

A decoy 18-sense	5'- CCGGG <u>GCTTTGATC</u> TTTGC - 3'
mismatch 18-sense	5'- CCGGGGCTT <u>G</u> IATCTTTGC - 3'
decoy 14-sense	5'- GGG <u>GCTTTGATC</u> TTT - 3'
mismatch 14-sense	5'- GGGGCTT <u>G</u> IATCTTT - 3'
decoy 12-sense	5'- GG <u>GCTTTGATC</u> TT - 3'
mismatch 12-sense	5'- GGCTT <u>G</u> IATCTT - 3'
decoy 9-sense	5'- <u>GCTTTGATC</u> - 3'
mismatch 9-sense	5'- GCTT <u>G</u> IATC - 3'
decoy 6-sense	5'- <u>CTTTGA</u> - 3'
mismatch 6-sense	5'- CTT <u>G</u> IA - 3'

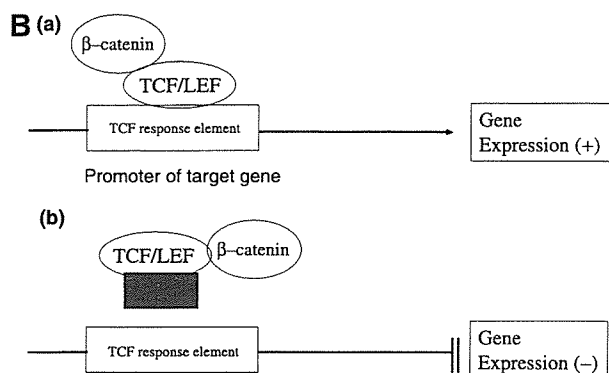


Figure 2. **A**, design of TCF decoy and two-base mismatch control DNA. Based on the consensus TCF-binding site *GCTTTGATC* mapped at 25 bp, 5' of the cyclic AMP-responsive element binding protein-binding site within the *cyclin D1* promoter, a series of oligodeoxynucleotide decoy constructs of various lengths were designed. Negative-control oligodeoxynucleotides with two base replacements were also prepared. **B**, theoretical scheme of TCF decoy strategy. **a**, normal β -catenin signaling. β -catenin/TCF complex binds to the promoter of a target gene and facilitates gene expression. **b**, inactivation of β -catenin signaling. The β -catenin/TCF complex may preferentially bind to the TCF decoy, which competitively inhibits target gene activation.

up to 96 hours in HEK293 and 72 hours in HCT116 (Fig. 4A and B). Similar results were obtained with the 18-mer oligodeoxynucleotide with two base replacements (data not shown).

Effects of a Series of Mismatch DNA Constructs on TCF Decoy

It remained possible that decoy DNA with some amount of mismatch to the TCF response element might still retain the ability to bind the TCF/ β -catenin complex. To pursue this possibility, we additionally designed and tested several control oligodeoxynucleotides with two, three, five, or eight base mismatches within the consensus sequence (*GCTTTGATC*; Fig. 5A). The results indicated that, as the number of mismatched bases in the decoy increased, competition with the natural TCF response sequence declined and TCF activity increased in HCT116 (Fig. 5B). When compared with a "scrambled" sequence (eight base mismatch) oligodeoxynucleotide, the TCF decoy significantly reduced the TCF promoter activity, and the

promoter activities of cyclin D1, c-myc, and MMP7 (Fig. 5C). RT-PCR assays indicated that the amounts of mRNA products of the downstream target genes were similar in cells 3 hours after addition of TCF decoy or

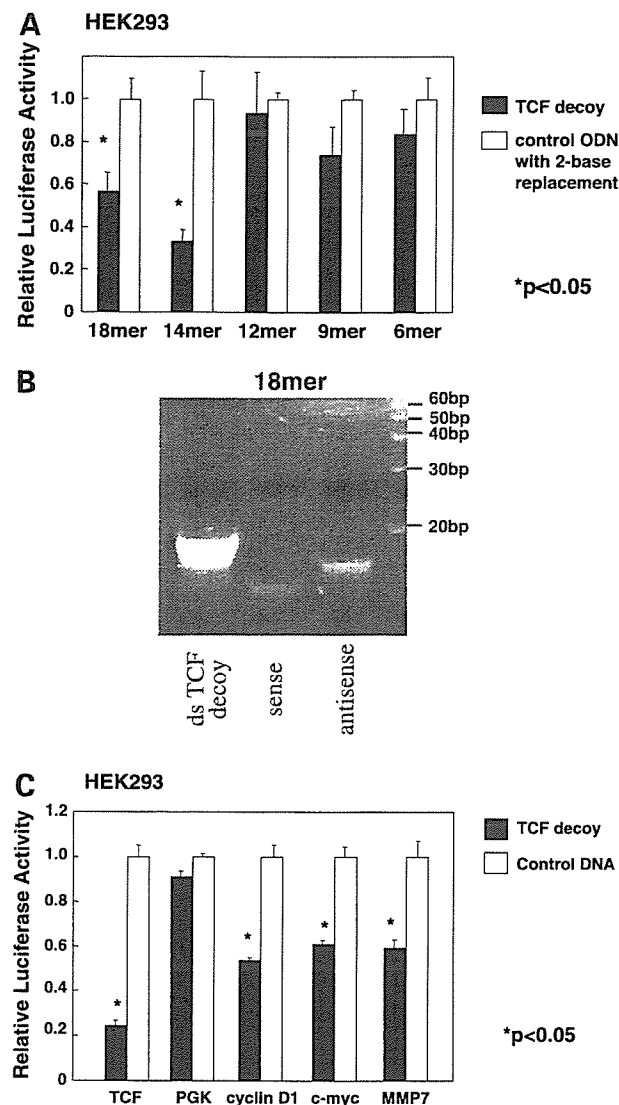


Figure 3. Inhibition of TCF activity in β -catenin-activated HEK293 cells. **A**, the HEK293 cells were transfected with β -catenin plasmid (1 μ g), TOPflash reporter plasmid (0.5 μ g), and double-stranded DNA (0.1 μ mol/L) using LipofectAMINE 2000. The 14- and 18-mer TCF decoy double-stranded oligodeoxynucleotides caused a significantly large reduction in TCF promoter activity ($P < 0.05$ for each). The 6-, 9-, or 12-mer TCF decoy showed no significant decrease. **B**, SDS-PAGE of 18-mer oligodeoxynucleotide. The double-stranded (ds) oligodeoxynucleotide TCF decoy appeared below 20 bp, whereas single sense strand oligodeoxynucleotide and single antisense strand oligodeoxynucleotide appeared at the lower levels. The upper black bands below 30 bp are blue dye. **C**, the 18-mer TCF decoy at 0.1 μ mol/L inhibited TCF activity and promoter activities of cyclin D1, c-myc, and MMP7. *, $P < 0.05$, statistically significant. The PGK/*luc* plasmids contain the firefly luciferase gene under control of the PGK promoter. TCF decoy did not affect PGK-driven luciferase expression.

scrambled oligodeoxynucleotide. However, at 24 hours posttreatment, mRNA levels for cyclin D1, MMP7, and PPAR δ decreased in cells transfected with the TCF decoy, but not in cells transfected with the scrambled oligodeoxynucleotide (Fig. 5D).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay showed that the labeled TCF probe bound to the nuclear extracts from HCT116 (Fig. 6, lane 2). Unlabeled TCF probe competitively blocked the binding (lane 3). The TCF decoy decreased the binding (lane 5) when compared with the scrambled oligodeoxynucleotide (lane 4).

