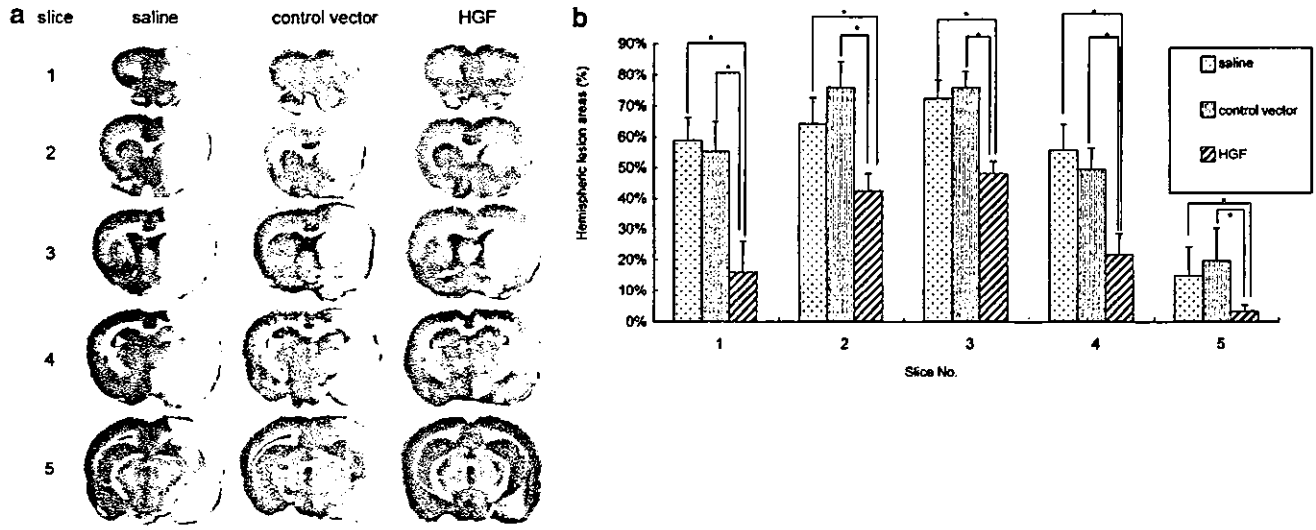

Figure 2. a, Reduction of infarcted area by HGF gene transfer: coronal sections stained with TTC at 24 hours after MCA occlusion. Red region shows intact area; white region shows infarcted area. b, Quantification of hemispheric lesion area 24 hours after ischemia. Corrected HLA was calculated as HLA (%)=[LT−(RT−RI)]/LT×100. LT indicates left hemisphere; RT, area of right hemisphere; RI, infarcted area; Saline, rats injected with saline; Control vector, rats transfected with control vector; HGF, rats transfected with human HGF vector. Number of slice is consistent with that in Figure 3. *$P<0.05$. n=6 for each group.

Results

Reduction of Infarcted Area by In Vivo Transfer of Human HGF Gene Into Subarachnoid Space

To test for successful gene transfer via the subarachnoid space, the concentrations of human HGF and rat HGF in CSF were measured by ELISA at 5 and 12 days after gene transfer (Figure 1, a and b). On day 5, human HGF could be detected in the CSF of rats transfected with human HGF vector, whereas human HGF protein could not be detected in control rats. The increase in human HGF protein in CSF continued up to 12 days after transfection. Interestingly, an increase in rat endogenous HGF was also observed in rats transfected with human HGF vector compared with control ($P<0.01$). Because upregulation of the receptor of HGF, c-met, has been reported in the central nervous system after human HGF gene transfer,[17] immunohistochemical staining for c-met was also examined. Consistently, upregulation of c-met was observed in the cerebral cortex as well as the brainstem and cerebellum of rats transfected with human HGF vector (Figure 1c). During the experimental periods, there was no abnormal activity, such as convulsion, after gene transfer into the subarachnoid space.

Given the successful gene transfer, we also investigated whether HGF could reduce ischemic injury in the right MCA occlusion model. The infarcted area in coronal sections was clearly detected by staining with TTC at 24 hours after MCA occlusion in rats transfected with control vector. Importantly, the ischemic area was reduced significantly in rats transfected with human HGF gene compared with control vector ($P<0.01$; Figure 2). There was no significant difference in infarcted area between rats transfected with control vector and sham-operated rats. Histological examination demonstrated a vague ischemic boundary in rats transfected with human HGF gene compared with control vector, consistent with the findings in coronal sections stained with TTC. Interestingly, numerous TUNEL-positive cells were observed in rats transfected with control vector, whereas a significant decrease in TUNEL-positive cells was detected in rats transfected with HGF gene ($P<0.01$; Figure 3). There was no significant difference in mean blood pressure and rectal temperature among the groups.

In addition, we investigated the effect of overexpression of HGF on capillary density in the cerebral cortex. As expected, the capillary density in rats transfected with human HGF vector showed more complex patterns than with control vector (Figure 4). As shown in Figure 5, in each region, the scores of area and length of vessels were significantly higher in rats transfected with human HGF vector compared with control vector ($P<0.01$). Importantly, there was no leakage through the BBB in rats transfected with human HGF vector, whereas destruction of the BBB was reported previously in the ischemic brain. To assess functional activity, we measured neurological severity score. As shown in Figure 6, neurological score was decreased significantly in rats transfected with human HGF vector compared with control vector ($P<0.01$). There was no significant difference in neurological severity score between rats transfected with control vector and sham-operated rats.

Inhibition of Destruction of BBB by Transfer of HGF Gene

Finally, we studied the side effects of overexpression of HGF, because overexpression of VEGF was reported to stimulate cerebral edema.[27,28] The infarcted hemisphere at 24 hours after MCA occlusion contained more water than the intact hemisphere ($P<0.01$; Figure 7). Unlike those with VEGF, the water content in the brain of rats transfected with human HGF was significantly decreased compared with control vector ($P<0.05$; Figure 7). Finally, we further checked the leakage of Evans blue dye to assess the extent of BBB destruction. Leakage of Evans blue dye was clearly detected in a wide

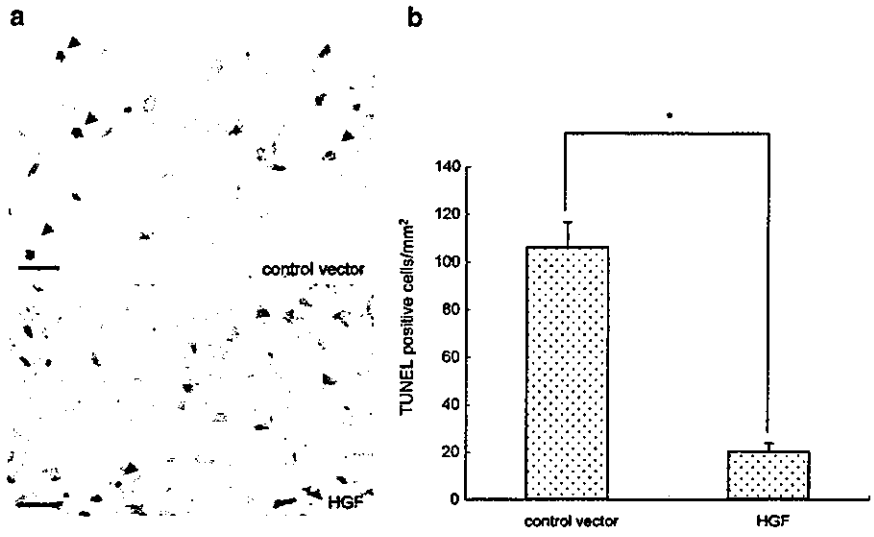

Figure 3. a, TUNEL staining of coronal sections of cerebral cortex in boundary of infarct area after 1 day of focal cerebral ischemia. Bar=100 μm. b, Number of TUNEL-positive cells. Control vector indicates rats transfected with control vector; HGF, rats transfected with human HGF vector. *$P<0.01$ vs control group. n=6 for each group.

area of brain in rats transfected with control vector (Figure 8a). There was no significant difference in infarcted area between rats transfected with control vector and sham-operated rats. Of importance, leakage of Evans blue dye was significantly less in rats transfected with human HGF vector than control vector, especially in the cerebral cortex ($P<0.01$; Figure 8).

Discussion

Disruption of blood flow to the brain initiates a cascade of events that produces neuronal death and leads to neurological dysfunction. Therefore, to prevent brain injury, numerous studies have focused on the development of neuroprotective agents that effectively prevent delayed neuronal death after transient forebrain ischemia.[17,29] Recently, HGF has been the center of interest in neuroprotective substances, because HGF works as a survival factor for embryonic motor neurons.[30] Moreover, sensory and sympathetic neurons and their precursors respond to HGF with increased differentiation, survival, and axonal outgrowth.[30] The broad spectrum of HGF activities and its observed synergy with other neurotrophic factors suggest that the major role of HGF is to potentiate the response of developing neurons to specific signals. In addition, HGF is a well-known potent angiogenic growth factor in various models, including a brain hypoperfusion model. However, the clinical usefulness of such neuroprotective agents as HGF is quite limited because of the presence of the BBB, which makes the central nervous system relatively inaccessible to circulating proteins and peptides. One method to overcome this limitation is to use a drug delivery system into the central nervous system. In particular, we focused on gene transfer into the subarachnoid space, because intrathecal injection into the cisterna magna with a needle involves no systemic anesthesia, no burr hole, and no pain. Using the HVJ-envelope vector, the reporter gene was transferred into the widespread meninges and adventitial cells of arteries by intrathecal injection via the cisterna magna and was not transferred to other organs except the brain.[19] In the present

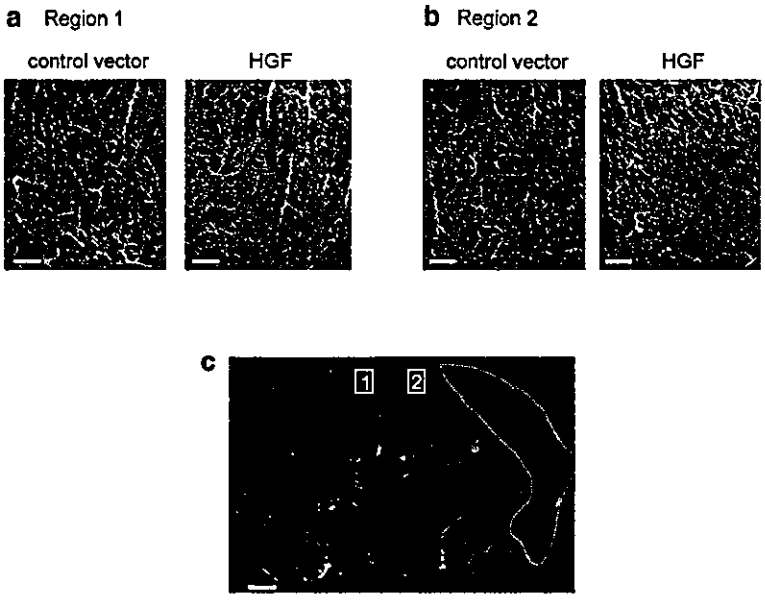

Figure 4. Vascular patterns in cerebral cortex at 24 hours after MCA occlusion. Region of interest was set at 2 points on surface of cerebral cortex (c). Rats transfected with human HGF gene showed a significant increase in capillary density in both intact (a) and infarcted (b) hemispheres. Dotted line in c represents infarcted region. a and b, bar=100 μm; c, bar=1 mm. Control vector indicates rats transfected with control vector; HGF, rats transfected with human HGF vector.

