodent models.[21,22,25,53] Because the higher concentration of HGF is more effective for survival and neurite extension in in vitro study,[17,52] several improvements, such as modification of the HVJ-envelope vector and HGF plasmid, are required to achieve better outcome.

Perspectives

Overall, the present study is the first to demonstrate that HGF gene therapy delayed for as long as 7 days improved the outcome from ischemic stroke through the reconstitution of the neuronal network and improvement in the microcirculation. In clinical use, the present study might be attractive to support the application of HGF for the treatment of the patients in the chronic stage of brain infarction. Although most of the previous reports demonstrated the effectiveness of growth factors before the insult or within several hours of the onset by the inhibition of apoptosis and extension of the ischemic lesion,[9,10,12,21,22] it is difficult to administer them in time in most patients. Additionally, some patients improve their cognitive dysfunction spontaneously within several days after cerebral infarction. Also, the intracisternal injection is too difficult in the acute stage of cerebral infarction, because it is possible that the brain edema is worsened by intracisternal injection itself. In contrast, the present study is more closed to the real clinical situation for the treatment of the patients with chronic brain stroke. Although additional study is necessary to determine whether other growth factors are effective or not in the chronic stage, gene therapy using HGF may provide new therapeutic options for treatment after cerebral ischemia.

Acknowledgments

This work was partially supported by a grant-in-aid from the Organization for Pharmaceutical Safety and Research, a grant-in-aid from the Ministry of Public Health and Welfare, a grant-in-aid from Japan Promotion of Science, and a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government.

References

1. Hunter AJ, Mackay KB, Rogers DC. To what extent have functional studies of ischaemia in animals been useful in the assessment of potential neuroprotective agents? *Trends Pharmacol Sci*. 1998;19:59–66.
2. Belayev L, Alonso OF, Busto R, Zhao W, Ginsberg MD. Middle cerebral artery occlusion in the rat by intraluminal suture. Neurological and pathological evaluation of an improved model. *Stroke*. 1996;27:1616–1623.
3. Lin TN, Te J, Lee M, Sun GY, Hsu CY. Induction of basic fibroblast growth factor (bFGF) expression following focal cerebral ischemia. *Brain Res Mol Brain Res*. 1997;49:255–265.
4. Arai S, Kinouchi H, Akabane A, Owada Y, Kamii H, Kawase M, Yoshimoto T. Induction of brain-derived neurotrophic factor (BDNF) and the receptor trk B mRNA following middle cerebral artery occlusion in rat. *Neurosci Lett*. 1996;211:57–60.
5. Kitagawa H, Sasaki C, Zhang WR, Sakai K, Shiro Y, Warita H, Mitsumoto Y, Mori T, Abe K. Induction of glial cell line-derived neurotrophic factor receptor proteins in cerebral cortex and striatum after permanent middle cerebral artery occlusion in rats. *Brain Res*. 1999;834:190–195.
6. Lennmyr F, Ata KA, Funa K, Olsson Y, Terent A. Expression of vascular endothelial growth factor (VEGF) and its receptors (Flt-1 and Flk-1) following permanent and transient occlusion of the middle cerebral artery in the rat. *J Neuropathol Exp Neurol*. 1998;57:874–882.
7. Hayashi T, Abe K, Sakurai M, Itoyama Y. Inductions of hepatocyte growth factor and its activator in rat brain with permanent middle cerebral artery occlusion. *Brain Res*. 1998;799:311–316.
8. Hayashi T, Abe K, Itoyama Y. Reduction of ischemic damage by application of vascular endothelial growth factor in rat brain after transient ischemia. *J Cereb Blood Flow Metab*. 1998;18:887–895.
9. Shirakura M, Inoue M, Fujikawa S, Washizawa K, Komaba S, Maeda M, Watabe K, Yoshikawa Y, Hasegawa M. Postischemic administration of Sendai virus vector carrying neurotrophic factor genes prevents delayed neuronal death in gerbils. *Gene Ther*. 2004;11:784–790.
10. Shimamura M, Sato N, Oshima K, Aoki M, Kurinami H, Waguri S, Uchiyama Y, Ogihara T, Kaneda Y, Morishita R. Novel therapeutic strategy to treat brain ischemia: overexpression of hepatocyte growth factor gene reduced ischemic injury without cerebral edema in rat model. *Circulation*. 2004;109:424–431.
11. Date I, Takagi N, Takagi K, Kago T, Matsumoto K, Nakamura T, Takeo S. Hepatocyte growth factor attenuates cerebral ischemia-induced learning dysfunction. *Biochem Biophys Res Commun*. 2004;319:1152–1158.
12. Zhang WR, Sato K, Iwai M, Nagano I, Manabe Y, Abe K. Therapeutic time window of adenovirus-mediated GDNF gene transfer after transient middle cerebral artery occlusion in rat. *Brain Res*. 2002;947:140–145.
13. Nagayama T, Nagayama M, Kohara S, Kamiguchi H, Shibuya M, Katoh Y, Itoh J, Shinohara Y. Post-ischemic delayed expression of hepatocyte growth factor and c-Met in mouse brain following focal cerebral ischemia. *Brain Res*. 2004;999:155–166.
14. Kovacs Z, Ikezaki K, Samoto K, Inamura T, Fukui M. VEGF and flt. Expression time kinetics in rat brain infarct. *Stroke*. 1996;27:1865–1873.
15. Nakamura T, Nawa K, Ichihara A. Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem Biophys Res Commun*. 1984;122:1450–1459.
16. Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, Tashiro K, Shimizu S. Molecular cloning and expression of human hepatocyte growth factor. *Nature*. 1989;342:440–443.
17. Honda S, Kagoshima M, Wanaka A, Tohyama M, Matsumoto K, Nakamura T. Localization and functional coupling of HGF and c-Met/HGF receptor in rat brain: implication as neurotrophic factor. *Brain Res Mol Brain Res*. 1995;32:197–210.
18. Sun W, Funakoshi H, Nakamura T. Localization and functional role of hepatocyte growth factor (HGF) and its receptor c-met in the rat developing cerebral cortex. *Brain Res Mol Brain Res*. 2002;103:36–48.
19. Kokuzawa J, Yoshimura S, Kitajima H, Shinoda J, Kaku Y, Iwama T, Morishita R, Shimazaki T, Okano H, Kunisada T, Sakai N. Hepatocyte growth factor promotes proliferation and neuronal differentiation of neural stem cells from mouse embryos. *Mol Cell Neurosci*. 2003;24:190–197.
20. Miyazawa T, Matsumoto K, Ohmichi H, Katoh H, Yamashima T, Nakamura T. Protection of hippocampal neurons from ischemia-induced delayed neuronal death by hepatocyte growth factor: a novel neurotrophic factor. *J Cereb Blood Flow Metab*. 1998;18:345–348.
21. Yoshimura S, Morishita R, Hayashi K, Kokuzawa J, Aoki M, Matsumoto K, Nakamura T, Ogihara T, Sakai N, Kaneda Y. Gene transfer of hepatocyte growth factor to subarachnoid space in cerebral hypoperfusion model. *Hypertension*. 2002;39:1028–1034.
22. Hayashi K, Morishita R, Nakagami H, Yoshimura S, Hara A, Matsumoto K, Nakamura T, Ogihara T, Kaneda Y, Sakai N. Gene therapy for