Effects of the TCF Decoy on Cell Growth

Using LipofectAMINE 2000 as a vehicle, scrambled oligodeoxynucleotides at various concentrations ranging 0.025 to 0.5 μ mol/L were administered for their potential cytotoxicity to HCT116. As concentration increased, scrambled oligodeoxynucleotide displayed a dose-dependent cytotoxicity (Fig. 7A). Thus, 0.025 to 0.5 μ mol/L oligodeoxynucleotide was apparently toxic and even 0.1 μ mol/L oligodeoxynucleotide exhibited a cytotoxicity at late time points of 72 to 96 hours. At 0.05 μ mol/L, a TCF decoy significantly inhibited cell growth when compared with the scrambled oligodeoxynucleotide (mismatch-8) and two base

mismatch oligodeoxynucleotide (mismatch-2a) at 24 and 48 hours (Fig. 7B). Significant inhibition was also noted at 72 and 96 hours between a TCF decoy and the scrambled oligodeoxynucleotide. On the other hand, no inhibitory effect by the TCF decoy was observed in HEK293 cells (Fig. 7C).

Discussion

The strategy of using DNA response element decoys to block transcription factors was originally reported for use of E2F and nuclear factor- κ B decoys in vascular occlusion disease (18, 19), and with signal transducers and activators of transcription-3, activator protein-1, and nuclear factor- κ B decoys in cancer therapy (17, 20, 21). Although a TCF decoy was theoretically possible (28), it was not shown before the work we report here. As mentioned in the Introduction, various human malignancies have alterations of genes in the Wnt signaling pathway. Colorectal cancer cells often harbor mutations in the APC gene and/or β -catenin gene. These mutations result in accumulation of β -catenin, leading to β -catenin/TCF-mediated transactivation (2). Accumulation of β -catenin has also been reported in other gastrointestinal cancers that have a low frequency of mutation in the APC and β -catenin genes. These include

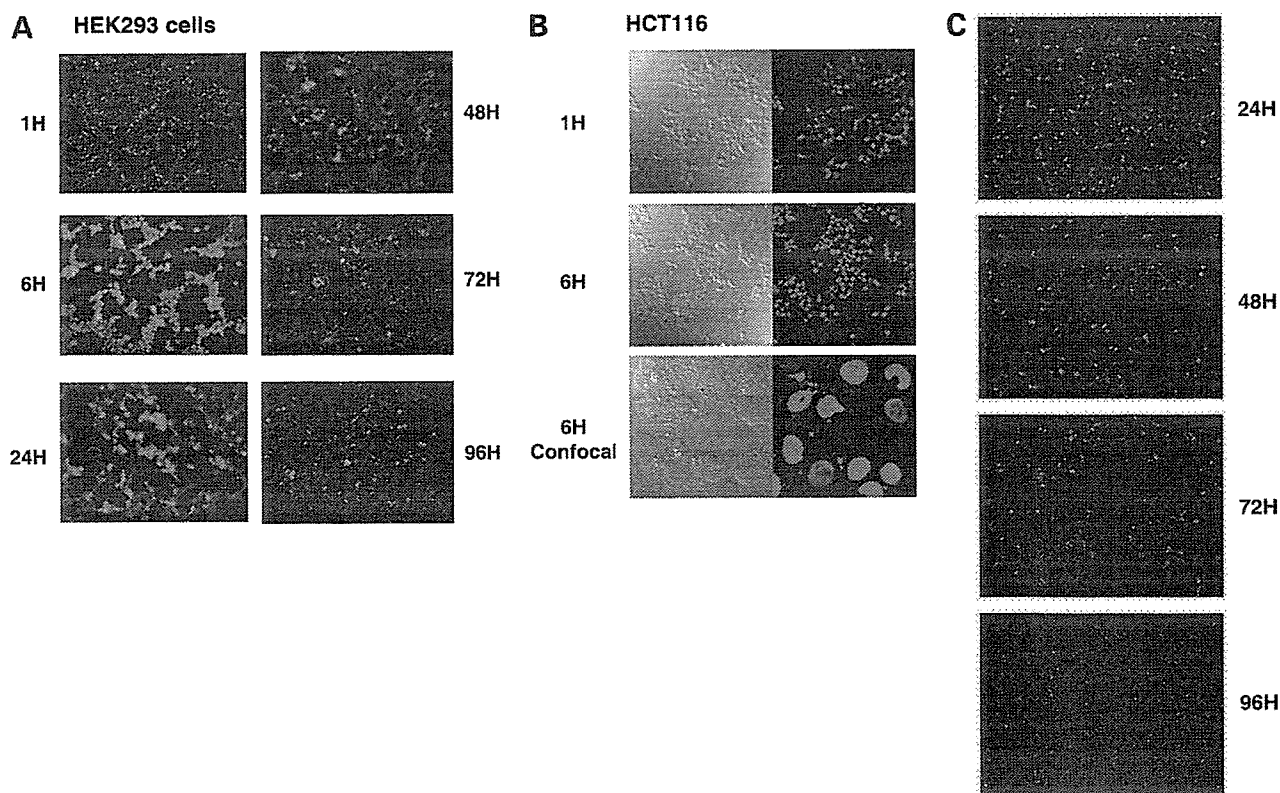


Figure 4. Incorporation of FITC-conjugated TCF decoy (18-mer). **A**, in HEK293 cells, almost 100% of the FITC-conjugated TCF decoy (0.1 μ mol/L) was detected in the cell nuclei at 6 h after transfection. The decoy was detectable for up to 96 h. **B**, in HCT116 colon cancer cells, the labeled decoy (0.1 μ mol/L) appeared in the cells as early as 1 h after transfection and it was detected in almost all cells at 6 h. Confocal laser microscopy clearly revealed an incorporation of decoy into nucleus. The decoy was detectable for up to 72 h.

an estimated 68% of esophageal squamous cell carcinomas, 46% of gastric cancers, and 68% of hepatocellular carcinomas (29–31). These findings suggest involvement of other regulatory proteins, such as Axin and ICAT (32–34). Studies have been done to correct some abnormalities of Wnt signaling in human cancers. These include gene delivery by transfection of the wild-type full-length *APC* gene, restoration of the central third of the *APC* gene containing the β -catenin binding domain, introduction of a small interfering RNA to block β -catenin transcription, and overexpression of Axin (35–38). The strategy of introducing a TCF decoy DNA is attractive because it is applicable to a broad range of gastrointestinal cancers that display TCF hyperactivity, irrespective of the particular molecular abnormality in the Wnt pathway.