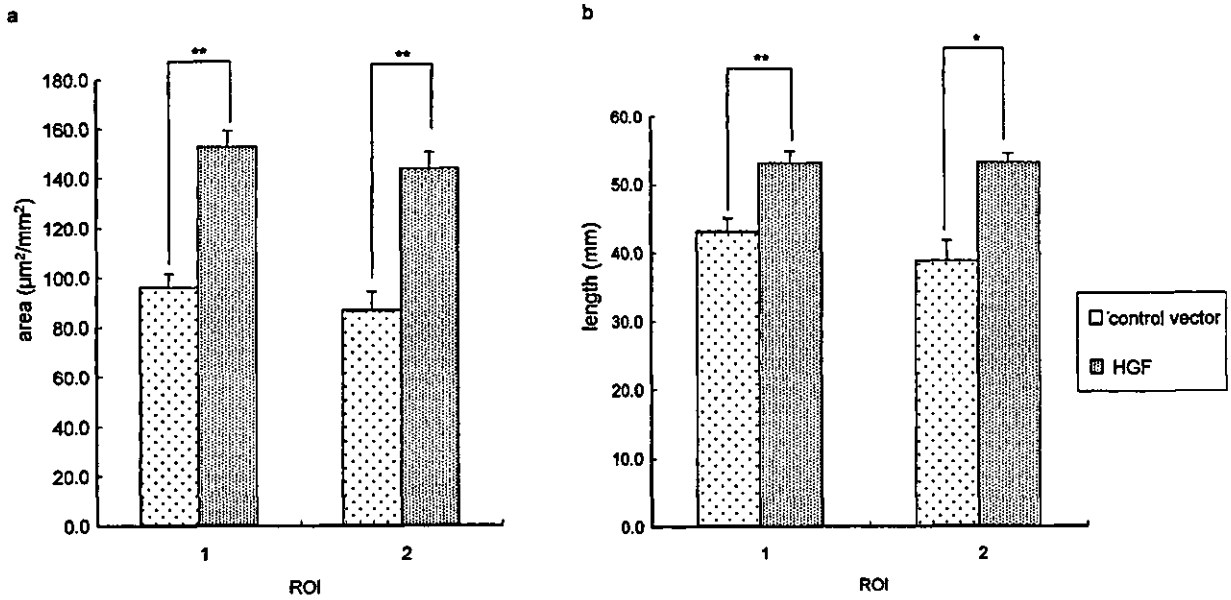

Figure 5. Capillary density as assessed by quantitative analysis at 24 hours after MCA occlusion. Control vector indicates rats transfected with control vector; HGF, rats transfected with human HGF vector. Number of ROI is same as in Figure 4. *$P<0.05$, **$P<0.01$ vs control vector (n=7 in each group).

study, human HGF protein was detected in the CSF after HGF gene transfer. In addition to the increase in human HGF, rat HGF concentration was increased ≈10-fold compared with the nontreatment group. We speculate that the secreted human HGF in CSF from the brain surface, such as the meninges, augmented the secretion of rat endogenous HGF in an autocrine–paracrine manner. Importantly, c-met, the receptor for HGF, was increased by transfection not only at the site of injection but also in the cerebral cortex. Recently, we reported that activation of the transcription factor ets-1 plays a pivotal role in the upregulation of HGF and c-met by HGF.[31] Although the present study cannot elucidate the contribution of upregulation of endogenous HGF, the positive feedback of HGF might play a role in the sustained beneficial effects of HGF.

Given the neurotrophic and angiogenic character of HGF, the present study demonstrated that (1) pretreatment with HGF gene transfer prevented ischemic injury in the rat MCA occlusion model, associated with a significant reduction in apoptotic cells; (2) overexpression of HGF reduced abnormal neurological findings, accompanied by a significant increase in capillary density; and (3) in vivo transfer of HGF gene reduced the destruction of the BBB without the exacerbation of cerebral edema. The region rescued by HGF gene transfer was primarily in the ischemic boundary region around the infarcted area, the so-called ischemic penumbra. In fact, the boundary zone in rats transfected with human HGF gene

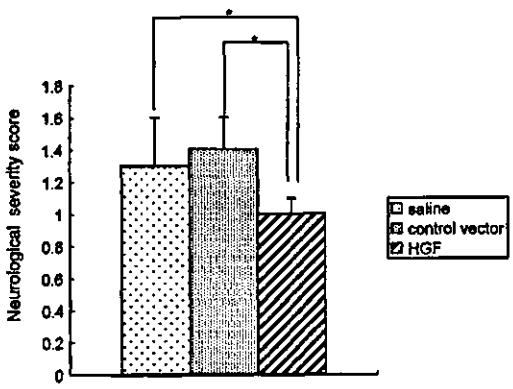

Figure 6. Neurological severity score at 24 hours after MCA occlusion. Saline indicates rats injected with saline; Control vector, rats transfected with control vector; HGF, rats transfected with human HGF vector. *$P<0.05$ (n=7, each group).

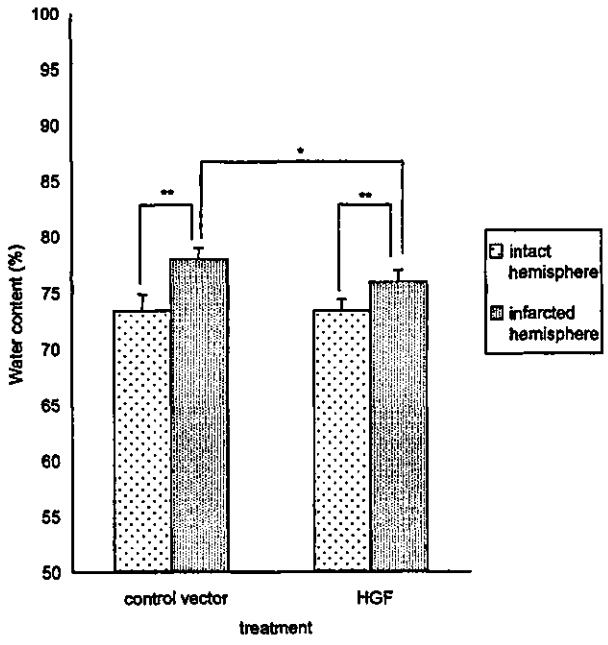

Figure 7. Water content after 24 hours of cerebral ischemia. Water content (WC) was calculated as WC (%)=(wet wt−dry wt)/(wet wt)×100. *$P<0.05$ (n=7 in each group).

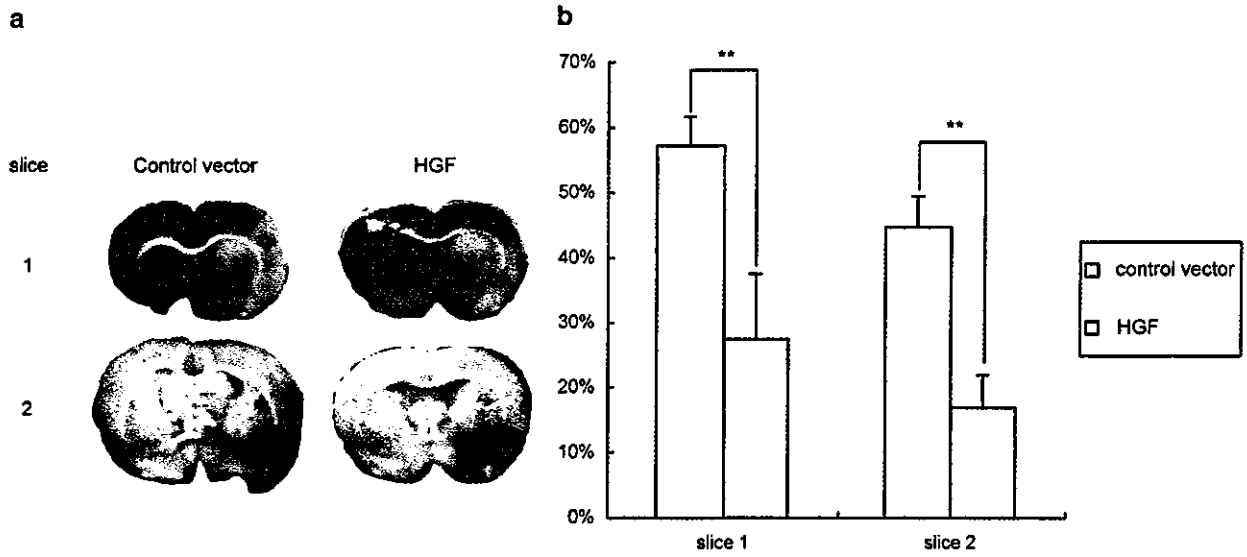

Figure 8. a, Coronal sections at 9 hours after MCA occlusion. Evans blue dye was injected at 6 hours after occlusion. To identify infarcted area, slice 1 was stained with TTC. Blue area indicates leakage of Evans blue dye. b, Percent area of Evans blue dye. Degree of leakage (%) was calculated as leakage (%)=[LT−(RT−RB)]/LT×100. RB indicates area stained blue. $^{**}P<0.01$ vs control vector (n=7 in each group).

demonstrated a larger area of mixed intact and dead neurons and fewer TUNEL-positive neurons. A neuroprotective effect of HGF has been reported in vitro and in vivo.[15–17,30,32,33] The mechanism of the neuroprotective effects of HGF is inhibition of apoptosis via the MAP kinase pathway[32] and the phosphatidylinositol-3 kinase/Akt pathway.[16,33] Another possible mechanism for the reduction of the infarct area might be the development of collateral circulation. An angiogenic effect of HGF was reported in a rat cerebral hypoperfusion model[34] and rat transient focal cerebral ischemia model,[9] in addition to the present study. Because the prevention of cerebral infarction was observed at 24 hours after transfection, the neuroprotective action of HGF might be contributed largely to the present results.

In the progression to human clinical gene therapy, severe side effects could be important. In particular, although recombinant VEGF is effective to reduce infarct volume when administered on the brain surface,[10] early postischemic (1 hour) administration of recombinant VEGF165 to ischemic rats significantly increased BBB leakage, hemorrhage, and ischemic lesions.[27] Thus, it is noteworthy to document the effects of HGF on leakage of the BBB. As a result, an increase in area and length of vessels in rats transfected with human HGF gene was observed without BBB leakage, suggesting that the collateral development induced by angiogenesis may contribute in part to the reduction of cerebral infarction volume. Moreover, it is important to examine whether HGF exacerbates cerebral edema after ischemic injury. The present study clearly demonstrated that overexpression of HGF did not exacerbate cerebral edema, in contrast to reported studies in which VEGF augmented cerebral edema and leakage of the BBB.[27,28,35] Indeed, human gene therapy in patients with limb ischemia using VEGF also demonstrated lower-limb edema as a side effect,[36] whereas human gene therapy using HGF plasmid DNA did not. The difference in edema formation between HGF and VEGF might be a result of the effects on vascular smooth muscle cells (VSMCs). HGF stimulates the migration of VSMC without their multiplication, but VEGF does not stimulate either the migration or the proliferation of VSMCs because of the lack of VEGF receptors on VSMCs.[37] Thus, angiogenesis induced by VEGF is featured as a delay in the maturation of blood vessels. In contrast, because HGF simultaneously stimulates the migration of both endothelial cells and VSMCs, the blood vessels might mature in a well-coordinated way, thereby avoiding the release of blood-derived cells into the extracellular space. From these viewpoints, HGF might be useful to treat cerebral ischemia. Because experimental conditions may not be ideal to test the clinical usefulness, further studies are necessary in advancing toward human gene therapy.

Overall, HGF gene transfer using HVJ-envelope vector reduced ischemic injury without exacerbation of cerebral edema or BBB leakage. Although the prevention of cerebral infarction is not advanced enough for human gene therapy, proof of the improvement of stroke-related symptoms might be necessary to promote new therapeutic options to treat cerebral ischemia using HGF gene therapy. In particular, because the safety issue of HVJ-envelop vector is unknown in human trials, continuous development of systems involving vectors, promoters, or alternative routes of administration may help to achieve human gene therapy for cerebrovascular disease in the future.