preventing neuronal death using hepatocyte growth factor: in vivo gene transfer of HGF to subarachnoid space prevents delayed neuronal death in gerbil hippocampal CA1 neurons. *Gene Ther.* 2001;8:1167–1173.
23. Tsuzuki N, Miyazawa T, Matsumoto K, Nakamura T, Shima K, Chigasaki H. Hepatocyte growth factor reduces infarct volume after transient focal cerebral ischemia in rats. *Acta Neurochir Suppl.* 2000;76: 311–316.
24. Kaneda Y, Nakajima T, Nishikawa T, Yamamoto S, Ikegami H, Suzuki N, Nakamura H, Morishita R, Kotani H. Hemagglutinating virus of Japan (HVJ) envelope vector as a versatile gene delivery system. *Mol Ther.* 2002;6:219–226.
25. Shimamura M, Morishita R, Endoh M, Oshima K, Aoki M, Waguri S, Uchiyama Y, Kaneda Y. HVJ-envelope vector for gene transfer into central nervous system. *Biochem Biophys Res Commun.* 2003;300: 464–471.
26. Koike H, Morishita R, Iguchi S, Aoki M, Matsumoto K, Nakamura T, Yokoyama C, Tanabe T, Ogihara T, Kaneda Y. Enhanced angiogenesis and improvement of neuropathy by cotransfection of human hepatocyte growth factor and prostacyclin synthase gene. *Faseb J.* 2003;17:779–781.
27. Petullo D, Masonic K, Lincoln C, Wibberley L, Teliska M, Yao DL. Model development and behavioral assessment of focal cerebral ischemia in rats. *Life Sci.* 1999;64:1099–1108.
28. Modo M, Stroemer RP, Tang E, Veizovic T, Sowniski P, Hodges H. Neurological sequelae and long-term behavioural assessment of rats with transient middle cerebral artery occlusion. *J Neurosci Methods.* 2000; 104:99–109.
29. Chen J, Zhang C, Jiang H, Li Y, Zhang L, Robin A, Katakowski M, Lu M, Chopp M. Atorvastatin induction of VEGF and BDNF promotes brain plasticity after stroke in mice. *J Cereb Blood Flow Metab.* 2005;25: 281–290.
30. Nedergaard M, Gjedde A, Diemer NH. Hyperglycaemia protects against neuronal injury around experimental brain infarcts. *Neurol Res.* 1987;9: 241–244.
31. Takamatsu H, Tatsumi M, Nitta S, Ichise R, Muramatsu K, Iida M, Nishimura S, Umemura K. Time courses of progress to the chronic stage of middle cerebral artery occlusion models in rats. *Exp Brain Res.* 2002; 146:95–102.
32. DeVries AC, Nelson RJ, Traystman RJ, Hurn PD. Cognitive and behavioral assessment in experimental stroke research: will it prove useful? *Neurosci Biobehav Rev.* 2001;25:325–342.
33. Robinson RG. Differential behavioral and biochemical effects of right and left hemispheric cerebral infarction in the rat. *Science.* 1979;205: 707–710.
34. O'Kane EM, Stone TW, Morris BJ. Distribution of Rho family GTPases in the adult rat hippocampus and cerebellum. *Brain Res Mol Brain Res.* 2003;114:1–8.
35. Schabitz WR, Berger C, Kollmar R, Seitz M, Tanay E, Kiessling M, Schwab S, Sommer C. Effect of brain-derived neurotrophic factor treatment and forced arm use on functional motor recovery after small cortical ischemia. *Stroke.* 2004;35:992–997.
36. Stroemer RP, Kent TA, Hulsebosch CE. Enhanced neocortical neural sprouting, synaptogenesis, and behavioral recovery with D-amphetamine therapy after neocortical infarction in rats. *Stroke.* 1998;29:2381–2395.
37. Albrecht PJ, Murtie JC, Ness JK, Redwine JM, Enterline JR, Armstrong RC, Levison SW. Astrocytes produce CNTF during the remyelination phase of viral-induced spinal cord demyelination to stimulate FGF-2 production. *Neurobiol Dis.* 2003;13:89–101.
38. Tada T, Nguyen JB, Hitoshi Y, Watson NP, Dunn JF, Ohara S, Nagano S, Kosai K, Israel MA. Diffuse encephaloventriculitis and substantial leukoencephalopathy after intraventricular administration of recombinant adenovirus. *Neurol Res.* 2005;27:378–386.
39. Maina F, Klein R. Hepatocyte growth factor, a versatile signal for developing neurons. *Nat Neurosci.* 1999;2:213–217.
40. Modo M, Stroemer RP, Tang E, Patel S, Hodges H. Effects of implantation site of stem cell grafts on behavioral recovery from stroke damage. *Stroke.* 2002;33:2270–2278.
41. Casu MA, Wong TP, De Koninck Y, Ribeiro-da-Silva A, Cuello AC. Aging causes a preferential loss of cholinergic innervation of characterized neocortical pyramidal neurons. *Cereb Cortex.* 2002;12:329–337.
42. Olenik C, Barth H, Just I, Aktories K, Meyer DK. Gene expression of the small GTP-binding proteins RhoA, RhoB, Rac1, and Cdc42 in adult rat brain. *Brain Res Mol Brain Res.* 1997;52:263–269.
43. Royal I, Lamarche-Vane N, Lamorte L, Kaibuchi K, Park M. Activation of cdc42, rac, PAK, and rho-kinase in response to hepatocyte growth factor differentially regulates epithelial cell colony spreading and dissociation. *Mol Biol Cell.* 2000;11:1709–1725.
44. Kornack DR, Rakic P. Cell proliferation without neurogenesis in adult primate neocortex. *Science.* 2001;294:2127–2130.
45. Rakic P. Adult neurogenesis in mammals: an identity crisis. *J Neurosci.* 2002;22:614–618.
46. Jiang W, Gu W, Brannstrom T, Rosqvist R, Wester P. Cortical neurogenesis in adult rats after transient middle cerebral artery occlusion. *Stroke.* 2001;32:1201–1207.
47. Zhang RL, Zhang ZG, Zhang L, Chopp M. Proliferation and differentiation of progenitor cells in the cortex and the subventricular zone in the adult rat after focal cerebral ischemia. *Neuroscience.* 2001;105:33–41.
48. Shimazaki K, Yoshida K, Hirose Y, Ishimori H, Katayama M, Kawase T. Cytokines regulate c-Met expression in cultured astrocytes. *Brain Res.* 2003;962:105–110.
49. Wei L, Erinjeri JP, Rovainen CM, Woolsey TA. Collateral growth and angiogenesis around cortical stroke. *Stroke.* 2001;32:2179–2184.
50. Alexandrov AV, Hall CE, Labiche LA, Wojner AW, Grotta JC. Ischemic stunning of the brain: early recanalization without immediate clinical improvement in acute ischemic stroke. *Stroke.* 2004;35:449–452.
51. van Bruggen N, Thibodeaux H, Palmer JT, Lee WP, Fu L, Cairns B, Tumas D, Gerlai R, Williams SP, van Lookeren Campagne M, Ferrara N. VEGF antagonism reduces edema formation and tissue damage after ischemia/reperfusion injury in the mouse brain. *J Clin Invest.* 1999;104: 1613–1620.
52. Hamanoue M, Takemoto N, Matsumoto K, Nakamura T, Nakajima K, Kohsaka S. Neurotrophic effect of hepatocyte growth factor on central nervous system neurons in vitro. *J Neurosci Res.* 1996;43:554–564.
53. Oshima K, Shimamura M, Mizuno S, Tamai K, Doi K, Morishita R, Nakamura T, Kubo T, Kaneda Y. Intrathecal injection of HVJ-E containing HGF gene to cerebrospinal fluid can prevent and ameliorate hearing impairment in rats. *Faseb J.* 2004;18:212–214.