In the initial screening step, we established the β -catenin activated model in nontumor HEK293 cells. This system allowed us to focus on the role of excess β -catenin as the primary, or possibly the only variable triggering oncogenicity. This is superior to screening using tumor cells, in which other genetic abnormalities might affect experiments with the TCF decoy. Another advantage of this system is that we were able to adjust the level of TCF activity by varying the amount of β -catenin gene transfected into the HEK293 cells. We hypothesized that the β -catenin/TCF level of tumor cells might be too high to be down-regulated competitively by the TCF decoy, so that the effect of decoy may be obscured or not observable in such a situation.

Using this system, we found that relatively long 14- and 18-mer double-stranded oligodeoxynucleotides inhibited the TCF activity, whereas shorter double-stranded oligodeoxynucleotides did not, possibly because of strand separation or nucleolytic degradation of the shorter oligodeoxynucleotides. The 14-mer double-stranded oligodeoxynucleotide strongly inhibited TCF activity in our early tests, but it was progressively less inhibitory in later experiments. Analyses by SDS-PAGE showed that the 14-mer double-stranded oligodeoxynucleotide was not stable to prolonged storage. By contrast, the 18-mer double-stranded oligodeoxynucleotides retained duplex structure stably even after extended storage. For subsequent experiments, therefore, we used 18-mer double-stranded oligodeoxynucleotide that stably formed double-stranded DNA even after long storage.

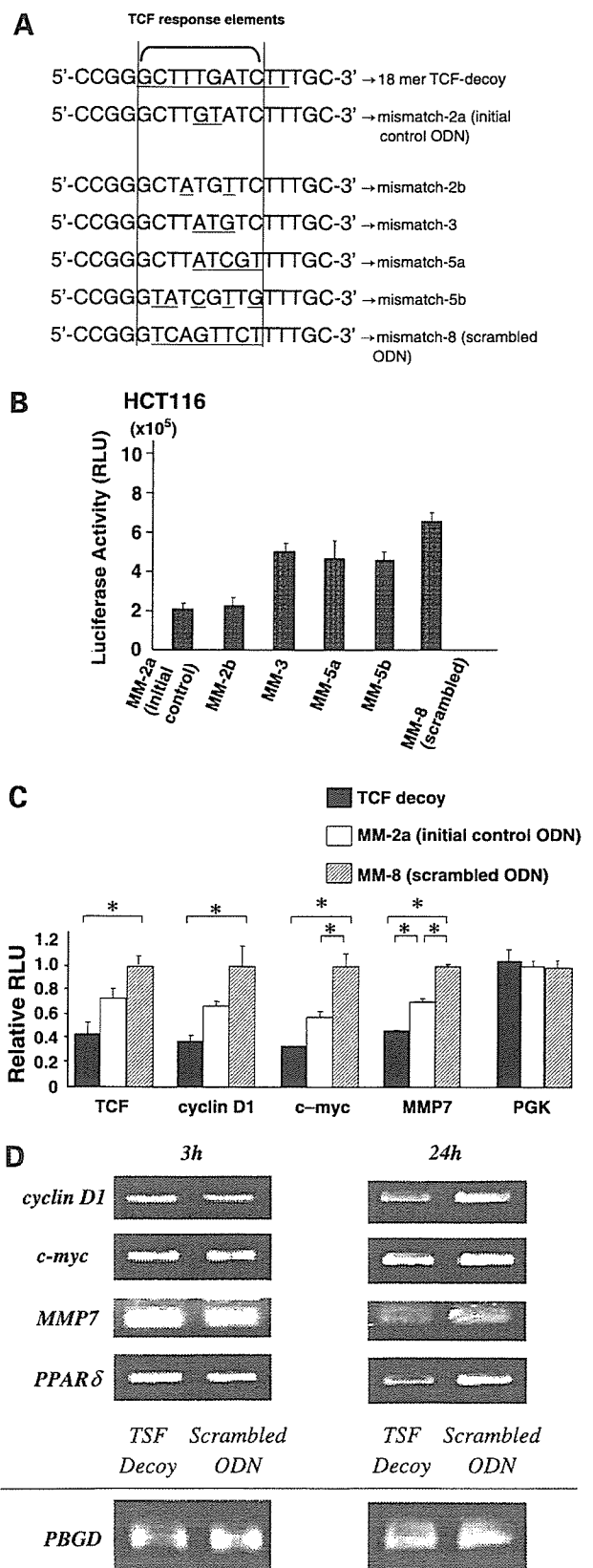


Figure 5. Construction of mismatch DNAs and their effects on TCF activity. **A**, besides the original control 18-mer oligodeoxynucleotide with two base replacements (mismatch-2a), a series of additionally synthesized oligodeoxynucleotides that have two, three, five, or eight base mismatches within the consensus sequence (GCTTTGATC) were designed. Mismatch-8 with eight base replacements was termed scrambled oligodeoxynucleotide. **B**, as the number of mismatched bases increased, TCF activity increased in HCT116. *MM*, mismatch (concentration, 0.1 μ mol/L). **C**, the TCF decoy at 0.1 μ mol/L, when compared with scrambled DNA (*MM-8*), significantly inhibited TCF activity and promoter activities of downstream target genes. *, statistical significance. **D**, RT-PCR assays indicated that the amounts of RNA products of the target genes were similar between cells 3 h after transfection with TCF decoy or scrambled DNA at 0.1 μ mol/L. At 24 h posttreatment, cyclin D1 RNA as well as c-myc, MMP7, and PPAR δ RNA levels were decreased in cells that received the TCF decoy.