Acknowledgments

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The HVJ-Envelope as an Innovative Vector System for Cardiovascular Disease

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Abstract: Recently promising results of gene therapy clinical trials have been reported for treatment of peripheral vascular and cardiovascular diseases using various angiogenic growth factors and other therapeutic genes. Viral vector and non-viral vector systems were employed in preclinical studies and clinical trials. Adenoviral vector and naked plasmid have been used most in the clinical studies. HVJ (hemagglutinating virus of Japan or Sendai virus)-liposome vector, a hybrid non-viral vector system with fusion of inactivated HVJ virus particle and liposome, has developed and demonstrated high transfection efficiency in preclinical studies of many different disease models, including a wide range of cardiovascular disease models. However, some limitations exist in the HVJ-liposome technology, especially in the scalability of its production. Recently an innovative vector technology, HVJ envelope (HVJ-E) has been developed as a non-viral vector, consisting of HVJ envelope without its viral genome, which is eliminated by a combination of inactivation and purification steps. HVJ-E is able to enclose various molecule entities, including DNA, oligonucleotides, proteins, as single or multiple therapeutic remedies. The therapeutic molecule-included HVJ-E vector can transfect various cell types in animals and humans with high efficiency. In this review, vector technology for cardiovascular disease and the biology of HVJ-E vector technology is discussed.

INTRODUCTION

Gene therapy, as an approach to treat diseases, uses vectors carrying therapeutic gene or genes. In the cardiovascular area, naked plasmid DNA and adenoviral vectors have been used most for gene therapy of ischemic heart disease (IHD) and lower extremity ischemia (LEI) with angiogenic growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), hypoxia inducible factor 1 (HIF-1) and hepatocyte growth factor (HGF). Adenoviral vectors demonstrated relative high transduction efficiency in skeletal muscle and myocardium compared to that of naked plasmid. However, the replication-deficient adenoviral vector system has its deficiencies for gene therapy applications, such as size limitation, viral toxicity and immunogenicity. The adenoviral vectors have been employed in a significant number of clinical trials with extensive safety considerations. In contrast, it has been considered safer for naked plasmid DNA as the vector carrying VEGF, FGF or HGF to treat IHD or LEI in the clinical trials. However, naked plasmid DNA is generally unstable while it is taken up by endocytosis. The *in-vivo* transfection efficiency of naked plasmid DNA also needs to be improved. Most non-viral vectors are much less efficient in delivery of genes into cells *in-vivo* as compared to recombinant viral vectors. In most cases the introduced DNA with non-viral vectors is taken up by endocytosis mechanism of the host cells and gets into lysosomes, resulting in rapid degradation. Therefore, there has been a demand to develop an improved non-viral vector technology, which can deliver genes efficiently and perform high efficacy with high safety in humans. Upon such a demand, HVJ (Hemagglutinating Virus of Japan)-liposome vectors were developed and then a further improved vector system called HVJ-envelope (HVJ-E) technology was innovated (Kaneda *et al.*, 2002) in order to overcome the deficiencies of both viral and other current non-viral vector systems.

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CURRENT GENE THERAPY VECTORS FOR CARDIOVASCULAR DISEASE

Since gene therapy emerged as a new approach to the treatment of cardiovascular disease in the late 1980s and early 1990s (Swain 1989; Nabel *et al.*, 1991), some promising results from gene therapy clinical trials of cardiovascular diseases have been reported recently, which are summarized in (Table 1).

Diseases and Target Genes

A majority of the reported clinical trials, 15 clinical trials out of the 19 clinical trials listed in (Table 1), focused on therapeutic angiogenesis for IHD or LEI caused by coronary artery disease (CAD) or peripheral artery disease (PAD). The early Phase I and Phase I/II clinical trials, using VEGF165 (Losordo *et al.*, 1998; Vale *et al.*, 2000; Huwer *et al.* 2001; Lathi *et al.* 2001; Sarkar *et al.*, 2001; Freedman *et al.*, 2002), VEGF121 (Rosengart *et al.*, 1999; Rajagopalan *et al.* 2001; Rajagopalan *et al.*, 2002), VEGF167 (Huwer *et al.*, 2001),

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Table 1. Clinical Trials of Gene Therapy for Cardiovascular Diseases

Disease Indication	Target Gene	Vector	Delivery	Clinical Trial	References
Angina (CMI)	VEGF165	Plasmid DNA	Intra-myocardial injection, invasive surgery.	Phase I (5 patients, OL); Phase I (13 patients, OL); Phase I (30 patients, OL); Phase I (7 patients, OL).	Losordo *et al.*, 1998; Vale *et al.*, 2000; Lathi *et al.*, 2001; Sarkar *et al.*, 2001.
PAD	VEGF165	Plasmid DNA	Intra-muscular injection	Phase I (34 patients).	Freedman *et al.*, 2002.
Angina (CAD)	VEGF121	Recombinant adenovirus	Intra-myocardial injection, invasive surgery (with CABG for Phase IA)	Phase II (71 patients, DB, R); Phase IA/IB (15/6 patients, OL).	Stewart 2002; Rosengart *et al.*, 1999.
IC or RP (PAD)	VEGF121	Recombinant adenovirus	Intra-muscular injection	Phase I (6 patients, OL).	Rajagopalan *et al.*, 2001; Rajagopalan et al, 2002.
Angina (CMI)	VEGF-2	Plasmid DNA	Intra-myocardial injection by catheter.	Phase I (6 patients, SB); Phase I/II (19 patients, DB, R).	Vale *et al.*, 2001; Losordo *et al.*, 2002.
Angina (CAD)	VEGF165, VEGF167	Plasmid DNA	Intra-myocardial injection, invasive surgery.	Phase I (24 patients, OL).	Huwer *et al.*, 2001.
LLI	VEGF	Recombinant adenovirus or plasmid/liposome	Catheter intra-arterial infusion after PTA	Phase II (54 patients, DB, R).	Makinen *et al.*, 2002.
Angina (CAD)	FGF-4	Recombinant adenovirus	Single intra-coronary injection.	Phase I/II (79 patients, DB, R).	Grines *et al.*, 2002.
RP or TN (PAD)	FGF-1	Plasmid DNA	Intra-muscular injection	Phase I (51 patients, OL).	Comerota *et al.*, 2002.
IC (PAD)	FGF-2	Plasmid DNA	Intra-arterial infusion	Phase II/III (190 patients, DB, R).	Lederman *et al.*, 2002.
Restenosis	Anti-c-myc	Single strand ODN	Intra-coronary local delivery after coronary stent implantation	Phase I/II (85 patients, DB, R).	Kutryk *et al.*, 2002.
Vein graft failure of PABG or CABG	E2F-decoy	Double strand ODN	*Ex-vivo* treatment of vein grafts prior to CABG	Phase I/II (41 patients, DB, R); Phase II (200 patients, DB, R).	Mann *et al.*, 1999; Terashima *et al.*, 2002.
Homozygous familial hypercholesterilemia	LDLR	Recombinant retrovirus	*Ex-vivo* primary hepatocyte transduction and implantation back to liver.	Phase I (5 patients, OL).	Raper *et al.*, 1997.

CMI indicates chronic myocardial ischemia; PAD, peripheral artery disease; CAD, coronary artery disease; IC, intermittent claudication; RP, rest pain; TN, tissue necrosis; LLM, lower-limb ischemia; PABG, peripheral artery bypass grafting; CABG, coronary artery bypass grafting; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; LDLR, low density lipoprotein receptor; ODN, oligodeoxynucleotide; PTA, percutaneous transluminal angioplasty; OL, open labeled; DB, double blind; SB, single blind; R, randomized.

VEGF-2 (Vale *et al.*, 2001; Losordo *et al.*, 2002), FGF-1 (Comerota *et al.*, 2002), FGF-2 (Lederman *et al.*, 2002) and FGF-4 (Grines *et al.*, 2002), demonstrated general safety in the therapeutic genes and the delivery procedures, and also promising indication in clinical efficacy. Two recent reports on double-blind randomized Phase II clinical trials, using VEGF genes to treat CAD patients (Stewart *et al.*, 2002; Makinen *et al.*, 2002), demonstrated statistical significant efficacy of the therapeutic angiogenesis gene therapy that warrants Phase III pivotal clinical trial.

Coronary restenosis, a vasoproliferative disease, was treated with antisense single-stranded oligodeoxynucleotides (ODN) anti-c-myc, targeting the cell cycle regulator c-myc, in a double blind and randomized phase I/II clinical trial (Kutryk *et al.*, 2002). Vein grafts were treated with intra-operative *ex-vivo* transfection of double-stranded ODN decoy for the DNA-binding site of E2F, a transcription factor necessary for the expression of genes that are involved in proliferation of smooth muscle cells, in a double blind and randomized phase I/II (Mann *et al.*, 1999) clinical trail for

peripheral artery bypass grafting and a phase II (Terashima *et al.*, 2002) clinical trial for coronary artery bypass grafting. The *ex-vivo* transfection of vein grafts with E2F ODN decoy for the artery bypass grafting was safe, feasible, and effective in ODN transfection of the vein grafts with potential therapeutic benefits on reduction of bypass-graft failure. Homozygous familial hypercholesterolemia was treated with low density lipoprotein receptor (LDLR) gene in a phase I clinical trial (Raper *et al.*, 1997).

There have also been many cardiovascular diseases under preclinical and clinical studies, demonstrating the potential of novel gene therapy remedies with different target genes. In the field of therapeutic angiogenesis, hypoxia inducible factor 1-α (HIF-1α) has entered phase I clinical trials for CAD and PAD patients (Rasmussen *et al.*, 2002). Hepatocyte growth factor (HGF) has also demonstrated angiogenic efficacy in preclinical studies and entered phase I clinical trials for PAD patients (Morishita 2002). The genes of nitric oxide synthases (iNOS and eNOS) (Chen *et al.*, 2002 & references therein), tissue factor pathway inhibitor (Yin *et al.*, 2002), anti-monocyte chemoattractant protein-1 (Usui *et al.*, 2002) and C-type natriuretic peptide (Ohno *et al.*, 2002) have been tested in preclinical studies to prevent restenosis after coronary intervention (Rutanen *et al.*, 2002 & references therein). The genes of anti-monocyte chemoattractant protein-1 (Inoue *et al.*, 2002), heme oxygenase-1 (Juan *et al.*, 2001) and dominant-negative Rho-kinase (Morishige *et al.*, 2001) have been tested in various animal models for the treatment of hypercholesterolemia and arteriosclerosis (Kawashiri and Rader, 2000 & references therein). The genes of prostacyclin synthase (Suhara *et al.*, 2002), antisense angiotensin II type I receptor (Pachori *et al.*, 2002), antisense angiotensinogen (Makino *et al.*, 1998; Wang *et al.*, 2001), antisense β1-adrenergic receptor (Zhang *et al.*, 2000) and eNOS (Lin *et al.*, 1997; Champion *et al.*, 1999) have been tested for the treatment of hypertension. The genes of HGF (Miyagawa *et al.*, 2002), antisense phospholamban (Eizena *et al.*, 2000; del Monte *et al.*, 2002), and sarcoplasmic reticulum Ca^{2+}-ATPase (del Monte *et al.*, 2001) have been tested for the treatment of heart failure. Moreover, expression of KCNE3 gene, encoding a regulatory subunit of pore-forming potassium channel, in the left ventricular cavity of a guinea pig model shortened the QT interval of electrocardiogram, demonstrating the potential for treatment of cardiac arrhythmias and sudden cardiac death (Mazhari *et al.*, 2002; Zhao *et al.*, 2002). Over-expression of a G1 cell cycle regulator gene, cdk inhibitor p16INK4a, demonstrated the suppression of left ventricular hypertrophy in a rat model (Nozato *et al.*, 2002).

In addition to the target gene, the delivery method and the vector system are vital for the success of cardiovascular gene therapy.