Review Article

Non-viral vectors for cancer therapy

Yasufumi Kaneda[1,3] and Yasuhiko Tabata[2]

[1]Division of Gene Therapy Science, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565–0871; and [2]Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawara-cho, Shogoin, Sakyo-ku, Kyoto 606–8507, Japan

(Received December 27, 2005/Revised January 23, 2006/Accepted January 24, 2006/Online publication March 28, 2006)

Cancers are diverse and often resistant to therapeutic strategies. Gene therapy has yet to meet the promise of a breakthrough in cancer therapy. There are several barriers to overcome in cancer gene therapy. One of the biggest challenges is the design of appropriate vectors. Numerous viral and non-viral methods for gene transfer have been developed for human gene therapy, but both viral and non-viral vectors have limitations and advantages. In this review article, recent improvements in the development of non-viral vectors for delivering gene therapy for the treatment of cancer will be discussed. (*Cancer Sci* 2006; 97: 348–354)

Gene therapy provides a novel strategy for cancer treatment, although it is still not capable of eradicating cancer in humans. The biggest challenge in gene therapy for the treatment of cancer is the development of appropriate vectors. Numerous viral and non-viral (synthetic) methods for gene transfer have been developed.[1–3] Generally, viral vectors are more efficient for gene delivery and gene expression than non-viral methods. However, viral vectors pose more risk than non-viral vectors. Moreover, viral vectors are not available for drug delivery, whereas non-viral vectors are capable of delivering anticancer reagents, as well as synthetic oligonucleotides, antibodies and RNA, in addition to therapeutic genes. From this perspective, non-viral vector systems are a favorable means by which to deliver cancer therapy. However, a number of barriers exist, including mechanisms which protect our body from the invasion of exogenous molecules. Non-viral vectors must be capable of overcoming these barriers in order to effectively deliver cancer therapy.

Endocytosis-mediated delivery

Liposomes

Although liposomes enable targeted delivery of macromolecules, a low and variable efficiency of gene transfer was observed during the early days of liposome development. The synthesis of cationic lipids produced a revolutionary improvement in gene transfer efficiency.[4] This led to the development of a new model of delivery involving liposome/DNA complexes or lipoplexes. Prior to this, DNA was incorporated into liposomes, however, lipoplexes enabled electrostatic interactions between negatively charged DNA and positively charged cationic liposomes. Numerous cationic lipids with improved transfection efficiency now exist, thus reducing the cytotoxicity of lipoplexes.[3] However, DNA is taken up into cells by endocytosis during lipoplex-mediated transfection.[5] The main problem with endocytosis-mediated delivery is that therapeutic molecules are prone to degradation within endosomes or lysosomes, as shown in Figure 1. Lipids may offer protection against the degradation of therapeutic molecules before they reach the cytoplasm. A neutral lipid, DOPE is capable of facilitating the endosomal release of DNA.[6] This discovery led to the use of a mixture of cationic lipids and DOPE for lipofection. Further analysis of various lipids has revealed that a 1 : 1 mixture of N-[1-(2,3-dimyristyloxy) propyl]-N,N-dimethyl-N-(2-hydroxyethyl) ammonium bromide and cholesterol is capable of destabilizing the endosome membrane more effectively than DOPE.[7] To further protect therapeutic molecules delivered by liposomes, DNA is now conjugated with cationic molecules. For example, protamine sulfate[5] or adenovirus mμ protein[8] are conjugated with DNA, after which the newly formed complexes are incorporated into or mixed with cationic liposomes. It is difficult to evaluate the efficiency of liposome-mediated gene delivery using cultured cells *in vitro* since the results are not consistent with those observed *in vivo*.[9] In spite of improvements in the membrane permeability of more complex cationic complexes, simple cationic liposomes remain more popular in clinical trials of cancer therapy. With regard to these trials, cationic liposomes containing HLA-B7 and β-2 microglobulin genes induce antitumor immunity in HLA-B7-negative melanoma patients, thus, a number of institutions have performed clinical trials of a liposomal drug (Allovectin-7) for the treatment of metastatic melanoma.[10] Delivery of the β-interferon gene by cationic liposomes has been evaluated to treat patients suffering from glioblastoma in Japan.[11] Several trials have also evaluated delivery of various anticancer agents using liposomes in humans.[12,13] As a result, liposomes are now considered safe for use in humans.

The requirement for nuclear transport of plasmid DNA poses a significant barrier to effective gene expression following gene therapy using non-viral vectors.[14] A number

[3]To whom correspondence should be addressed.
E-mail: kaneday@gts.med.osaka-u.ac.jp
Abbreviations: AS-ODN, antisense oligodeoxynucleotides; CDDP, *cis*-diamminedichloroplatinum (II); DOPE, dioleoylphosphatidylethanolamine; EBV, Epstein-Barr virus; FITC, fluorescent isothiocyanate; HGF, hepatocyte growth factor; HSV-TK, herpes simplex virus-thymidine kinase; HVJ, hemagglutinating virus of Japan; MSC, mesenchymal stem cells; ODN, oligodeoxynucleotides; PEG, polyethylene glycol; siRNA, short interfering RNA; UV, ultraviolet.

doi: 10.1111/j.1349-7006.2006.00189.x
© 2006 Japanese Cancer Association

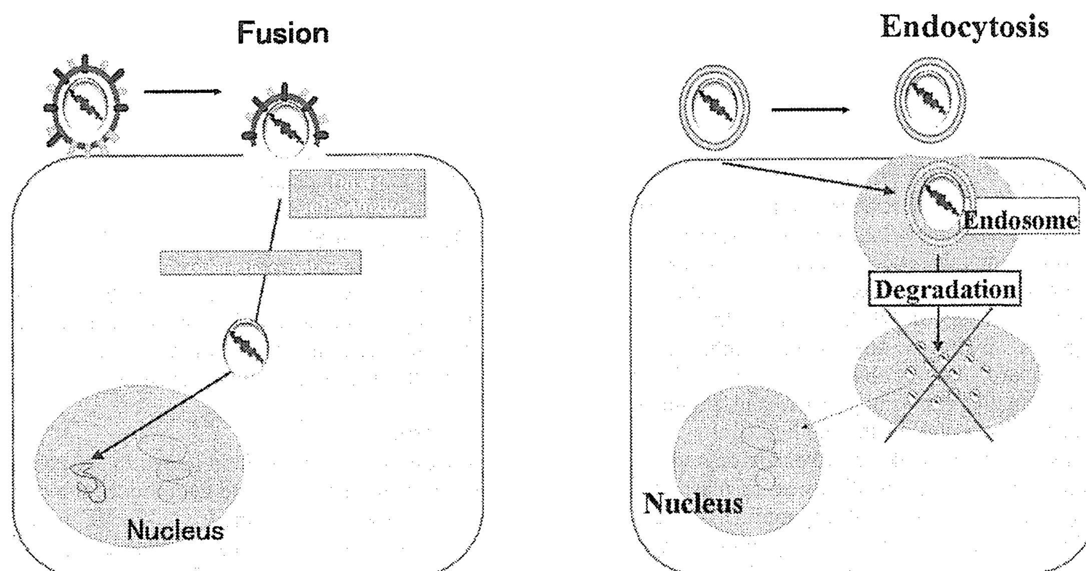


Fig. 1. Pathway by which therapeutic molecules are introduced into cells by liposomes or hemagglutinating virus of Japan (HVJ)-liposomes. Molecules are delivered by liposomes into cells by endocytosis by way of the endocytotic pathway, which makes cells susceptible to degradation. However, molecules delivered by HVJ-liposomes are directly introduced into the cytoplasm by membrane fusion.

of trials have evaluated the success of nuclear transport of exogenous DNA using non-viral vectors, such as liposomes.[15,16] Incorporation of the viral machinery capable of mediating nuclear transport of exogenous DNA into non-viral vectors might enhance the migration of exogenous DNA into the nucleus. From this standpoint, more work is needed to reproduce the viral capability of transporting DNA into the nucleus.