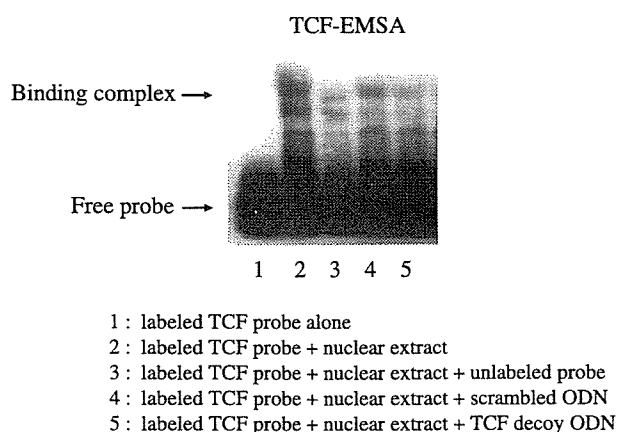


Figure 6. Electrophoretic mobility shift assay. The following reaction mixtures were run on 6% native polyacrylamide gels. Nuclear extracts containing active β -catenin/TCF complex were prepared from HCT116. Lane 1, labeled TCF probe (1.5 pmol) alone. Lane 2, labeled TCF probe + nuclear extract (5 μ g). 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 antiviral antibody response that may lessen its

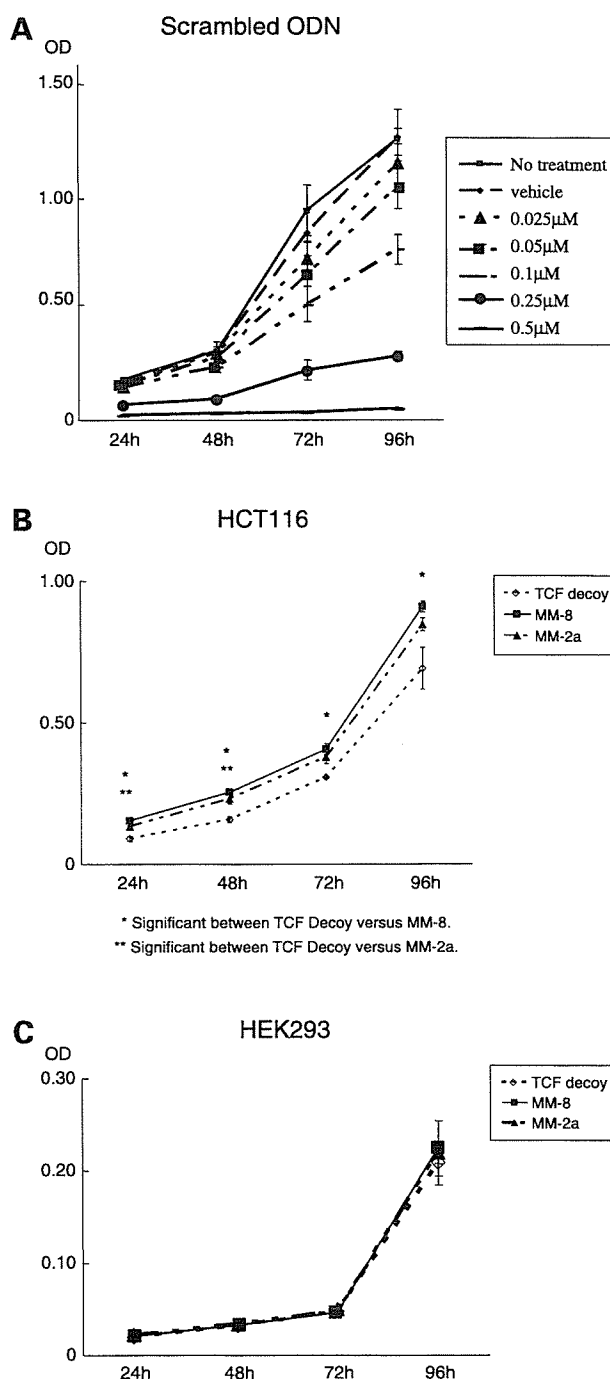


Figure 7. Growth assays. Twelve hours after transfection, cells were harvested and then seeded at 4,000 cells per 100 μ L medium into 96-well plates. Cell growth was determined using WST-1 assay kit (Dojindo Laboratories; ref. 27) at indicated time point after transfection. **A**, dose-dependent cytotoxicity 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 μ mol/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.

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

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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 fibrillary 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 humans¹ 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 MCAo¹³ and up to 14 days in FGF³ 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)-

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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/cm²) 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 protocol²⁷ 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% H₂O₂ 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

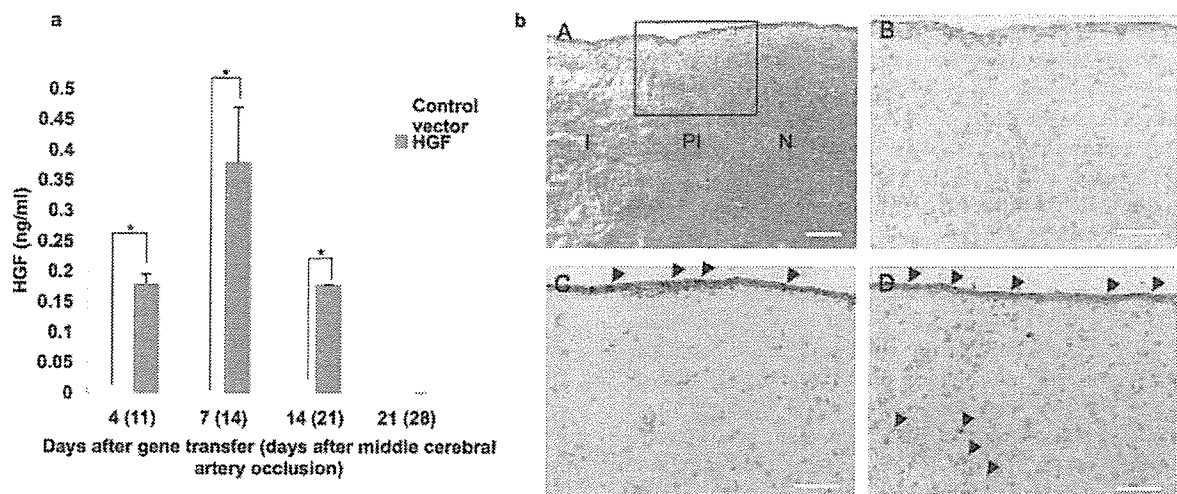


Figure 1. (a) Concentrations of human HGF in cerebrospinal fluid at 4, 7, 14, and 21 days after gene transfer (11, 14, 21, and 28 days after middle cerebral artery occlusion). Control vector indicates rats transfected with control vector (n=4); HGF, rats transfected with HGF vector (n=4). * $P<0.01$ 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 naphthol 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 \pm 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