Delivery Method

Most of the reported clinical trials, 16 clinical trials out of the 19 clinical trials, employed various *in-vivo* local delivery methods, such as intra-myocardial direct injection with invasive surgery (7 clinical trials) (Losordo *et al.*, 1998; Rosengart *et al.* 1999; Vale *et al.*, 2000; Lathi *et al.*, 2001; Sarkar *et al.*, 2001; Huwer *et al.*, 2001; Stewart *et al.*, 2002), intra-myocardial injection with catheter (2 clinical trials) (Vale *et al.*, 2001; Losordo *et al.*, 2002), local direct intra-muscular injection (3 clinical trials) (Rajagopalan *et al.*, 2001; Rajagopalan *et al.*, 2002; Freedman *et al.*, 2002; Comerota *et al.*, 2002), local intra-coronary delivery (2 clinical trials) (Grines *et al.*, 2002; Kutryk *et al.*, 2002), local intra-arterial infusion (2 clinical trials) (Lederman *et al.*, 2002; Makinen *et al.*, 2002). *Ex-vivo* delivery methods were used in 3 reported clinical trials. In the clinical trials of PREVENT (Mann *et al.*, 1999) and PREVENT II (Terashima *et al.*, 2002) for the treatment of vein graft failure, the ODN E2F-decoy was delivered to the vein grafts by *ex-vivo* pressure-mediated transfection prior to grafting of the CABG surgery. The LDLR gene was delivered to the autologous hepatocyte culture by *ex-vivo* transduction before implantation back to patients' liver in the phase I clinical trial to treat homozygous familial hypercholesterolemia (Raper *et al.*, 1997).

Although *ex-vivo* was the choice of delivery method for many early gene therapy clinical trials, it became less favorable for the later gene therapy clinical trials because most cardiovascular diseases need to be treated *in-vivo* and also because of the cost of individualized *ex-vivo* process and the difficulties in scaling-up the *ex-vivo* process for commercial manufacturing. In some cases, such as the *ex-vivo* transfection of vein graft immediately prior to CABG surgery (Mann *et al.*, 1999; Terashima *et al.*, 2002), it can be attractive and efficacious.

Because of toxicity and safety concerns, none of the clinical trials in (Table 1) used the *in-vivo* systemic delivery. However, in most cases effective local delivery requires specific procedures and delivery devices, such as invasive surgeries, catheters, imaging instruments, etc., which may cause additional complications of adverse incidents and are more costly. Development of targeting vector technology can make *in-vivo* systemic delivery safer, more effective and economically sound. At that time *in-vivo* systemic delivery may become a more attractive choice for cardiovascular gene therapy.

Vector System

The naked plasmid DNA or ODN was the most frequently used vector system in the reported gene therapy clinical trials on cardiovascular disease and the adenovirus was the choice of viral vector system. As listed in (Table 1), naked plasmid DNA or ODN was used by 13 clinical trials, replication-deficient recombinant adenovirus was used by 5 clinical trials, only one clinical trial used liposome and one clinical trial used replication-deficient recombinant retrovirus.

In preclinical studies, adeno-asociated virus (AAV) has been tested as the vector system to deliver therapeutic genes in a mouse ischemic heart model (Su *et al.*, 2002) and in a rat hind limb ischemia model (Shimpo *et al.*, 2002). It was also demonstrated that a lentivirus vector can successfully deliver genes into adult cardiac myocytes *in-vitro* and *in-vivo* (Martin *et al.*, 2002). In addition to the non-viral vector technologies, such as liposomes and cationic polymers, some physical treatments, such as *in-vivo* electroporation (Nakano *et al.*, 2001) and endovascular therapeutic ultrasound

(Amabile *et al.*, 2001), have demonstrated the enhancement of plasmid DNA delivery efficiency into tibialis anterior muscles and femoral arteries in animal models.

The prominent concerns in regards to the gene therapy vectors in clinical use are always the issue of safety, especially for the viral vector systems. That may be the reason for the majority of reported clinical trials to choose naked DNA or ODN as the vector system. The potential of generation of replication competent virus (e.g. replication competent retrovirus, replication competent adenovirus) during *in-vitro* packaging or *in-vivo* application, the potential of insertional mutagenesis and germline mutations by integrating viral vectors, such as retrovirus, AAV and lentivirus, the potential of acute and chronic toxicities of the viral components carried by the viral vectors, and the potential of adverse effects due to over expression or unspecific expression of transgenes in non-targeted tissues or organs are a few of the top concerns on the list of safety issues. On the other hand, efficiency of the gene delivery is the major challenge for naked DNA-based vector technology. Although many promising non-viral vectors and gene delivery-enhancing technologies, such as liposomes, *in-vivo* electroporation and ultrasound, have been developed; most of them are still in early preclinical studies except liposomes, which have been used in some early clinical trials. The emergence of many technical hurdles and safety-toxicity issues with clinical use of the non-viral vectors and gene delivery-enhancing technologies is largely responsible for slowing the development of these approaches.

An ideal vector system should combine the gene delivery efficiency of a viral vector and the safety profile of the naked DNA. The HVJ-liposome vector and HVJ-E non-viral vector are candidates of such ideal vector systems as described in the rest of this review.

HVJ-LIPOSOME VECTOR

Hemagglutinating virus of Japan (HVJ) or Sendai virus is a member of the murine paramyxovirus family, containing a single-stranded RNA virus genome with an envelope. The HVJ-envelope contains two glycoproteins, HN (hemagglutinating neuraminidase) and F (fusion protein) proteins, which possess hemagglutinating and fusion activity respectively (Fig. 1). These HVJ-envelope proteins are

- **Murine paramyxovirus discovered in Japan (1950s)**
- **Cell fusion activity (monoclonal Antibody, chromosome mapping)**
- **Viral genome : single-strand RNA (minus strand)**
- **Envelope proteins : two glycoproteins (F, HN)**
- **Diameter : 150-600nm (average 300nm)**

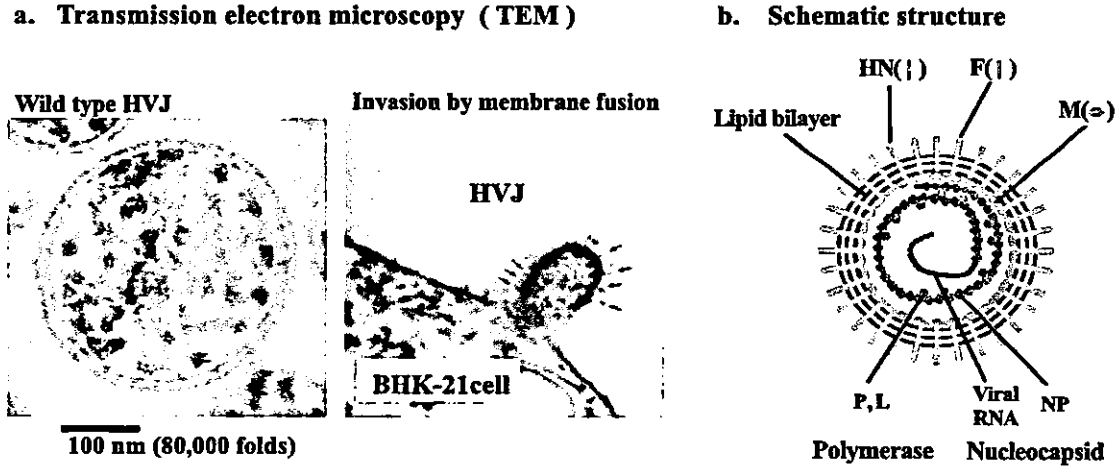

Fig. (1). Structure of hemagglutinating virus of Japan (HVJ)

a. Transmission electron microscopic observation of HVJ.

HVJ was discovered at Sendai Japan in 1952 as a pathogen for rodent animals, so it also called "Sendai virus" (box). HVJ is belongs to paramyxovirus group and its structure resembles influenza virus (upper panel). The envelope portion is a lipid bilayer derived from host cell membrane and dense materials inside envelope are nucleocapsid, which contains minus strand RNA genome and nucleocapsid protein (left panel). The average size of viral particles is 300 nm (left panel). HVJ is nonpathogenic for human, though it is able to infect human cells. The major character of HVJ is fusogenic activity (right panel). The spike proteins (F and HN) of viral envelope are indicated by arrow. Hybridoma cells producing monoclonal antibody is originally prepared using this activity by Dr. Köller and Dr. Milshtein in Cambridge University in 1970s. And this activity was also used for preparation of chimeric cells that were essential for chromosome mapping.

b. Schematic structure of HVJ

The viral particle of HVJ consists of three component, envelope, nucleocapsid and polymerase. Viral envelope is a lipid bilayer containing two glycoproteins: fusion (F) and hemagglutinating neuraminidase (HN) proteins. Nucleocapsid portion contains viral genome and nucleocapsid protein (NP). The virus particle contains two kinds of polymerases (P and L) and a matrix protein (M). The envelope portion of HVJ is used for the preparation of an HVJ-envelope vector. F and HN proteins are involved in the membrane fusion activity.

involved in cell fusion. HVJ virus is an enveloped large particle ranging from 300-600 nm in diameter. The viral particle is negatively charged and attaches to sialic acid (the HVJ receptor), fuses with cell membrane, and releases its genome into cytoplasm directly, rather than via the endocytosis.

HVJ-liposome gene transfer technology was developed in late 1980s (e.g. Kaneda *et al.*, 1987) and early 1990s (e.g. Tomita *et al.*, 1993; Morishita *et al.*, 1993) to introduce nucleic acid, ODN, and protein with high efficiently. The molecules included in HVJ-liposomes are delivered directly into various types of mammalian cells by means of the virus-cell fusigenic character of HVJ (Fig. 2) (Dzau *et al.* 1996). The first generation of HVJ-liposome was constructed by a combination of inactivated viral particles and multi- or uni-lamellar cationic liposomes to produce a non-viral gene transfer system. The HVJ-liposomes can deliver nucleic acids (e.g. Hirano *et al.*, 1998) or ODN (e.g. Morishita *et al.*, 1994) more efficiently than other non-viral vectors (e.g. liposomes). Moreover, the ODN delivered by HVJ-liposome were accumulated in the nucleus rapidly and persisted up to 2 weeks, whereas liposome-mediated delivery of ODN did not result in nuclear accumulation and rapidly decayed within a few days (Morishita *et al.* 1994), demonstrating the advantage of fusigenic gene delivery over endocytotic gene delivery. With modification of liposome composition from cationic to anionic, the second generation HVJ-AVE (artificial viral envelope) liposome showed a 5- to 10-fold higher gene expression in liver and muscle than the first generation HVJ-liposome vector. In addition, the high level of gene expression in muscle delivered by HVJ-AVE persisted as long as 30 days (Saeki *et al.* 1997). Delivered by HVJ-AVE liposome, the Fas-ligand protected the liver transplantation in rats from graft rejection for 20 day (Li *et al.*, 1998) similar to the protection achieved by adenovirus-delivered Fas-ligand (Okuyama *et al.*, 1998), implying the delivery efficiency of HVJ-AVE liposomes in liver was comparable to that of adenoviral vector. A more recent development of the HVJ-liposome technology was the reconstituted HVJ-fusion liposomes (Suzuki *et al.*, 2000b), which reconstituted purified fusion proteins from the HVJ-envelope into liposomes and demonstrated the gene delivery efficiency comparable to the HVJ-liposomes both *in-vitro* and *in-vivo*.