Polymers

Polymers used as non-viral vectors to enhance gene expression can be divided into two categories based on biodegradability. Various cationized non-biodegradable polymers have been evaluated with regard to their success of delivering DNA into cells, resulting in improved gene expression. These include linear cationized polymers of poly(ethyleneimine)[17] and poly-L-lysine.[18] Others are poly(N-ethyl-4-vinylpyridinium bromide), poly(dimethylaminoethyl methacrylate), chitosan, and dimethylaminodextran, or cationic polymers of branched poly(amidoamine) dendrimer and branched poly(ethyleneimine). Generally, because DNA is a large and negatively charged molecule, it has difficulty attaching to the negatively charged cell membrane for internalization. It is well recognized that cationized polymers readily form complexes with negatively charged DNA through electrostatic interactions. This condenses the DNA and creates a positive net electric charge under appropriate conditions. This facilitates cell attachment and subsequent internalization by means of endocytosis. In order to promote the internalization of DNA into cells, several cell receptor ligands have been used to take advantage of receptor-mediated endocytosis. For example, a folate can be covalently attached to a cationized polymer in order to promote DNA transfection. This folate-bound cationized polymer results in selective delivery and internalization of DNA into tumors.[19] Similarly, selective delivery and internalization of DNA into tumors can be achieved with transferrin-bound cationized polymers.[20] Galactose-bound cationized polymers enable direct delivery and internalization of DNA into the liver through the asialoglycoprotein receptor, to which galactose binds.[21] Successful use of the asialoglycoprotein receptor for DNA targeting suggests that polysaccharides might serve as useful non-viral carriers. We have designed cationized derivatives of the polysaccharides pullulan or dextran with spermine for complexation with plasmid DNA. Enhanced gene expression is observed with cationized polysaccharides, compared to commercially available cationic liposomes. Gene expression is reduced by pretreatment of cells with a natural ligand for the asialoglycoprotein receptor, asialofetuin, which clearly supports receptor-mediated endocytosis of cationized polysaccharide-plasmid DNA complexes. Efficient transfection of rat bone marrow-derived MSC with adrenomedullin plasmid DNA can be achieved using a cationized polysaccharide vector. Transplantation of adrenomedullin-transfected MSC into an ischemic site following rat myocardial infarction achieves a superior therapeutic effect to transplantation of MSC alone (Y. Tabata, unpublished data, 2005).

Biodegradable polymers have been used to achieve controlled-release of DNA, thus enhancing and prolonging gene expression. Controlled-release technology increases and prolongs the concentration of DNA around an injection site. Several reports describe the controlled-release of DNA from the matrixes of various biodegradable polymers, including poly(D,L-lactic acid-coglycolic acid), poly(lactic acid)-poly

© 2006 Japanese Cancer Association

(ethylene glycol), poly(2-aminoethyl propylene phosphate), poloxamer, poly(ethylene-covinyl acetate), silk-elastinlike polymer, atelocollagen, and gelatin. Shea *et al.* describe the sustained release of plasmid DNA encoding platelet-derived growth factor from a poly(D,L-lactic acid-coglycolic acid) matrix, leading to enhanced deposition of extracellular matrix and blood vessel formation *in vivo*.[22] Controlled-release of plasmid DNA by an atelocollagen minipellet enhances gene expression and consequent therapeutic effects in animal models of disease. We have prepared cationized gelatin by chemically introducing amine residues to the carboxyl groups of gelatin, subsequently cross-linked by exposure to various concentrations of glutaraldehyde in order to produce several cationized gelatin hydrogels with different propensities toward degradation and release of DNA. Each DNA-containing cationized gelatin hydrogel significantly enhances gene expression, compared to that observed following injection of a solution containing plasmic DNA, and prolongs the duration of gene expression. Using the hydrogel system, the release of plasmid DNA is likely driven by degradation of the vector alone, as opposed to diffusion of plasmid DNA following injection. The fact that plasmid DNA becomes immobilized within the hydrogel through polyionic complexation with the cationized gelatin, makes it all the more likely that plasmid DNA is released as a result of degradation of the hydrogel carrier. Data regarding the release of plasmid DNA over time further support this hydrogel degradation theory. Plasmid DNA is more stable when complexed with a hydrogel, and the controlled-release of DNA from plasmid DNA-cationized gelatin complexes deposits greater concentrations of plasmid DNA around cells, resulting in increased efficacy of gene transfection. As degradation of the hydrogel determines the rate of release of plasmid DNA, it is possible to achieve controlled-release of plasmid DNA using any shape of hydrogel carrier. Controlled-release technology enhances the biological activity of an antitumor DNA plasmid[23] of NK4, which is a protein composed of the NH_2-terminal hairpin and subsequent four-kringle domains of HGF. NK4 is a known HGF antagonist, which inhibits the ability of HGF to promote metastasis and angiogenesis. Subcutaneous injection of hydrogel microspheres containing NK4 plasmid DNA into nude mice injected with ascitic AsPC-1 tumor cells significantly prolongs mouse survival, compared with mice injected with NK4 plasmid DNA in the solution form. Thus, the capability to achieve controlled-release of DNA is a promising technology to enhance the *in vivo* biological effects of plasmid DNA.

Following administration of polymers or liposomes into the body, the generally rapid uptake of vectors by the mononuclear phagocyte system can prevent drugs from reaching their desired site of action if they are not being targeted to mononuclear phagocyte system tissues and organs. One effective way to tackle this problem is to modify the surface of drug carriers with PEG or PEG-like polymers. It is well known that cationized polymers or liposomes modified with PEG interact electrostatically with DNA to form complexes with a core-shell micelle structure. PEGylated vectors containing plasmid DNA circulate in the blood longer than free plasmid DNA. Drug carriers modified with PEG or PEG-like polymers enable DNA to accumulate within tumors or at sites of inflammation due to characteristic changes in the vasculature, including increased vascular permeability and a relative lack of lymph vessels, the so-called enhanced permeability and retention effect.[24] Pronectin F +, an artificial protein with repeated RGDS sequences, has been cationized and modified with PEG. When intravenously injected into mice with subcutaneous masses of Meth-AR-1 fibrosarcoma, PEG-modified cationized Pronectin F + complexed with plasmid DNA demonstrates significantly greater gene expression within tumors than PEG-free cationized Pronectin F + containing plasmid DNA and free plasmid DNA.[25]

Physical methods

Non-viral gene delivery vectors enable delivery of plasmid DNA into target cells. A number of methods to physically force DNA into cells have also been developed. Two such methods are electroporation and ultrasound.