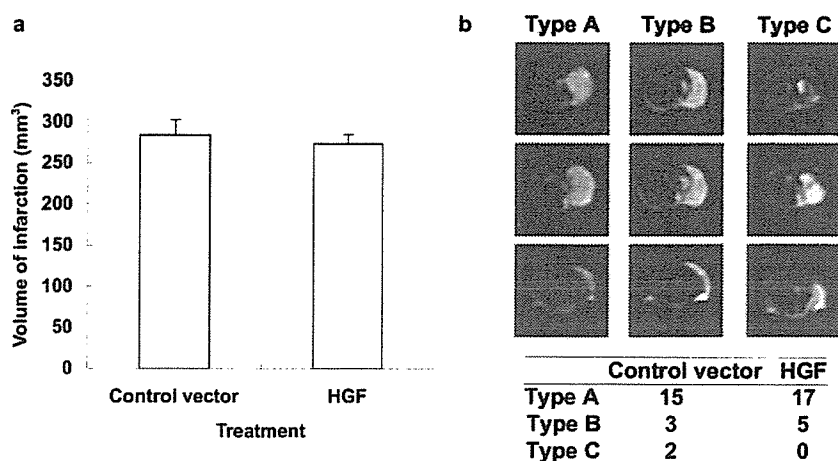


Figure 2. Magnetic resonance images of brain. (a) Volume of infarction in all rats calculated in T2-weighted images. Control vector indicates rats transfected with control vector (n=20); HGF, rats transfected with HGF vector (n=22). (b) Typical T2-weighted image of coronal section of rat brain. The images 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 $87.1 \pm 8.1\%$, HGF $81.0 \pm 4.3\%$; 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

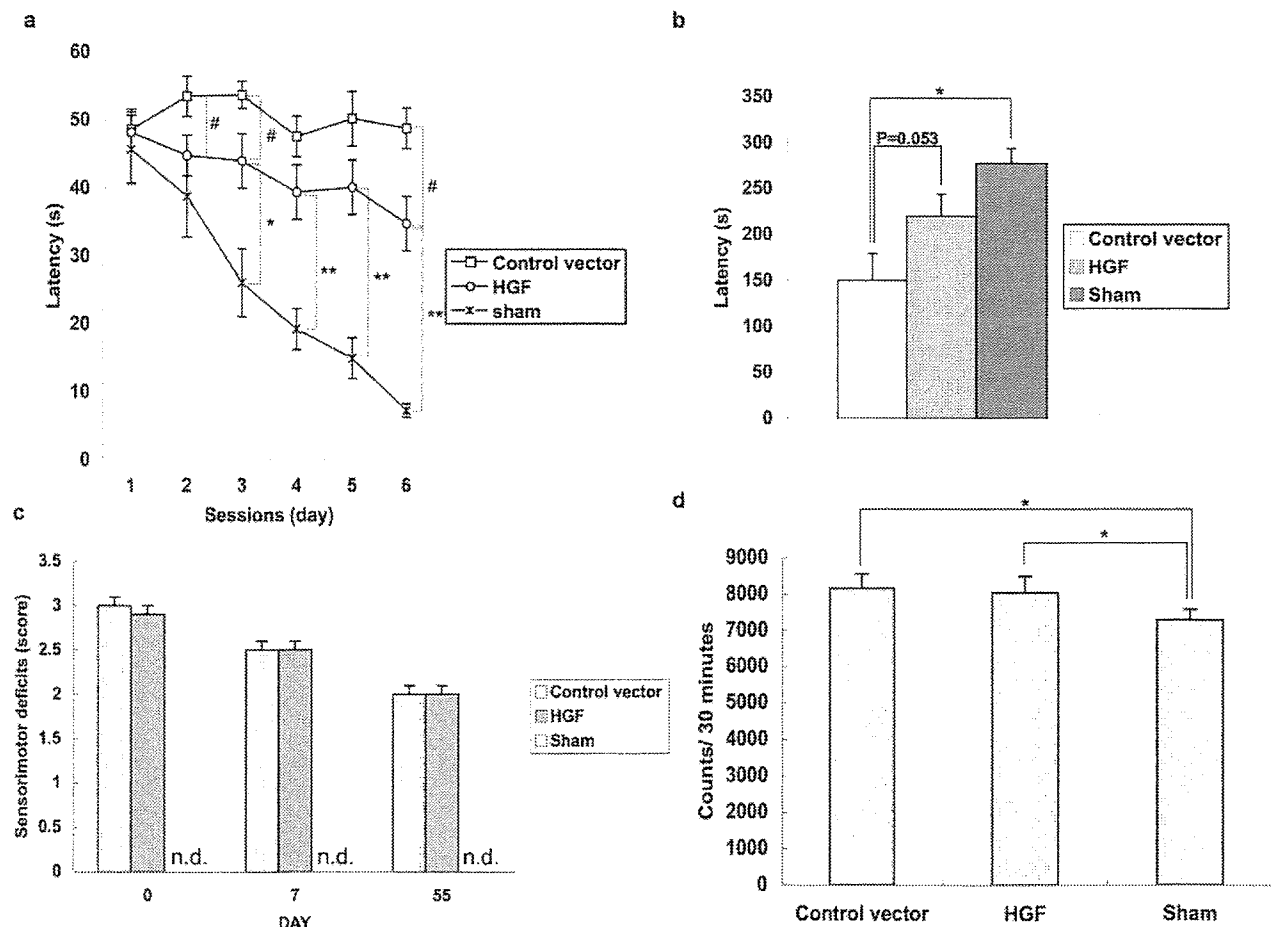


Figure 3. Learning and memory in the chronic stage of cerebral infarction. (a) Hidden platform test in MWM test in all rats. Although rats subjected to middle cerebral artery occlusion hardly reached the hidden platform as compared with sham-operated rats, rats transfected HGF vector could reach faster 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.