The HVJ-liposome system has exhibited therapeutic potential in various animal models for different disease indications such as liver cirrhosis (Ueki *et al.*, 1999), arthritis (Tomita *et al.*, 1999), transplantation rejection (Li *et al.* 1998) and cancer (Zhou *et al.*, 1999). More extensively HVJ-liposome technology has been tested as the vehicle for delivery of genes and ODNs in a variety of cardiovascular diseases, including vein graft failure (Suzuki *et al.*, 1997a; Matsumoto *et al.*, 1998; Mann *et al.*, 1995; Suzuki *et al.*, 2000a), restenosis (Morishita *et al.*, 1993; Morishita *et al.*; 1994, Morishita *et al.*, 1995; Yonemitsu *et al.*, 1996; Yonemitsu *et al.*, 1997; Morishita *et al.*, 1998; Aoki *et al.*, 1999; Morishita *et al.*, 2000), hypertension (Tomita *et al.*, 1993; Tomita *et al.*, 1995; Nakamura *et al.*, 1999),

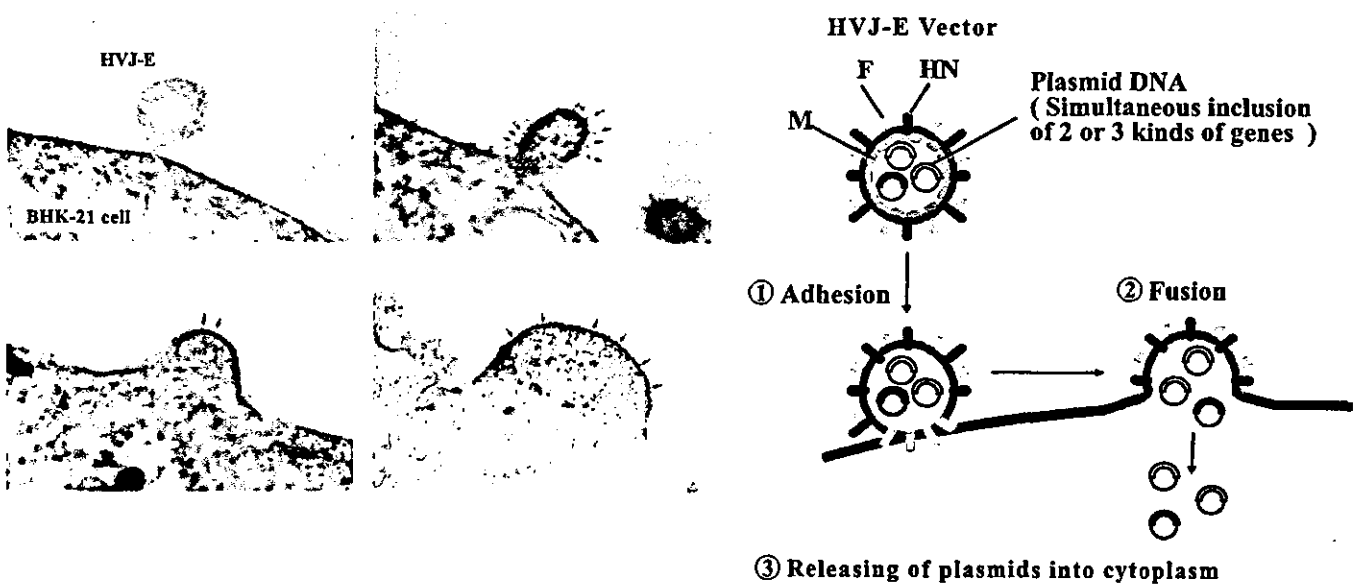

Fig. (2). Mechanism of gene transfer mediated by HVJ-E vector

a. Transmission electron microscopic observation of membrane fusion between HVJ and target cell (BHK-21).
The fusogenic activity of HVJ is utilized for the delivery of incorporated materials. Similar to wild type virus, HVJ envelope vector attaches to the cell surface and directly injects the incorporated materials into target cell cytoplasm (BHK-21). The reaction occurs within a few seconds.

b. Simultaneous gene transfer by HVJ-E vector
Direct injection of incorporated materials by membrane fusion permits the simultaneous gene transfer to identical target cells. Immediately after the attachment of HVJ-E particle containing two or three kinds of plasmid vector to the target cells, membrane fusion between vector and target cell occurs and plasmid DNAs are released into the cytoplasm of target cells.

myocardial protection (Suzuki *et al.*, 1997b; Sawa *et al.*, 1997; Sawa *et al.*, 1998; Suzuki *et al.*, 1999) and therapeutic angiogenesis (Aoki *et al.*, 2000).

Remarkably, the HVJ-liposome vectors could be administered repeatedly into rat liver without decreasing the level of gene expression, implying low immunogenicity and low pathogenicity (Hirano *et al.*, 1998). A safety study with repetitive intramuscular administration and single intravenous injection into cynomologus monkeys demonstrated the safety, feasibility, and therapeutic potential of the HVJ-AVE liposome vector for humans (Tsuboniwa *et al.*, 2001).

HVJ ENVELOPE (HVJ-E) VECTOR TECHNOLOGY

In the course of developing a vector technology for *in-vivo* gene delivery with high efficiency and low toxicity, which are critical to the success of therapeutic goals, HVJ-liposome hybrid vector has been utilized successfully in many preclinical studies as mentioned above. However, compared to wild type HVJ viruses, the HVJ-liposome has lower fusion activity probably due to the dilution of HVJ-envelope proteins by hybridizing with liposomes. In addition, there are substantial technical hurdles for the development of a scalable process to produce large quantity of the HVJ-liposomes in supporting a real clinical application.

The HVJ-E vector technology has been developed to overcome these hurdles (Kaneda *et al.*, 2002). In contrast to a recombinant HVJ viral vector (e.g. Yonemitsu *et al.*, 2000), the HVJ-E is a non-viral vector system that consists of an envelope derived from wild type HVJ virus by inactivation and purification processes (Fig. 3). Without the viral genome in the HVJ-E vector, there are no replication and viral gene expression in the cells transfected with the HVJ-E vector, whereas the recombinant HVJ viral vector replicates and expresses viral genes after its infection of cells as illustrated in (Fig. 4). A comparison of the characteristics between recombinant HVJ and HVJ-E vectors is listed in (Table 2). Virus replication and viral gene expression of the recombinant HVJ vector cause serious toxicity concerns and high immunogenicity, which make it less desirable for repeated administration of the recombinant HVJ vector. In contrast, when plasmid DNA carrying luciferase gene was delivered by HVJ-E in the mice, which had been immunized twice with HVJ-E vector, the luciferase expression in the immunized mice was as high as in the naïve mice, which were first time injected with luciferase-included HVJ-E (data not shown). It indicates that repeated administration is possible for the HVJ-E vector to deliver therapeutic genes.

Fusion between HVJ-E vector envelope and cell membrane, as shown in the transmission electron microscopy pictures of Fig. 2 (data not published), occurs within only 3-5 seconds immediately after the attachment of

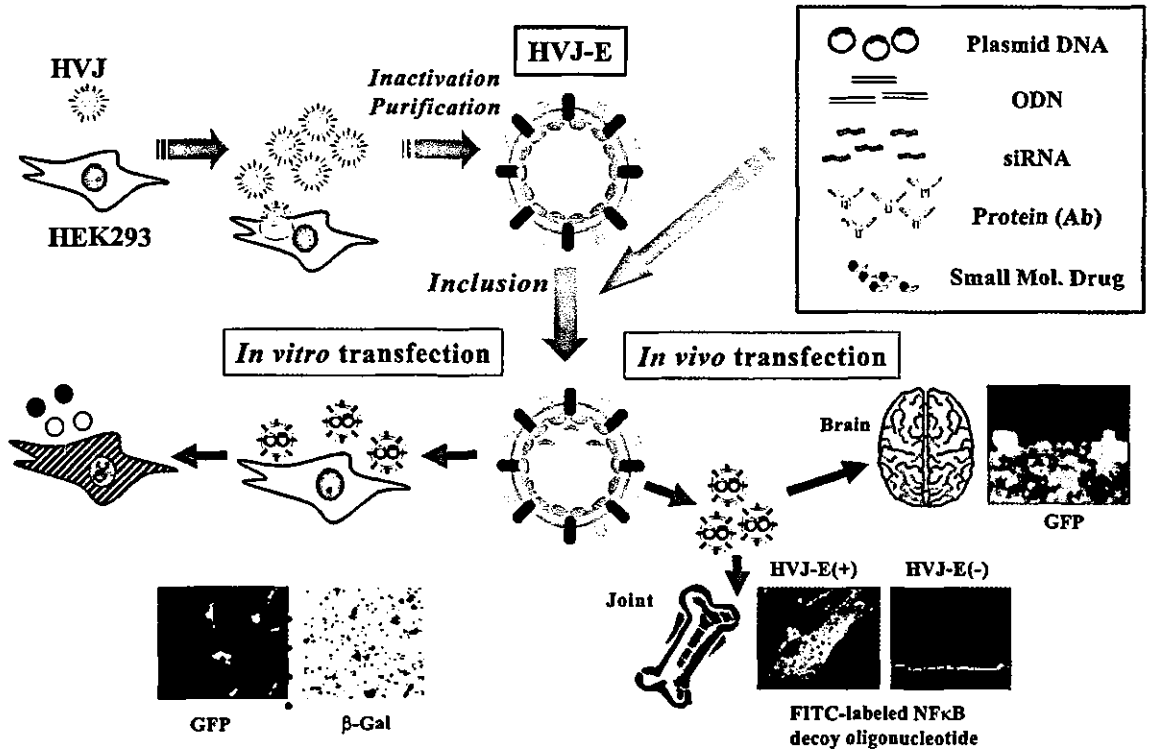

Fig. (3). Creation of HVJ-E vector with ability to transfect *in vitro* and *in vivo*.

HVJ particles are produced by human HEK293 cells. Empty HVJ envelop (HVJ-E) vector particles are prepared by inactivation of viral genome with chemical agent and removal of viral genome by purification. Various biomolecules, including plasmid DNA, oligonucleotides, protein and antibody, are incorporated into empty particles and used for transfection of many kinds of cells and organs. The left corner shows the BHK21 cells co-transfected with HVJ-E included GFP and β-Gal plasmid DNA, where both GFP and β-Gal expressed in the same cells. The right corner shows two *in vivo* HVJ-E transfection experiments: GFP expression in rat brain through carotid artery injection of EVJ-E included GFP plasmid; FITC-labeled NF-κB decoy double-stranded oligonucleotides penetrated into cartilage cells when included by HVJ-E. The major advantages of HVJ-E vector are summarized in the bottom text box.