Electroporation

In electroporation, an electric field increases the permeability of the cell membrane to facilitate the introduction of plasmid DNA into cells. Using this method, a 10- to 100-fold increase in gene expression over that obtained with administration of naked DNA alone is achieved.[26] This is a popular method for transfection *in vitro*. Skin and muscle are the main targets of *in vivo* transfection and two types of electrodes are used, plate-type and needle-type. The plate-type electrode yields more reproducible results, but the needle-type is more useful for transfection of various tissues. Electroporation poses a problem of tissue damage when high electrical currents are used. To solve this problem, it is now performed at lower voltages without a notable decline in transfection efficiency.[27] For cancer treatment, phase II trials of electro-chemotherapy are underway for head, neck, and skin cancer.[28]

Ultrasound

Ultrasound is also available for gene delivery and is more useful *in vivo* than *in vitro*.[29] This transfection method depends on the ability of ultrasound to induce cavitation. DNA is first mixed with contrast reagents, such as Optison and Levovist, which are gas-filled particles coated with lipids or albumin. Using ultrasound, microbubbles (1–100 μm in diameter) are then generated and ruptured. This is how ultrasound can be used to induce cavitation. It is thought that membrane permeability might be enhanced when the bubbles are ruptured by the energy of the ultrasound. An alternative theory is that a high-speed jet flow (>600 km/h) is produced which might enhance membrane permeability.[30] Either way, DNA is incorporated into target cells with recovery of cell membrane permeability within 60 s. Transfection efficiency increases with increases in the mechanical index, frequency and exposure time. However, these parameters are also linked to cell damage. Therefore, it is essential to determine optimum conditions for performing ultrasound. We currently use ultrasound with a mechanical index of 0.6, a frequency of 1 MHz, and 1 min of exposure to achieve transfection in skeletal muscle, and 10 s of exposure to achieve transfection in mouse embryo tissue using Ultax UX-301 (Celcom, Fukuoka, Japan). Applications for ultrasound-mediated gene delivery in cancer therapy are similar to the electroporation method.

doi: 10.1111/j.1349-7006.2006.00189.x
© 2006 Japanese Cancer Association

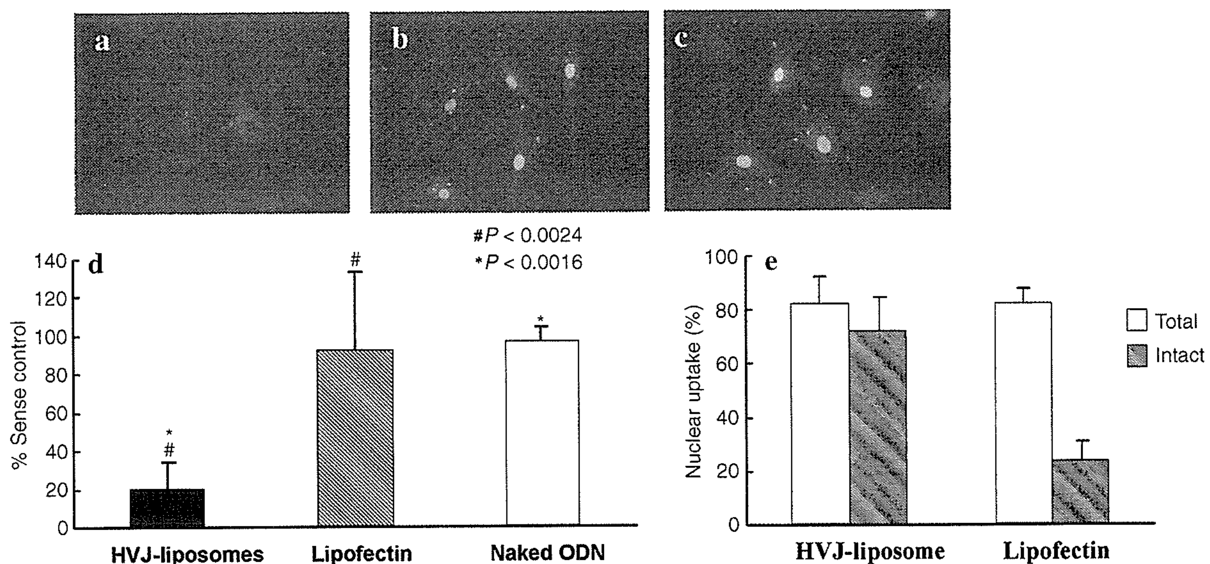


Fig. 2. Uptake of fluorescent isothiocyanate (FITC)-labeled AS-ODN against decorin into human fibroblasts demonstrated by fluorescence microscopy (a–c). No fluorescence was seen in cells administered antisense oligodeoxynucleotides (AS-ODN) alone (a), however, fluorescence was detected in the nuclei of most cells administered AS-ODN using cationic liposomes (Lipofectin) (b) or hemagglutinating virus of Japan (HVJ)-liposomes (c). Suppression of the decorin gene by AS-ODN in human fibroblasts was observed (d). The ratio of fully intact to total AS-ODN was examined in fluorescent cells using the fluorescence resonance energy transfer system, as described under 'Fusion liposomes' (e). Following administration using HVJ-liposomes, approximately 85% of cells contained intact AS-ODN, whereas only 30% of cells did following administration using Lipofectin.

Fusion-mediated delivery

Fusion liposomes

To avoid degradation prior to reaching the cytoplasm, fusion-mediated delivery systems have been developed, as shown in Figure 1. A fusigenic viral liposome with a fusigenic envelope derived from HVJ (Sendai virus) was first constructed.[31] HVJ is considered a mouse parainfluenza virus and is not a human pathogen. The virus is famous for inducing fusion with the cell membrane at neutral pH, and HN- and F-fusion proteins of the virus contribute to cell fusion.[32] HN binds to acetyl-type sialic acid and degrades the sugar chain with its neuraminidase activity. Then F associates with lipids, such as cholesterol within the cell membrane, to induce cell fusion. The F glycoprotein is first synthesized as inactive F0 in cells infected with HVJ, then cleaved by a host protease to produce the active F1 and F2 forms. The resulting F1 contains hydrophobic peptides of approximately 25 amino acids, which induce cell fusion. To achieve fusion-mediated gene transfer, DNA-loaded liposomes can be fused with UV-inactivated HVJ to form a fusigenic viral-liposome, the HVJ-liposome, which is 400–500 nm in diameter. Primitive HVJ-liposomes are constructed by fusion of liposomes with UV-inactivated HVJ. Reconstituted fusion liposomes can also be constructed. The HVJ virion is first completely lyzed with detergent, after which the lysate is mixed with DNA solution and various lipids are added to the mixture. By removing the detergent with dialysis or column filtration, reconstituted HVJ particles containing DNA can be constructed. Instead of using the entire HVJ virion, fusion proteins (F and HN) isolated from the virion can be mixed with the lipid/DNA mixture in the presence or absence of detergent. Reconstituted fusion liposomes are as effective as conventional HVJ-liposomes using fully intact HVJ virions in terms of delivery of FITC–ODN, as well as the luciferase gene, into cultured cells. The *LacZ* gene can also be directly transferred to mouse skeletal muscle using reconstituted fusion particles *in vivo*. Incubation with anti-F protein antibody at least 30 min prior to transfection reduces the efficiency of HVJ F protein-mediated gene delivery. However, a significant reduction in the efficiency of gene delivery is not observed when cells are incubated with wortmannin, an inhibitor of endocytosis, for 15 min prior to transfection.