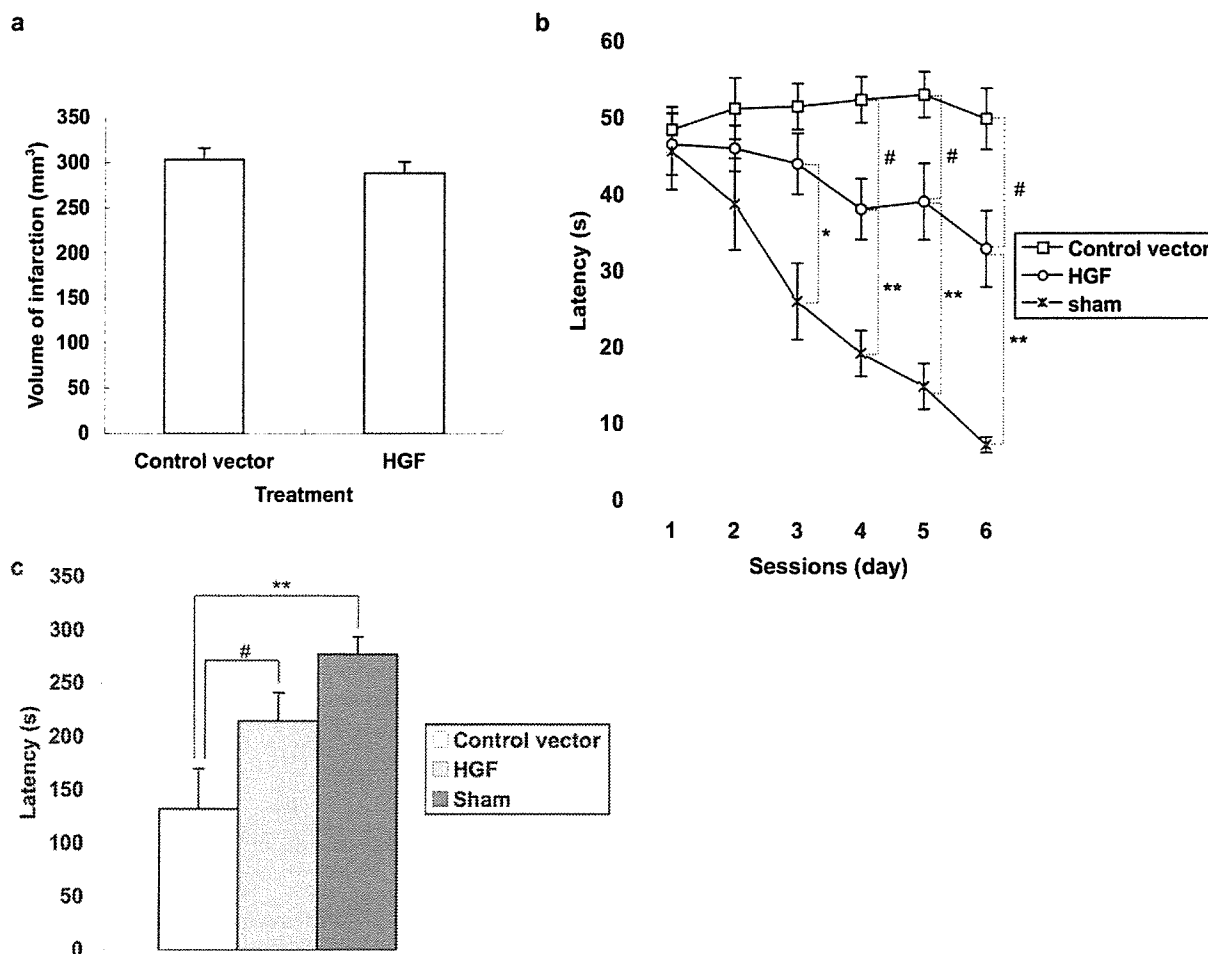


Figure 4. (a) Volume of infarction in type A rats. (b) Hidden platform test in MWM test in type A rats. (c) Retention trial in passive avoidance task in type A rats. Control vector indicates rats transfected with control vector ($n=15$); HGF, rats transfected with HGF vector ($n=17$); Sham, sham-operated 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 ischemia³⁶ 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-