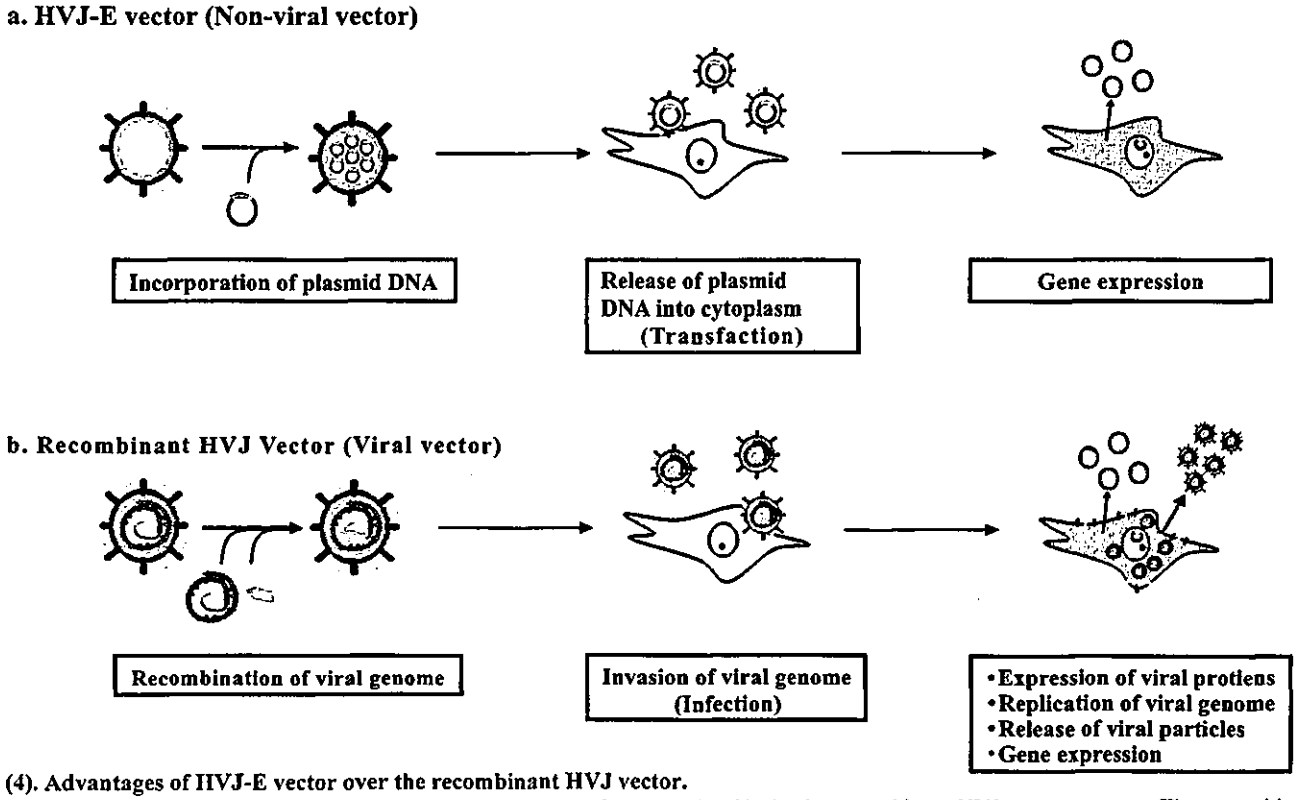

Fig. (4). Advantages of HVJ-E vector over the recombinant HVJ vector.
HVJ-E vector system has a lot of advantages over another type of vector using HVJ, the recombinant HVJ vector system. The recombinant HVJ vector system causes viral replication and production of viral proteins in target cells (lower panel). One viral protein, NP protein, is highly antigenic and strongly induces immune reaction *in vivo*. Therefore, the repeated injection of vector is difficult in case of recombinant HVJ vector system. So the major drawback of recombinant HVJ vector system is biosafety. In contrast, HVJ-E induces only the expression of transgene (upper panel) and can be used for the repetitive administration.

Table 2. Characteristics of HVJ-E and Recombinant HVJ Vectors

	HVJ-E	Recombinant HVJ
Replication of viral genome	No	Yes
Production of viral proteins	No	Yes
Release of virus particles	No	Yes
Toxicity	Low	Moderate
Immunogenicity	Low	High
Suitability for repeated administrations	Yes	Possible

the plasmid-containing HVJ-E vector to a cell surface. The plasmid was directly released into cytoplasm through the cell-HVJ-E fusion hole, but not through endocytosis. The plasmid is transported in cytoplasm, not taken into lysosomes. Thus the plasmid is not degraded by lysosomal enzymes, resulting in higher and more efficient gene expression in the host cells. Advantages of the HVJ-E vector technology are (1) rapid incorporation of therapeutic molecules into an envelope, eliminating recombinant DNA construction steps; (2) no viral replication and viral gene expression, eliminating the major safety concerns for viral vectors; (3) ability to include single therapeutic molecule entity as well as a mixture of different types of therapeutic molecular entities for combination therapies. Figure 5 shows that the HVJ-E vectors, containing GFP plasmid DNA, NF-κB decoy ODN, immunoglobulin G, and BSA respectively, introduced each molecule into cells at high efficiency (data not published).

HVJ-E vector can efficiently transfect various types of human and mammalian cells, such as BHK-21, SAS, HEK 293, HuH-7, K-562, as well as human aortic endothelial primary cells and rat aortic primary cells (Table 3, Kaneda *et al.*, 2002, and data not published). In animal studies, HVJ-E vectors deliver genes effectively in organs such as liver, brain, skin, uterus, tumor masses, lung and eye of animals including mouse, rat, rabbit and monkey (data not shown). The pictures in Fig. 3 (data not published) demonstrate high GFP expression in rat brain by administration of the HVJ-E via carotid artery and high transfection of a decoy FITC-labeled ODN into a rat cartridge tissue by intra joint administration of the HVJ-E. These indicate the powerful penetration activity of HVJ-E vectors.

In comparison to HVJ-liposome and liposome of lipofectin, HVJ-E shares many favorable characteristics with HVJ-liposome, such as high level of transgene expression and low cytotoxicity, whereas liposome exhibits much higher cytotoxicity. Nevertheless, HVJ-E vector possesses higher fusion activity reflected in more rapid transfection time and requires much simpler preparation process reflected

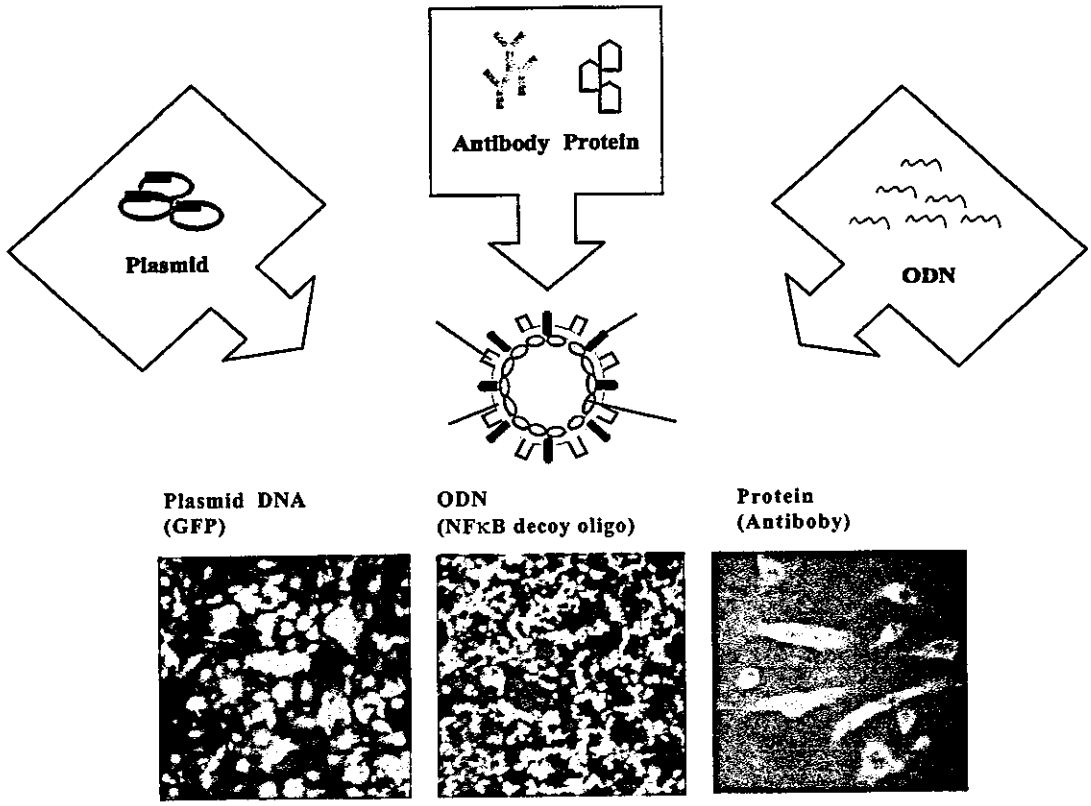

Fig. (5). HVJ-E vector as delivery system for various biomacromolecules
HVJ-E vector has a capability for delivering biomolecules and synthetic molecules with high molecular weight. Various kinds of biomolecules including plasmid DNA, antibody, enzyme, other proteins and oligonucleotide can be incorporated into the empty particles of HVH-E vector (upper panel). Lower pictures of fluorescence microscope demonstrate the transfection results of GFP expression vector (left), FITC-labeled oligonucleotides (center) and FITC-labeled antibody (right) delivered by HVJ-E vector. As shown in the pictures, over 90% of the target cells were transfected by HVJ-E vector.

Table 3. Transfection of Various Cells by HVJ-E

Cell type	Species	Source of cells	Transfection efficiency
Adherent cells			
HeLa	Human	Epitheoid carcinoma	+
293	Human	Primary embryonic kidney	+++
SAS	Human	Tongue squamous carcinoma	+++
HuH-7	Human	Hepatoma	+++
BHK-21	Hamster	Kidney	+++
Blood cells			
K-562	Human	Chronic myelogenous leukemia	++
CCRF-CEM	Human	Acute lymphoblastic leukemia	-
NALM-6	Human	T cell leukemia	+
Primary cells			
HAEC	Human	Aortic endothelial cells	++
RAC	Rat	Aortic cells	++

in the much shorter preparation time (Table 4, data not published).

Figure 6 illustrates a process for HVJ-E production. The HVJ-E is produced by cell culture followed by downstream processes, including inactivation, purification and inclusion of therapeutic molecules into the envelope particles. Wild type HVJ is produced in a suspension culture of cloned 293 cells in serum free medium in a bioreactor. The viral particles were collected and inactivated by the treatment with beta-propiolactone and then purified by column chromatography. The purified HVJ-E particles were treated with a mild detergent and then mixed with the molecules of interests for inclusion. The included HVJ-E vectors are further purified with a buffer exchange into final formulation

Table 4. Characteristics of Transfection Mediated by HVJ-E, HVJ Liposome, and Liposome (Lipofectin) (*In Vivo* and *In Vitro*)

	HVJ liposome	HVJ-E	Liposome (Lipofectin)
Gene expression level	+_++	++_+++	+_+++
Homogeneity of gene expression	+++	+++	+
Cell Toxicity	-	-	++++
Time necessary for gene expression	16 hrs	16 hrs	48 hrs
Time necessary for transfection	2 hrs	5 min	4 _ 24 hrs
Capability of multiple gene transfection	+++	+++	+
Sample preparation time	4 hrs	15 min	40 min