It is expected that molecules of interest might be protected from degradation within endosomes and lysosomes by fusion-mediated delivery. Fluorescence is detected in nuclei following the introduction into human fibroblast cells of FITC–AS-ODN against the decorin gene using either HVJ-liposomes or lipoplex (Lipofectin) (Fig. 2a–c). However, decorin expression is suppressed following delivery of FITC–AS-ODN by HVJ-liposomes, but not lipoplex (Fig. 2d). Using the fluorescence resonance energy transfer system shown in Fig. 2(e), more than 85% of ODN labeled with two different fluorescent dyes at their 5′ and 3′ ends remain intact within the nucleus following delivery by HVJ-liposomes, compared to 30% following delivery using Lipofectin.[33]

A similar approach to enhance the efficiency of gene transfer uses fusion peptides derived from influenza virus hemagglutinin for receptor-mediated gene delivery. Combining transferrin/poly-L-lysine/DNA complexes with the hemagglutinin peptide increases gene transfer efficiency by more than 1,000-fold in cultured cancer cells, compared to gene transfer in the absence of this peptide.[34]

© 2006 Japanese Cancer Association

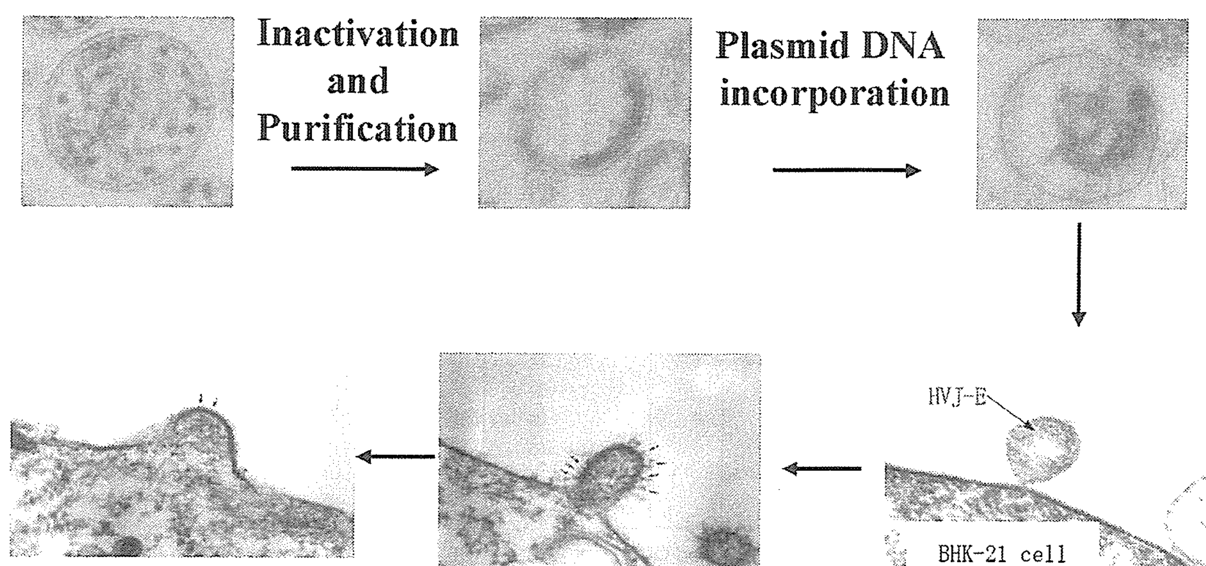


Fig. 3. Development of the hemagglutinating virus of Japan (HVJ) envelope vector and fusion with the cell membrane. Inactivated HVJ was purified through a column procedure and mixed with plasmid DNA in the presence of a mild detergent. After centrifugation, plasmid DNA was incorporated into empty particles. When the HVJ envelope vector attached to the cell membrane, fusion occurred in 10 s.

The use of HVJ-liposomes for delivering cancer treatment has been investigated in animal models. A melanoma-associated antigen gene or RNA injected into skeletal muscle or the spleen successfully evokes tumor-immunity to prevent melanoma growth.[35] A radio-inducible HSV-TK gene driven by an Egr-1 promoter enhances the efficacy of radiotherapy against hepatocellular carcinoma when delivered by HVJ-anionic liposomes.[36]

To achieve sustained gene expression, HVJ-liposomes can be combined with an EBV replicon plasmid containing the *cis*-acting oriP (the latent viral DNA replication origin) sequence and the *trans*-acting EBV nuclear antigen-1 gene.[37] The EBV replicon plasmid enhances transcriptional activation and enables stable retention of the plasmid DNA. Using an EBV replicon plasmid containing the HSV-TK gene, suicide gene therapy is more effective against melanoma in tumor-bearing mice.[38]

It is well known that silencing of transgene expression occurs in host cells, despite insertion of transgenes into the host genome.[39] Although limited or silenced transgene expression following transfection is a major problem with human gene therapy, the mechanism(s) by which transgene expression is regulated has yet to be determined. It is thought that histone methylase, acetylase and deacetylase can regulate transcription by modifying chromatin.[40] Histone deacetylase inhibitors can lead to a recovery of transgene expression after silencing.[41] We discovered a novel histone deacetylase, FR901228, which was originally developed as an anticancer drug by Astellas Pharma (Tokyo, Japan), capable of amplifying exogenous DNA expression both *in vitro* and *in vivo*. As the drug was first developed as an anticancer compound, synergistic anticancer effects are obtained when suicide gene therapy is combined with administration of FR901228. Co-injection of the HSV-TK gene and FR901228, followed by gancyclovir (GCV) administration, significantly suppresses tumor growth in mice, compared with gene therapy alone. Furthermore, more than 50% of tumor-bearing mice become tumor-free and survive for an extended period of time.[42] Thus, combination therapy is a promising and practical approach for cancer treatment.

HVJ envelope vector

There are some drawbacks to using HVJ-liposomes, even though the vector is widely used for gene transfer both *in vitro* and *in vivo*. One disadvantage of using HVJ-liposomes is the difficulty of isolating and producing both inactivated HVJ and DNA-loaded liposomes. Another limitation is that the fusion capacity of HVJ-liposomes decreases to approximately 2% that of native HVJ due to a reduction in the density of fusion proteins on the surface of HVJ-liposomes. To simplify the vector system and to increase the efficiency of gene delivery, we incorporated plasmid DNA into inactivated HVJ particles without liposomes.[43]

As shown in Fig. 3, HVJ can be inactivated by exposure to β-propiolactone (0.0075–0.001%) or UV irradiation (99 mJ/cm^2), after which purification by ion-exchange column chromatography and gel filtration can be performed. The HVJ envelope has a diameter of 280 nm, and its zeta potential is approximately –5 mV.

Exogenous plasmid DNA can be incorporated into inactivated HVJ by treatment with mild detergent and centrifugation (10 000 *g*, 5–10 min). Many detergents, such as Triton X-100, NP-40, and deoxycholate, are available for preparation of the HVJ envelope vector. Without detergent treatment, DNA does not become incorporated into the viral particle. The DNA trapping efficiency of the HVJ envelope vector using this method is approximately 15–20%. Electron microscopy can be used to confirm incorporation of DNA into all inactivated HVJ particles. A 14 kb DNA plasmid is the largest piece of DNA delivered thus far, with a trapping efficiency of approximately 18%.

Similar quantities and molar ratios of F and HN fusion proteins are identified within HVJ envelope vectors as in