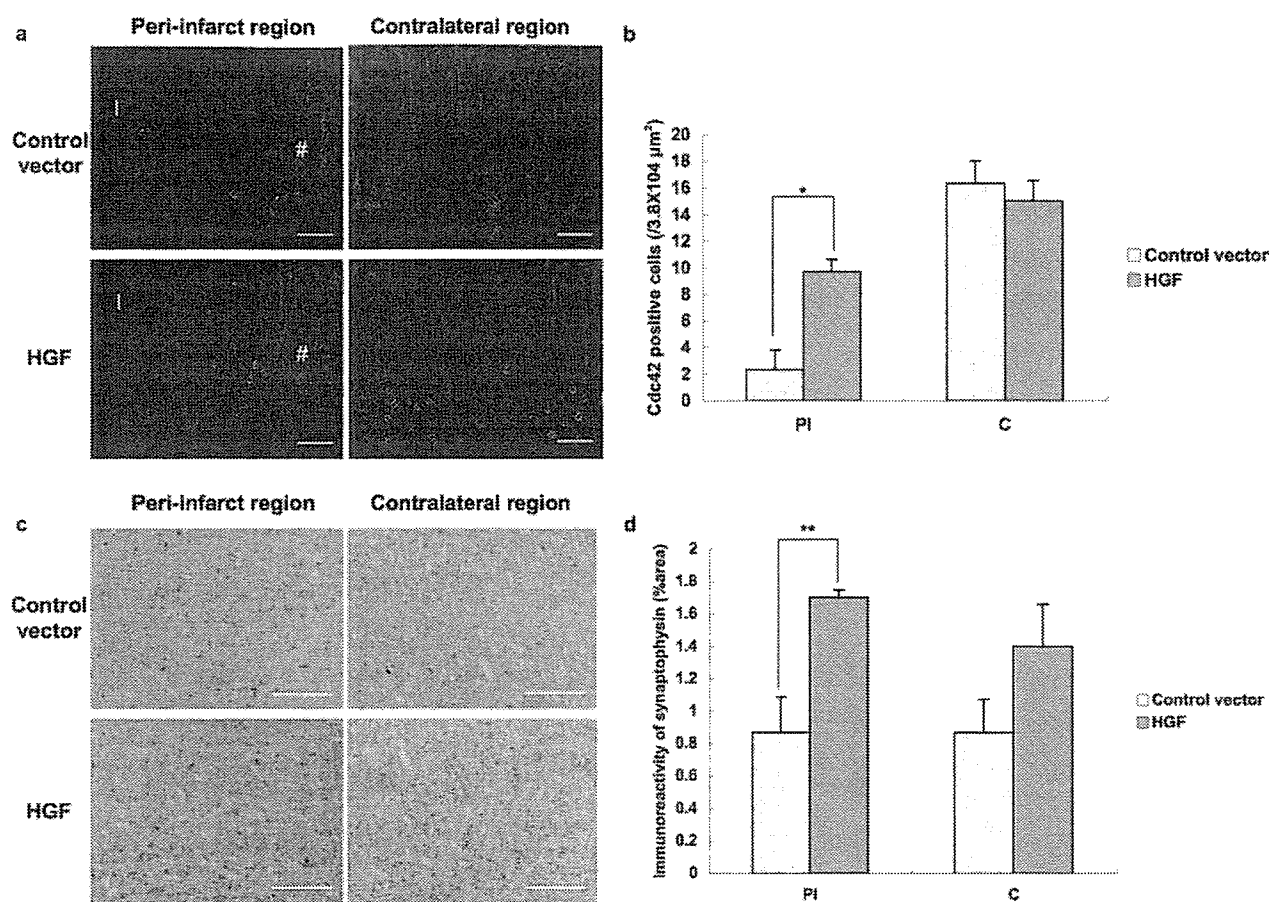


Figure 5. (a) Representative images of immunohistochemical staining for Cdc42 on day 14 in rats transfected with control and HGF vector. The number of cells immunoreactive for Cdc42 was significantly increased in the pyramidal neurons in the peri-infarct region (#) of rats transfected with HGF vector. 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

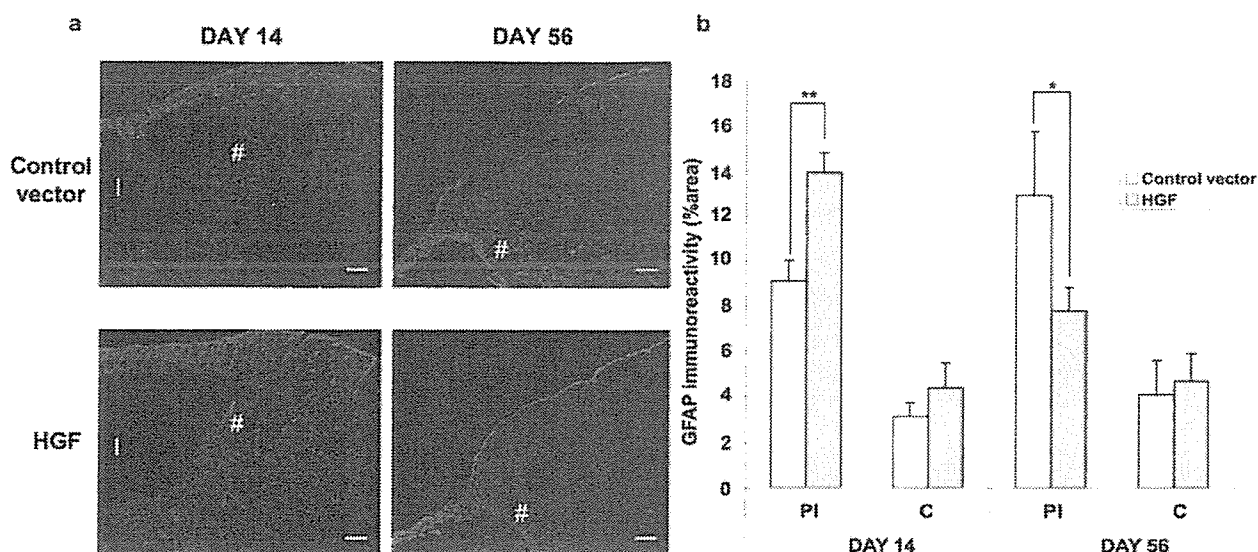


Figure 6. (a) Representative images of immunohistochemical staining for GFAP in ipsilateral neocortex on day 14 and 56 in rats transfected with control and HGF vector. Positive staining for GFAP was increased on day 14 and decreased on day 56 in the peri-infarct region (#) in rats transfected with HGF vector. I indicates infarct region (n=4 in each group; bar=100 μ m.) (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 lamellipodia, 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 al⁴⁶ showed the existence of neurogenesis, but Zhang et al⁴⁷ 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.