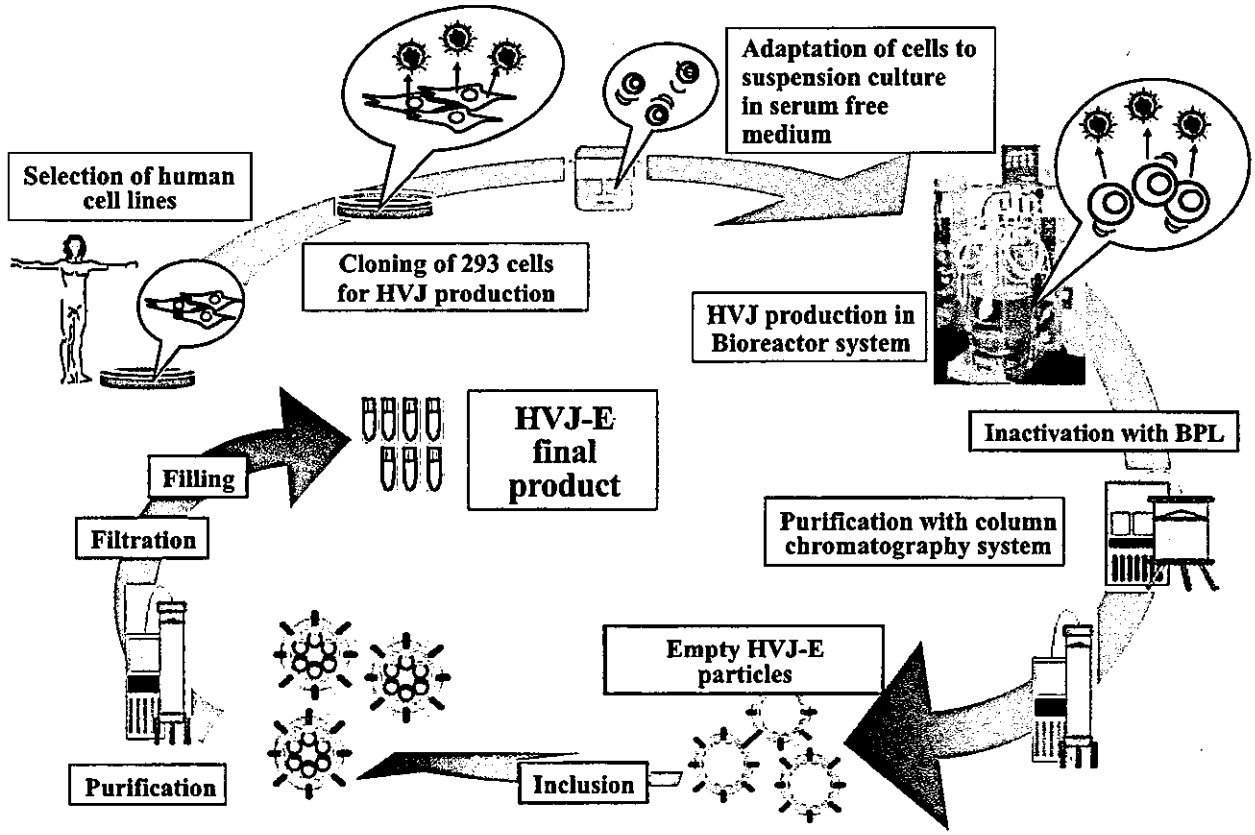

Fig. (6). Process development and manufacturing of HVJ-E vector for clinical application.
A GMP production process of HVJ-E vectors has been developed for clinical use such as treatment of cardiovascular diseases. After the screening of human cell lines suitable for GMP production, cloning of parental 293 cells was conducted. Cloned 293 cells have been adapted to serum-free/animal protein-free medium in suspension culture and used for HVJ production in stirred tank bioreactor. This automated bioreactor system is able to scale up to 100L or larger. After inactivation of the HVJ viral genome, the HVJ-E is purified by multiple steps of filtration and column chromatography to remove viral genome, viral proteins, host cell-derived proteins and host cell nucleic acids. After inclusion to incorporate various biomolecules, the biomolecule-included HVJ-E is further purified for removal of unincorporated materials, formulated, sterile-filtrated and subjected to final filling as HVJ-E final product.

buffer for either immediate application or storage. This is a scalable process that can meet future demands of large quantity HVJ-E production to supply real clinical applications.

With the versatility in inclusion of a wide range of different molecules and high transfection efficiency into a variety of cells and tissues both *in-vitro* and *in-vivo*, the HVJ-E vector technology not only can deliver various therapeutic molecular entities, such as therapeutic genes, ODNs or proteins, but can play an important role in functional genomics and proteomics, as well as in high throughput drug screening for the discovery of new target

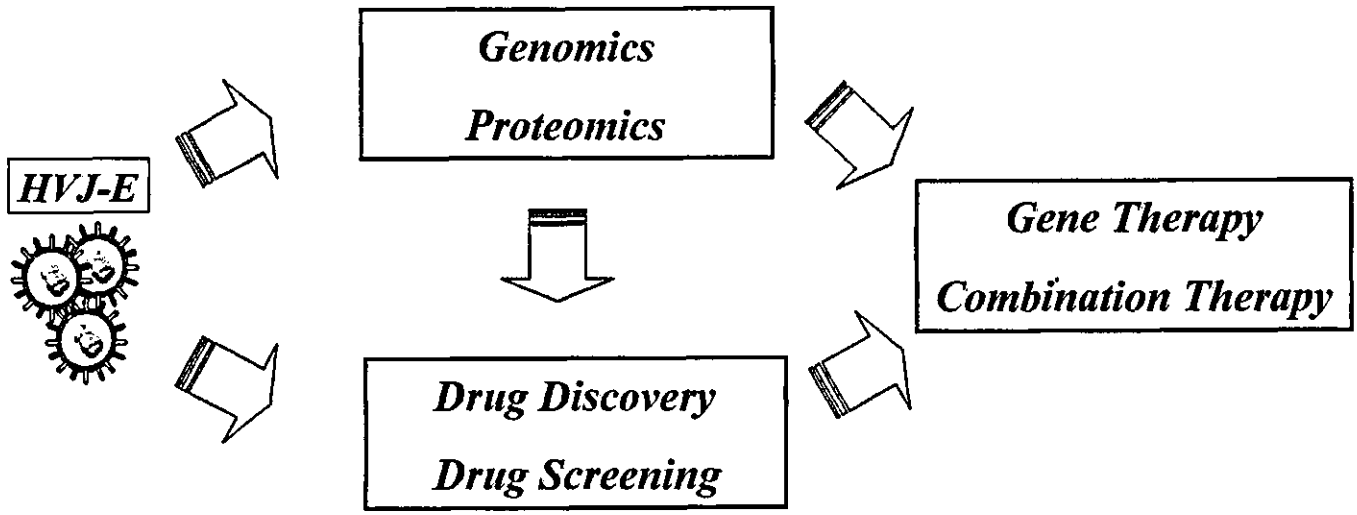

Fig. (7). Application of HVJ-E non-viral vector technology
HVJ-E non-viral vector system is useful tool for two fields, basic science and drug development. For genomics and proteomics analyses, cell array system (or vector array system) using HVJ-E vector in solid phase is under development (upper box). Drug delivery system (DDS) using HVJ-E vector is also developed in parallel (lower box). HVJ-E non-viral vector will become a tool for drug discovery and drug screening, since it could be used for both *in vivo* and *in vitro* delivery of various kinds of molecules including conventional drugs.

genes and new drugs (Fig. 7). As an emerging novel delivery system with no precedent case of clinical applications, systemic safety and toxicology studies are required for the clinical use of HVJ-E. Nevertheless, delivery by HVJ-E possibly allows repeated administration of therapeutic genes or therapeutic molecules and results in more persistent gene expression in comparison to other gene delivery technologies, the HVJ-E vector technology has the potential being not only safer but also more efficacious for the treatment of cardiovascular disease, as well as many other clinical applications.

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Pharmaceutical Research, Vol. 21, No. 7, July 2004 (© 2004) *Research Paper*

Suppression of the Progress of Disseminated Pancreatic Cancer Cells by NK4 Plasmid DNA Released from Cationized Gelatin Microspheres

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Purpose. NK4, composed of the NH_2-terminal hairpin and subsequent four-kringle domains of hepatocyte growth factor (HGF), acts as a potent angiogenesis inhibitor. This study is an investigation to evaluate the feasibility of controlled release of NK4 plasmid DNA in suppressing the tumor growth. Controlled release by a biodegradable hydrogel enabled the NK4 plasmid DNA to exert the tumor suppression effects.

Methods. Biodegradable cationized gelatin microspheres were prepared for the controlled release of an NK4 plasmid DNA. The cationized gelatin microspheres incorporating NK4 plasmid DNA were subcutaneously injected to tumor-bearing mice to evaluate the suppressive effects on tumor angiogenesis and growth.

Results. The cationized gelatin microspheres incorporating NK4 plasmid DNA could release over 28 days as a result of microspheres degradation. The injection of cationized gelatin microspheres incorporating NK4 plasmid DNA into the subcutaneous tissue of mice inoculated with pancreatic cancer cells prolonged their survival time period. An increase in the tumor number was suppressed to a significantly greater extent than free NK4 plasmid DNA. The controlled release of NK4 plasmid DNA suppressed angiogenesis and increased the cell apoptosis in the tumor tissue while it enhanced and prolonged the NK4 protein level in the blood circulation.

Conclusions. We conclude that the controlled release technology is promising to enhance the tumor suppression effects of NK4 plasmid DNA.

KEY WORDS: cationized gelatin; controlled release; microspheres; NK4; pancreatic cancer.

INTRODUCTION

Pancreatic cancer ranks as the eighth most frequent type of solid tumor arising worldwide and represents the fourth most frequent cause of death. Although early tumor diagnosis, improved surgical treatment, and multimodal therapeutic concepts have helped to reduce the mortality of patients with pancreatic cancer, the prognosis is still extremely poor; the overall 5-year survival rate is only 1–4% after the diagnosis (1). A chance of cure exists only for a minority of patients with locally limited and surgically resectable tumor. However, of patients who are radically treated by surgical curative resection, 70–80% will suffer from an incurable local relapse, distant metastases, and peritoneal carcinosis. Although a local relapse might be caused by incomplete resection, distant metastases and peritoneal carcinosis depend on dissemination of malignant cells (2). Their elimination is the aim of various adjuvant therapy concepts that are currently under investigation, including chemotherapy, immunotherapy, and gene therapy.

Hepatocyte growth factor (HGF) has been noted as the signal molecule that plays an important role in development, differentiation, and morphogenesis of living systems (3–5). Recently, some therapeutic trials of angiogenesis (6,7), chronic fibrotic diseases (8,9), and tissue regeneration (10,11) by this HGF have been performed to demonstrate the potential efficacy. On the other hand, HGF often acts in an autocrine fashion to induce and enhance the invasive, angiogenic, and metastatic functions of malignant tumors by way of the c-Met/HGF receptor (12–15). Therefore, it is highly expected that the molecular blocking of c-Met/HGF receptor effectively suppresses the invasive, angiogenic, and metastatic functions of tumor cells. Based on this concept, Date *et al.* have prepared an antagonist for HGF, which is composed of the NH_2-terminal hairpin domain of HGF α-subunit and the subsequent four kringles domains (NK4) (16). The NK4 binds to the c-Met/HGF receptor but does not induce tyrosine phosphorylation of c-Met. NK4 competitively inhibits some biological events driven by the c-Met/HGF receptor binding, such as the invasion and metastasis of distinct types of tumor cells and angiogenesis (16–18). The recombinant protein of NK4 has been used for tumor animal models to demonstrate the *in vivo* efficacy in tumor therapy (17–19), and the plasmid DNA of NK4 exhibited similar antitumor effects *in vivo* (20–24).

Based on the recent advent of genomics, new genes have been discovered and will become therapeutically available for various diseases in the near future. In this connection, gene therapy is expected as a new and promising therapeutic choice. Currently, several human clinical trials are proceeding to treat the cancer by using the viral vectors of retroviruses, adenoviruses, and adeno-associated viruses. In spite of the high transfection efficiency, the trials are limited by the adverse effects of virus itself, such as immunogenicity and toxicity or the possible mutagenesis of cells transfected. As the nonviral vectors, many types of cationized polymers (25) and cationized liposomes (26) have been explored to use plasmid DNAs. This approach is to enable the plasmid DNA to neutralize the anionic charge as well as to reduce the molecular size for enhanced efficiency of plasmid DNA transfection, which causes an increase in the gene expression. However, the shorter duration and the lower level of gene expression than viral vectors are important issues to be technologically improved. Moreover, a plasmid DNA, only when complexed with the nonviral vector and given to cells or injected into the body in the naked form, is degraded and inactivated by enzymes or cells with ease. One of the possible ways to tackle the issues is to permit the controlled release of plasmid DNA by combining with an appropriate carrier.

Gelatin has extensively been used for industrial, pharmaceutical, and medical applications and the biosafety is proved through its long clinical usage as a surgical biomaterials and drugs ingredient. Another unique advantage of gelatin is variation in the electrical nature, while the electric nature can

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be readily changed by the processing method of collagen (27). We have designed and explored the controlled release system of drugs on the basis of drug release governed by degradation of drug carrier. Drugs are immobilized into the biodegradable hydrogel of gelatin on the way of physicochemical interaction forces between the drug and gelatin molecules. In this release system, the drug immobilized is not released from the hydrogel unless the hydrogel carrier is degraded to generate water-soluble gelatin fragments. The drug release can be controlled only by changing the hydrogel degradation (28). We have demonstrated that the hydrogel system enables growth factors to release in a bioactive state and consequently enhance their biological functions, in marked contrast to the growth factor in the solution form. The growth factor used includes basic fibroblast growth factor (bFGF) (28), bone morphogenetic protein-2 (BMP-2) (29), transforming growth factor beta1 (TGF-beta1) (30), and HGF (31). In addition, the cationized gelatin of positive charge can readily be prepared by introducing amine residues to the carboxyl groups of gelatin. The plasmid DNA polyionically immobilized in the cationized gelatin hydrogel is released from the hydrogel only if the hydrogel is degraded to generate the water-soluble gelatin fragments (32,33). This study indicates that the cationized gelatin hydrogel enabled a NK4 plasmid DNA to achieve the controlled release and consequently exert the tumor-suppressive effects that were not observed for the plasmid DNA solution.