doi: 10.1111/j.1349-7006.2006.00189.x
© 2006 Japanese Cancer Association

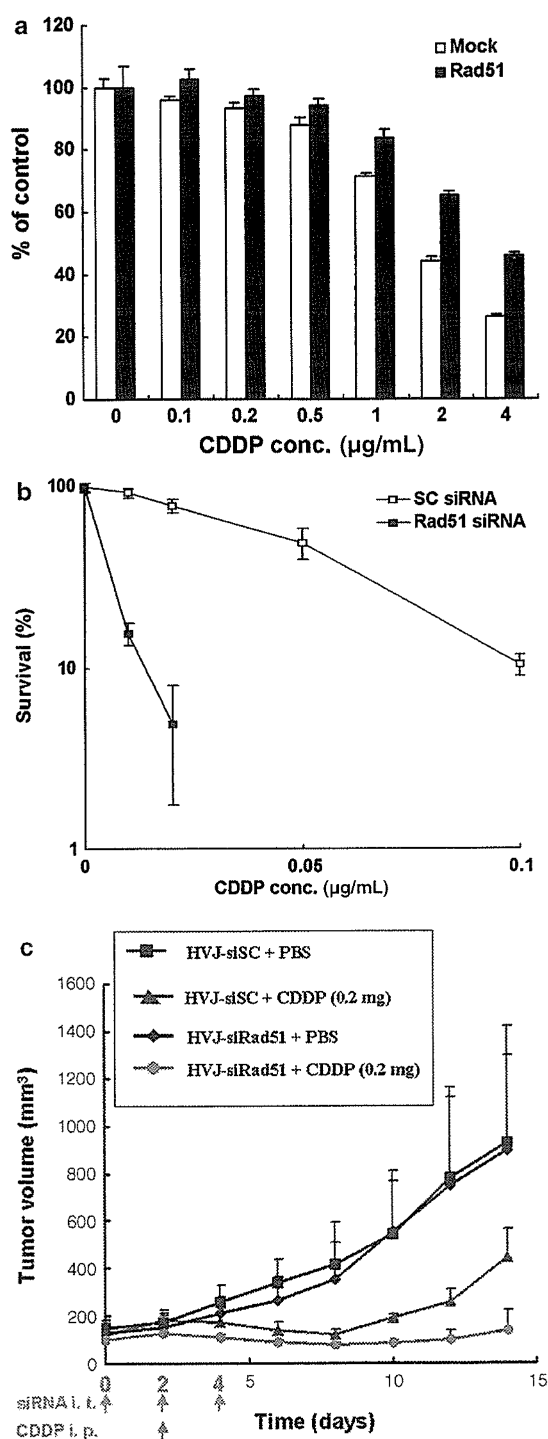


Fig. 4. Effect of *Rad51* on the sensitivity of cancer cells to *cis*-diamminedichloroplatinum (II) (CDDP). Over-expression of *Rad51* increased resistance to cell death caused by CDDP in HeLa cells (a). *Rad51* siRNA markedly inhibited colony formation of HeLa cells, compared with scrambled (SC) siRNA (b). When *Rad51* short interfering RNA (siRNA) was subcutaneously injected into HeLa tumor cell masses in nude mice three times using the hemagglutinating virus of Japan (HVJ) envelope vector, along with a single intraperitoneal administration of CDDP, significant suppression of tumor growth was observed (c).

native HVJ. Therefore, the HVJ envelope vector demonstrates similar fusion capacity as wild-type HVJ. Electron microscopic observations confirm fusion between the vector envelope and cell membrane within 3–5 s after attachment to the cell surface (Fig. 3).

The viral genome is eliminated from the HVJ envelope vector, therefore replication and viral gene expression are lacking in cells transfected with the HVJ envelope vector. Gene transfer to mouse muscle using the HVJ envelope vector is not diminished with repeated injections.[44] Following repeated injections, insufficient anti-HVJ antibody is generated to neutralize the HVJ envelope vector in mice, as unfortunately occurs with HVJ-liposomes.[45] This is probably the result of rapid fusion, such that fusion-mediated drug delivery is completed before sufficient binding of antibody to the vector.

The HVJ envelope vector is also available for drug delivery not involving gene therapy. Using the HVJ envelope vector, Cy3-labeled siRNA can be delivered into the cytoplasm of almost all cultured cells. Much attention has been paid to the use of siRNA for cancer treatment. However, it appears difficult to inhibit tumor growth using siRNA alone, especially *in vivo* because it is impossible to deliver siRNA into all cells of a tumor mass. A more practical approach to cancer therapy using siRNA might be to use siRNA to enhance the anticancer effects of chemotherapy or radiotherapy. CDDP, one of the most widely used anticancer drugs, inhibits cellular growth by inducing DNA double-strand breaks. However, cells can use DNA repair machinery to respond to DNA damage, thereby inducing resistance to anticancer drugs in human cancer cell lines. Rad51 plays a major role in homologous recombination repair machinery that is involved in the repair of double-strand DNA breaks generated by CDDP.[46] Overexpression of the human *Rad51* gene in HeLa cells induces resistance of HeLa cells to CDDP (Fig. 4a). When Rad51 siRNA is delivered to HeLa cells using the HVJ-E, Rad51 expression is completely knocked-out. As shown in Figure 4(b), colony numbers in the presence of 0.02 μg/mL CDDP are less than 10% of those observed in the absence of CDDP, following transfer of *Rad51* siRNA.[47] Combined treatment with cisplatin and *Rad51*siRNA significantly inhibits the growth of HeLa tumors (Fig. 4c). *Rad51* siRNA also increases the anticancer effects of another chemotherapeutic drug, bleomycin.

The HVJ envelope vector has enhanced transfection efficiency after conjugation with a biocompatible polymer[48] or magnetic beads.[49] Tissue-specific HVJ envelope vectors have been constructed (Y. Kaneda, unpublished data, 2005). A clinical grade HVJ envelope vector is currently being produced for use in clinical trials. Thus far, the virus has only been produced in chick eggs[32] however, egg-derived HVJ is difficult to use in clinical trials. It is also difficult to produce large amounts of the virus in cultured cells. However, we recently succeeded in producing large amounts of HVJ in human cells using animal product-free medium. Now we can produce more than $10^{(10)}$ particles/mL of culture medium of human cell-derived HVJ.[44] A pilot plant to commercially produce clinical grade HVJ envelope vector has already been established. Thus, a human cell-derived HVJ envelope vector is now ready for clinical use.