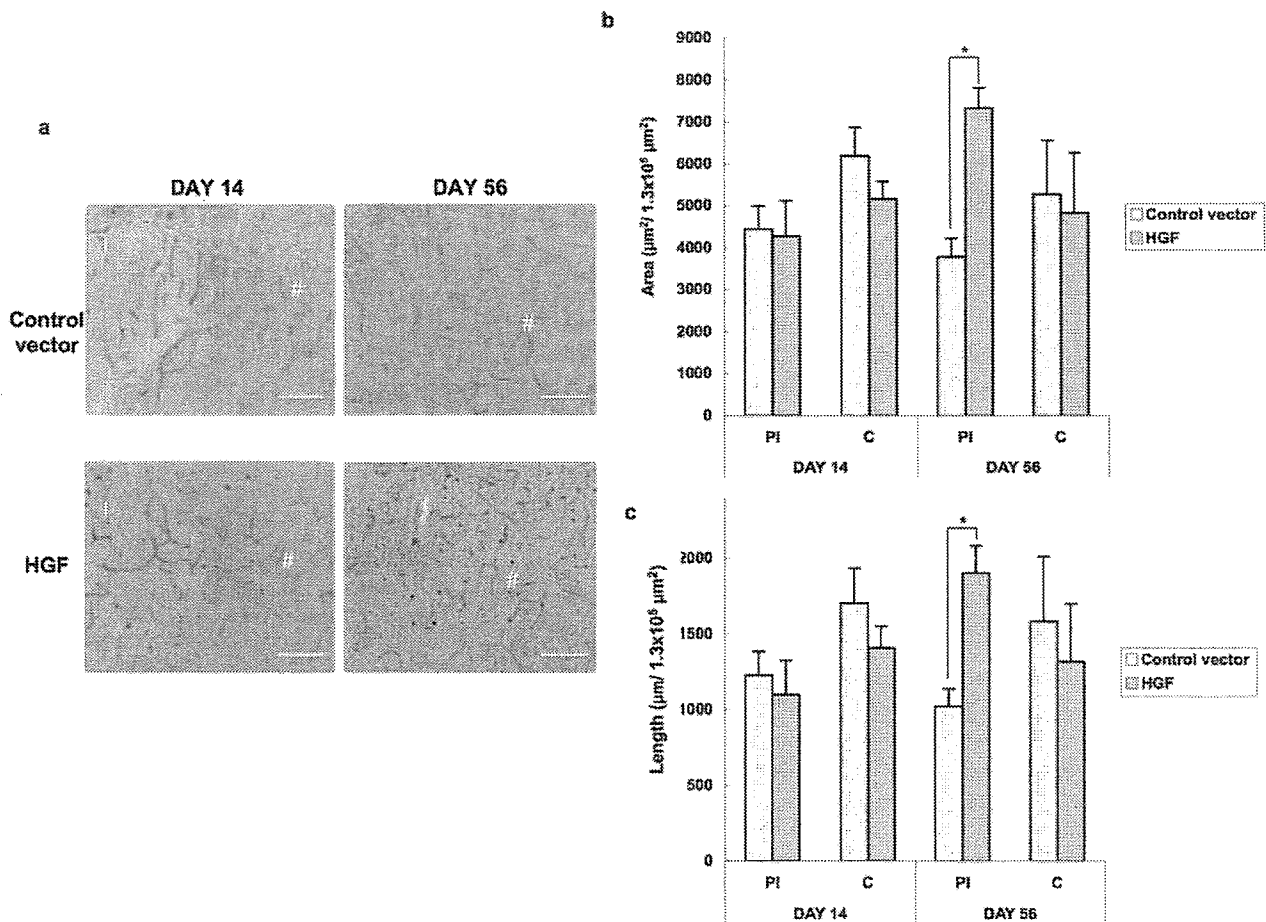


Figure 7. (a) Typical images of ALP staining in ipsilateral neocortex on day 14 in rats transfected with control and HGF vector. Coronal sections of ipsilateral neocortex stained for ALP. The structure of arteries in the peri-infarct region (#) was not different on day 14. However, the arteries in rats 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 neurons¹⁷ 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

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Non-viral vectors for cancer therapy

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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 m μ 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 (Allovecin-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

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Abbreviations: AS-ODN, antisense oligodeoxynucleotides; CDDP, *cis*-diaminedichloroplatinum (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.

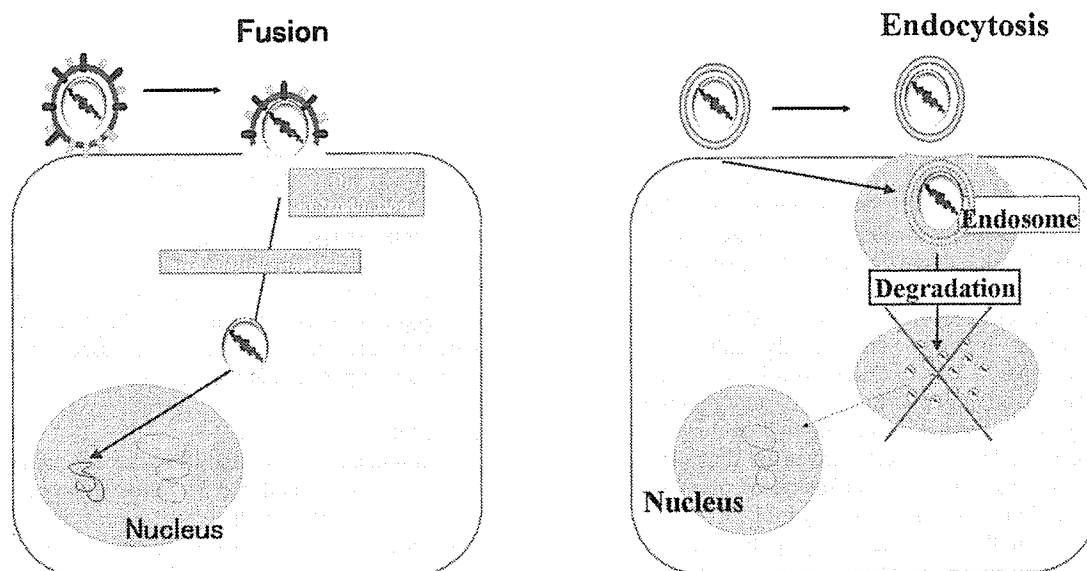


Fig. 1. Pathway by which therapeutic molecules are introduced into cells by liposomes or hemagglutinating virus of Japan (HVJ)-liposomes. Molecules are delivered by liposomes into cells by endocytosis by way of the endocytotic pathway, which makes cells susceptible to degradation. However, molecules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

(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 plasmid 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 NH₂-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 μ m 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.

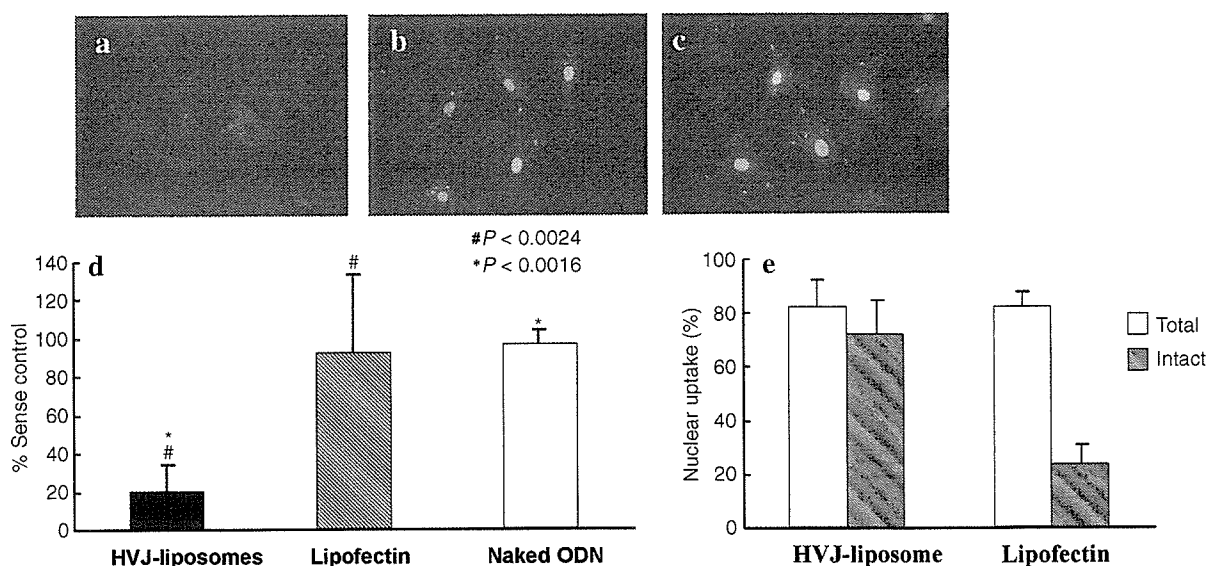


Fig. 2. Uptake of fluorescent isothiocyanate (FITC)-labeled AS-ODN against decorin into human fibroblasts demonstrated by fluorescence microscopy (a–c). No fluorescence was seen in cells administered antisense oligodeoxynucleotides (AS-ODN) alone (a), however, fluorescence was detected in the nuclei 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 lysed 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⁾