In this study, we applied the cationized gelatin hydrogel to the controlled release of expression plasmid for human NK4 to evaluate the suppressive effects on tumor angiogenesis and growth in tumor-bearing mice. The release mechanism driven by degradation of release carrier is quite different from that of plasmid DNA diffusion from the release carrier, which has been reported as the conventional release system of plasmid DNA (34,35). The results were compared to those with delivery of free plasmid to emphasize efficacy of the release system in enhancing the biological activity of NK4.

MATERIALS AND METHODS

Preparation of Cationized Gelatin Microspheres Incorporating NK4 Plasmid DNA

The carboxyl groups of gelatin, with an isoelectric point of 9.0 (MW 100,000) prepared by an acid process of pig skin (Nitta Gelatin Inc., Osaka, Japan), were chemically converted by introducing amino groups for cationization of gelatin (32,33). Ethylenediamine was added at a molar ratio of 50 moles per mole of carboxyl groups of gelatin into 250 ml of 100 mM phosphate-buffered solution containing 5 g of gelatin. Immediately after that, the solution pH was adjusted to 5.0 by adding 5 M HCl aqueous solution. Further, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt was added at a molar ratio of 3 moles per mole of carboxyl groups of gelatin. The reaction mixture was agitated at 37°C for 18 h and then dialyzed against double-distilled water (DDW) for 48 h at room temperature. The dialyzed solution was freeze-dried to obtain a cationized gelatin. When determined by the conventional 2,4,6-trinitrobenzene sulfonic acid (TNBS) method (36), the percentage of amino groups introduced into gelatin was 50.9 mol% per the carboxyl groups of gelatin. Gelatin microspheres were prepared by chemical cross-linking of gelatin in a water-in-oil emulsion state. Aqueous solution of 10 wt% cationized gelatin (10 ml) was preheated at 40°C and then added dropwise into 375 ml of olive oil preheated at 40°C, while an impeller stirring at 420 rpm was used for 10 min to yield a water-in-oil emulsion. The emulsion temperature was decreased to 4°C for the natural gelation of gelatin solution. The resulting microspheres were washed three times with cold acetone, collected by centrifugation (5000 rpm, 4°C, 5 min), fractionated in size by sieves with apertures of 70 and 100 μm, and air-dried at 4°C. The non–cross-linked and dried gelatin microspheres (50 mg) were placed in 25 ml of acetone/0.01 M HCl solution (7/3, vol/vol) containing 60 μl of 25 wt% glutaraldehyde solution and stirred at 4°C for 24 h to allow the cationized gelatin to cross-link. After washing by centrifugation with DDW, the microspheres were agitated in 25 ml of 100 mM aqueous glycine solution at room temperature to block the residual aldehyde groups of glutaraldehyde. The resulting microspheres were washed with DDW by centrifugation and freeze-dried. The average diameter of microspheres prepared was 75 μm.

To impregnate NK4 plasmid DNA (6.2 kbp) containing the cytomegalovirus immediate early enhancer-chicken β-actin hybrid (CAG) promoter, into cationized gelatin microspheres, 20 μl of 100 mM phosphate-buffered saline solution (PBS; pH 7.4) containing 100 or 200 μg of NK4 plasmid DNA was dropped onto 2 mg of the freeze-dried cationized gelatin microspheres, followed by incubation for 24 h at 4°C. The similar procedure other than using PBS without NK4 plasmid DNA was done to prepare empty cationized gelatin microspheres. NK4 plasmid DNA was completely incorporated into cationized gelatin microspheres by this impregnation procedure, as the volume of plasmid DNA solution (20 μl) is much smaller than that theoretically impregnated into the freeze-dried microspheres and, in fact, was found to be sorbed into the microspheres. The observation of the cryosection of microspheres incorporating fluorescent-labeled plasmid DNA by fluorescent microscopy revealed that the fluorescent-labeled plasmid DNA was localized homogeneously throughout the microspheres but not on the surface (data not shown)

Evaluation of *in Vivo* Degradation of Cationized Gelatin Microspheres

Cationized gelatin microspheres were radioiodinated using [^{125}I] Bolton-Hunter reagent (37). The ^{125}I-labeled cationized gelatin microspheres (2 mg/200 μl PBS/mouse) were subcutaneously injected into the back of ddY mice, 6 to 8 weeks old (Japan SLC, Inc., Hamamatsu, Japan) (6 mice/group). At 1, 3, 7, 14, 21, and 28 days after injection, the mouse skin and muscle containing the cationized gelatin microspheres injected were taken out to measure their radioactivity on a gamma counter. The radioactivity ratio of the sample to the cationized gelatin microspheres injected initially was measured to express the percentage of remaining radioactivity in the cationized gelatin microspheres. All the animal experiments were carried out according to the Institutional Guidance of Kyoto University on Animal Experimentation.

Evaluation of *in Vivo* NK4 Plasmid DNA Release from Cationized Gelatin Microspheres Incorporating NK4 Plasmid DNA

NK4 plasmid DNA was radioiodinated according to the method of Chan *et al.* (38). Cationized gelatin microspheres incorporating 100 μg of ^{125}I-labeled NK4 plasmid DNA were prepared similarly and subcutaneously injected to ddY mice at the injection volume of 200 μl (6 mice/group). As control, the PBS solution of ^{125}I-labeled NK4 plasmid DNA (100 μg/200 μl/mouse) was subcutaneously injected. The radioactivity was measured 1, 3, 7, 14, 21, and 28 days later. The percentage of remaining radioactivity in the NK4 plasmid DNA was similarly calculated.

In Vivo Experiments

The human pancreatic cancer cells, AsPC-1, suspension of 1×10^6 cells/200 μl, were transplanted to the peritoneal cavity of 6-week-old nude mice (BALBc nu/nu, Japan SLC, Inc.). For therapeutic treatment, 4 days later, 2 mg of cationized gelatin microspheres incorporating 100 or 200 μg of NK4 plasmid DNA, 200 μg of free NK4 plasmid DNA, 2 mg of empty cationized gelatin microspheres, and saline were subcutaneously injected into the back of nude mice (200 μl/mouse).

First, the survival of treated mice was evaluated every day to prepare the survival curve (10 mice/group). The number and weight of disseminated implants in the peritoneal cavity were recorded (5 mice/group per time point). The immunochemical section of disseminated nodule on day 28 was stained with an antibody against the von Willebrand factor (Dako, Glostrup, Denmark) to recognize blood vessels in the tumor tissue. The stained section was viewed on a light microscope to count the number of blood vessels from at least 10 fields randomly selected per section. The immunochemical section of disseminated nodule was stained with TdT (terminal deoxynucleotidyl transferase) mediated dUTP-biotin nick end labeling (TUNEL) method (39) by Apoptosis Detection Kit (ApopTag®, Intergren Company, NY, USA) to recognize apoptosis cells in the tumor tissue. The number of positive-stained cells was counted under a light microscope from at least 10 fields randomly selected per section.

In Vivo Assessment of Gene Expression Following Injection of Cationized Gelatin Microspheres Incorporating NK4 Plasmid DNA or lacZ Plasmid DNA

Cationized gelatin microspheres incorporating 100 or 200 μg of NK4 plasmid DNA and 200 μg of free NK4 plasmid DNA were injected into tumor-bearing mice by the similar procedure mentioned above. The mice were sacrificed by cervical dislocation 7, 14, 21, and 28 days after NK4 plasmid DNA treatment to evaluate gene expression. The level of NK4 protein expressed in the disseminated nodule and serum was measured by use of HGF EIA kit (Institute of Immunology Co., Ltd., Tokyo, Japan) (5 samples/group). Briefly, the samples of tumor were immersed and homogenized in a lysis buffer (Institute of Immunology Co., Ltd., Tokyo, Japan) at the buffer volume (μl)/sample weight (mg) ratio of 4:1 in order to normalize the influence of weight variance on the assay. The sample lysate (0.2 ml) was transferred to a centrifuge tube and centrifuged at 15,000 × *g* at 4°C for 15 min. The supernatant (50 μl) and serum were applied to the well of HGF EIA kit.

The tumor-bearing mice were sacrificed by cervical dislocation to collect the tissue around microspheres 7 days after injection of cationized gelatin microspheres incorporating 100 μg of lacZ plasmid DNA. The tissue samples were frozen and cut into 10-μm sections. The sections were fixed with 0.5% gluteraldehyde for 10 min, washed with PBS, and stained with X-gal using the standard procedure to detect β-galactosidase protein, while they were counterstained with eosin.

Statistical Analysis

All the data were expressed as the mean ± the standard derivation of the mean. Statistical analyses were performed based on the unpaired Student's *t* test (two-tailed), and the statistical difference between survival curves was determined with the generalized Wilcoxon test,; significance was accepted at $p < 0.05$.

RESULTS

In Vivo Release Profile of NK4 Plasmid DNA from Cationized Gelatin Microspheres

Radiotracing experiment (Fig. 1) revealed that the NK4 plasmid DNA was retained around the injected site of cationized gelatin microspheres incorporating NK4 plasmid DNA over the time period of 28 days, whereas free NK4 plasmid DNA injected was excreted more rapidly. A good correlation in the time profile of *in vivo* retention was observed between the NK4 plasmid DNA incorporated and mi-

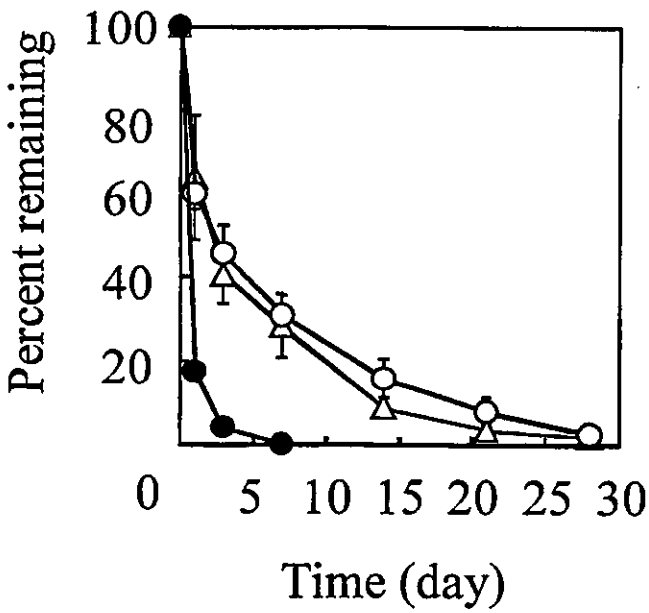

Fig. 1. The time course of radioactivity remaining of cationized gelatin microspheres incorporating ^{125}I-labeled NK4 plasmid DNA (○) and free ^{125}I-labeled NK4 plasmid DNA (●) or ^{125}I-labeled cationized gelatin microspheres (△) after subcutaneous injection into the back of mice. The microspheres enabled NK4 plasmid DNA to remain in the injected site for a longer time period than in the solution form. The *in vivo* retention profile of NK4 plasmid DNA was in good accord with that of microspheres as the release carrier, indicating the controlled release of NK4 plasmid DNA accompanied with the carrier degradation.