© 2006 Japanese Cancer Association

References

1 Mulligan RC. The basic science of gene therapy. *Science* 1993; **260**: 926–32.
2 Ledley FD. Nonviral gene therapy: the promise of genes as pharmaceutical products. *Hum Gene Ther* 1995; **6**: 1129–44.
3 Li S, Huang L. Nonviral gene therapy: promises and challenges. *Gene Ther* 2000; **7**: 31–4.
4 Wheeler CJ, Felgner PL, Tsai YJ *et al.* A novel cationic lipid greatly enhances plasmid DNA delivery and expression in mouse lung. *Proc Natl Acad Sci USA* 1996; **93**: 11454–9.
5 Wattiaux R, Laurent N, Wattiaux-De Cornick S, Jadot M. Endosomes, lysosomes: their implication in gene transfer. *Adv Drug Deliv Rev* 2000; **41**: 201–8.
6 Farhood H, Serbina N, Huang L. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim Biophys Acta* 1995; **1235**: 289–95.
7 El Ouahabi A, Thirry M, Pector V, Fuks R, Ruysschaert JM, Vandenbranden M. The role of endosome destabilizing activity in the gene transfer process mediated by cationic lipids. *FEBS Lett* 1997; **414**: 187–92.
8 Murray KD, Etheridge CJ, Shah SJ *et al.* Enhanced cationic liposome-mediated transfection using the DNA-binding peptide mu (mu) from the adenovirus core. *Gene Ther* 2001; **8**: 453–60.
9 Saeki Y, Matsumoto N, Nakano Y, Mori M, Awai K, Kaneda Y. Development and characterization of cationic liposomes conjugated with HVJ (Sendai virus): reciprocal effect of cationic lipid for *in vitro* and *in vivo* gene transfer. *Hum Gene Ther* 1997; **8**: 2133–41.
10 Bergen M, Chen R, Gonzalez R. Efficacy and safety of HLA-B7/beta-2 microglobulin plasmid DNA/lipid complex (Allovectin-7) in patients with metastatic melanoma. *Expert Opin Biol Ther* 2003; **3**: 377–84.
11 Yoshida J, Mizuno M, Fujii M *et al.* Human gene therapy for malignant gliomas (glioblastoma multiforme and anaplastic astrocytoma) by *in vivo* transduction with human interferon beta gene using cationic liposomes. *Hum Gene Ther* 2004; **15**: 77–86.
12 Siehl JM, Thiel E, Schmittel A *et al.* Ifosfamide/liposomal daunorubicin is a well tolerated and active first-line chemotherapy regimen in advanced soft tissue sarcoma: results of a phase II study. *Cancer* 2005; **104**: 611–7.
13 Krown SE, Northfield DW, Osoba D, Stewart JS. Use of liposomal anthracyclines in Kaposi's sarcoma. *Semin Oncol* 2004; **31** (6 Suppl. 13): 36–52.
14 Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ. Cellular and molecular barriers to gene transfer by a cationic lipid. *J Biol Chem* 1995; **270**: 18997–9007.
15 Mesika A, Kiss V, Brumfeld V, Ghosh G, Reich Z. Enhanced intracellular mobility and nuclear accumulation of DNA plasmids associated with a karyophilic protein. *Hum Gene Ther* 2005; **16**: 200–8.
16 Subramanian A, Ranganathan P, Diamond SL. Nuclear targeting peptide scaffolds for lipofection of nondividing mammalian cells. *Nat Biotechnol* 1999; **17**: 873–7.
17 Demeneix B, Behr J, Boussif O, Zanta MA, Abdallah B, Remy J. Gene transfer with lipospermines and polyethylenimines. *Adv Drug Deliv Rev* 1998; **30**: 85–95.
18 Wolfert MA, Schacht EH, Toncheva V, Ulbrich K, Nazarova O, Seymour LW. Characterization of vectors for gene therapy formed by self-assembly of DNA with synthetic block co-polymers. *Hum Gene Ther* 1996; **7**: 2123–33.
19 Sudimack J, Lee RJ. Targeted drug delivery via the folate receptor. *Adv Drug Deliv Rev* 2000; **41**: 147–62.
20 Qian ZM, Li H, Sun H, Ho K. Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. *Pharmacol Rev* 2002; **54**: 561–87.
21 Hashida M, Nishikawa M, Yamashita F, Takakura Y. Cell-specific delivery of genes with glycosylated carriers. *Adv Drug Deliv Rev* 2001; **52**: 187–96.
22 Shea LD, Smiley E, Bonadio J, Mooney DJ. DNA delivery from polymer matrices for tissue engineering. *Nat Biotechnol* 1999; **17**: 551–4.
23 Kushibiki T, Matsumoto K, Nakamura T, Tabata Y. Suppression of tumor metastasis by NK4 plasmid DNA released from cationized gelatin. *Gene Ther* 2004; **11** (15): 1205–14.
24 Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J Control Release* 2000; **65**: 271–84.
25 Hosseinkhani H, Tabata Y. PEGylation enhances tumor targeting of plasmid DNA by an artificial cationized protein with repeated RGD sequences, Pronectin. *J Control Release* 2004; **97**: 157–71.
26 Wells D. Gene therapy progress and prospects: electroporation and other physical mthods. *Gene Ther* 2004; **11**: 1363–9.
27 Liu F, Huang L. A syringe electrode device for simultaneous injection of DNA and electrotransfer. *Mol Ther* 2002; **3**: 323–8.
28 Hofmann GA, Deb SB, Nanda GS, Rabussay D. Electroporation therapy of solid tumors. *Crit Rev Ther Drug Carrier Syst* 1999; **16**: 523–69.
29 Taniyama Y, Tachibana K, Hiraoka K *et al.* Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle. *Gene Ther* 2002; **9**: 372–80.
30 Kim HJ, Greenleaf JF, Kinnick RR, Bronk JT, Bolander ME. Ultrasound-mediated transfection of mammalian cells. *Hum Gene Ther* 1996; **7**: 1339–46.
31 Kaneda Y, Saeki Y, Morishita R. Gene therapy using HVJ-liposomes: the best of both worlds. *Mol Med Today* 1999; **5**: 298–303.
32 Okada Y. Sendai virus-induced cell fusion. In: Duzgunes N, ed. *Methods in Enzymology*, Vol. 221. San Diego: Academic Press, 1993; 18–41.
33 Nakamura N, Hart DA, Frank CB *et al.* Efficient transfer of intact oligonucleotides into the nucleus of ligament scar fibroblasts by HVJ-cationic liposomes is correlated with effective antisense gene inhibition. *J Biochem* 2001; **129**: 755–9.
34 Wagner E, Plank C, Zatloukal K, Cotten M, Birnstiel ML. Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. *Proc Natl Acad Sci USA* 1992; **89**: 7934–8.
35 Zhou W-Z, Hoon DSB, Huang SKS *et al.* RNA melanoma vaccine: induction of anti-tumor immunity by human glycoprotein 100 mRNA immunization. *Hum Gene Ther* 1999; **10**: 2719–24.
36 Kawashita Y, Ohtsuru A, Miki F *et al.* Eradication of hepatocellular carcinoma xenografts by radiolabelled lipiodol-inducible gene therapy. *Gene Ther* 2005; **12**: 1633–9.
37 Saeki Y, Wataya-Kaneda M, Tanaka K, Kaneda Y. Sustained transgene expression *in vitro* and *in vivo* using an Epstein–Barr virus replicon vector system combined with HVJ liposomes. *Gene Ther* 1998; **5**: 1031–7.
38 Otomo T, Yamamoto S, Morishita R, Kaneda Y. EBV replicon vector system enhances transgene expression *in vivo*: applications to gene therapy for cancer. *J Gene Med* 2001; **3**: 345–52.
39 Jahner D, Stuhlmann H, Stewart CL *et al. De novo* methylation and expression of retroviral genomes during mouse embryogenesis. *Nature* 1982; **298**: 623–8.
40 Henikoff S, Ahmad K. Assembly of variant histones into chromatin. *Annu Rev Cell Dev Biol* 2005; **21**: 133–53.
41 Cheng X, Collins RF, Zhang X. Structural and sequence motifs of protein (histone) methylation enzymes. *Annu Rev Biophys Biomol Struct* 2005; **34**: 267–94.
42 Yamamoto S, Yamano T, Tanaka M *et al.* A novel combination of suicide gene therapy and histone deacetylase inhibitor for treatment of malignant melanoma. *Cancer Gene Ther* 2003; **10**: 179–86.
43 Kaneda Y, Nakajima T, Nishikawa T *et al.* Hemagglutinating virus of Japan (HVJ) envelope vector as a versatile gene delivery system. *Mol Ther* 2002; **6**: 219–26.
44 Kaneda Y, Yamamoto S, Nakajima T. Development of HVJ envelope vector and its application to gene therapy. In: Huang L, Hung M-C, Wagner E, eds. *Non-Viral Vectors for Gene Therapy.* London: Elsevier Academic Press, 2005; 307–32.
45 Hirano T, Fujimoto J, Ueki T *et al.* Persistent gene expression in rat liver *in-vivo* by repetitive transfections using HVJ-liposome. *Gene Ther* 1998; **5**: 459–64.
46 Aloyz R, Xu ZY, Bello V *et al.* Regulation of cisplatin resistance and homologous recombinational repair by the TFIIH subunit XPD. *Cancer Res* 2002; **62**: 5457–62.
47 Ito M, Yamamoto S, Nimura K, Hiraoka K, Tamai K, Kaneda Y. Rad51 siRNA delivered by HVJ envelope vector enhances the anticancer effect of cisplatin. *J Gene Med* 2005; **7**: 1044–52.
48 Mima H, Tomoshige R, Kanamori T *et al.* Biocompatible polymer enhances the *in vitro* and *in vivo* transfection efficiency of HVJ envelope vector. *J Gene Med* 2005; **7**: 888–97.
49 Morishita N, Nakagami H, Morishita R *et al.* Magnetic nanoparticles with surface modification enhanced gene delivery of HVJ-E vector. *Biochem Biophys Res Comm* 2005; **31**: 1121–6.

doi: 10.1111/j.1349-7006.2006.00189.x
© 2006 Japanese Cancer Association