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# HVJ-based non-viral gene transfer method: successful gene therapy using HGF and VEGF genes in experimental ischemia

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## 1. ABSTRACT

VEGF and HGF are pleiotropic factors that regulate cell growth, cell motility, and morphogenesis of various types of cells. The receptors of these growth factors are expressed in neurons and endothelial cells, and are identified as neurotrophic, neuroprotective, and angiogenic factors. Indeed, gene therapy using viral vectors encoding the VEGF or HGF gene has been reported to be effective for preventing the expansion of ischemic injury. However, the safety issue of viral vectors is a major problem in clinical application. To overcome this problem, we have developed an HVJ-based non-viral vector, which achieves high-efficiency transfection rates of viral vectors with the safety of liposomes. This review discusses the feasibility of gene therapy using an HVJ-based non-viral vector containing the VEGF or HGF gene for cerebral ischemia.

## 2. INTRODUCTION

Ischemic stroke is the one of the common causes of permanent disability of cognitive and sensorimotor function. Although thrombolysis and controlled hypothermia are an effective treatment in the acute stage, an effective treatment has not yet been established to enhance both neuroprotection and angiogenesis for the ischemic brain.

Vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) have been examined in the laboratory for the treatment of cerebral ischemia, primarily because of their pleiotropic actions, such as neurotrophic, neuroprotective, and angiogenic effects in the central nervous system. It is also known that VEGF and HGF are related to functional recovery in the ischemic brain, since

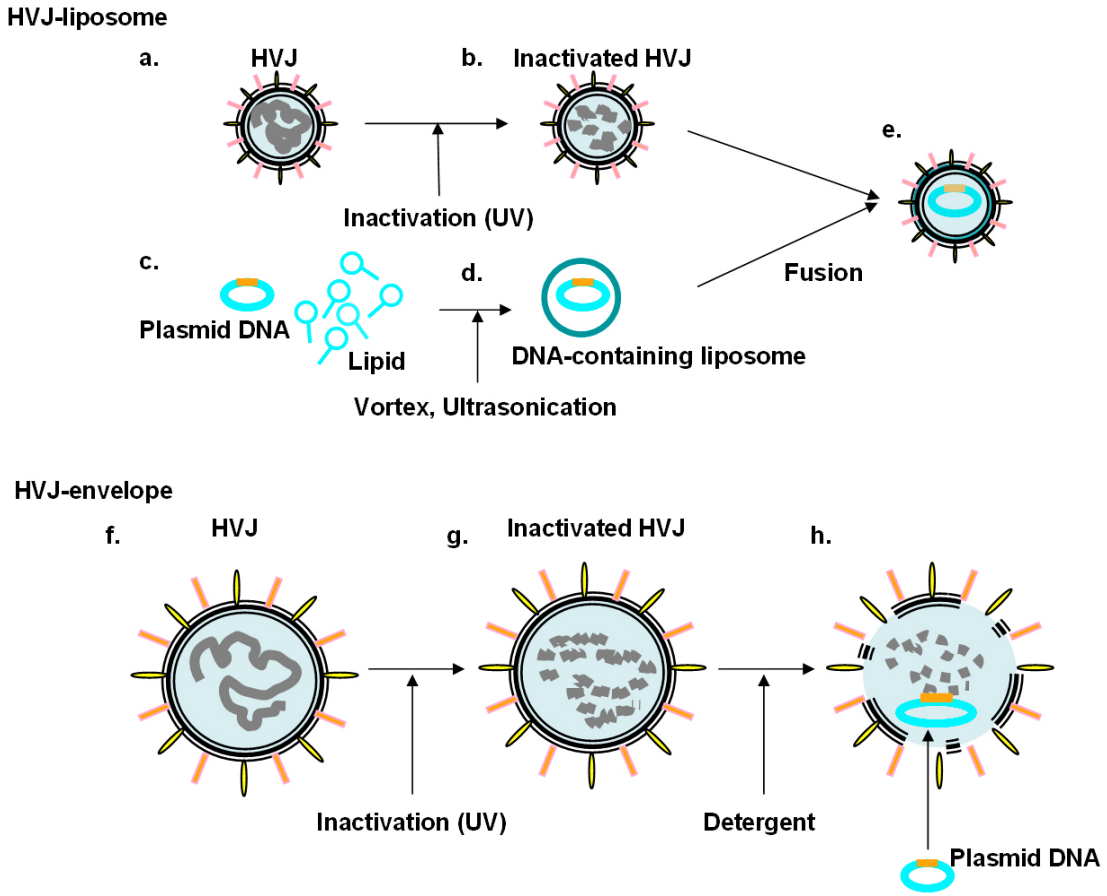


**Figure 1.** HVJ (hemagglutinating virus of Japan)-liposome and HVJ-envelope. HVJ-liposome: (a, b) HVJ is inactivated by UV. (c, d) Plasmid DNA is incorporated into liposome by vortex-ultrasonication. (e) The liposome is fused with UV-inactivated HVJ. HVJ-envelope: (f, g) HVJ is inactivated by UV. (h) The membrane permeability of HVJ is increased by detergent and the plasmid DNA is incorporated into inactivated HVJ.

their expression is up-regulated or down-regulated in the ischemic brain (1-6), and application of these growth factors has been shown to rescue ischemic brain in rodent models (7-11). From these viewpoints, VEGF and HGF are believed to be promising molecules that could achieve functional recovery after ischemic stroke.

However, it is hard to apply these growth factors clinically, since it is difficult to produce sufficient quantities of human quality grade recombinant protein at low cost. To overcome this problem, many researchers have tried gene therapy in a rodent cerebral ischemia model using viral or non-viral vectors such as adenovirus or cationic-liposomes, and also achieved rescue from ischemic injury. Similarly, we have shown the effectiveness of gene therapy using a so-called "HVJ-liposome" vector containing the HGF gene (12,13). The HVJ-liposome vector is a liposome combined with fusion proteins derived from the hemagglutinating virus of Japan (HVJ) (Figure 1), which appears more efficient than conventional liposomes and displays less immunogenicity and toxicity than viral vectors (14). We discuss the potential of HVJ-liposome vectors incorporating the VEGF or HGF gene for gene therapy in cerebral ischemia.

## 3. VEGF AND HGF IN ISCHEMIC BRAIN

VEGF is a 45 kDa basic heparin binding homodimeric glycoprotein, which is a secreted growth factor. There are four isoforms of VEGF; VEGF A, B, C, and D. VEGF A is the key growth factor for angiogenesis and neuroprotection. VEGF A also has isoforms: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$. The receptors of VEGF are FLT-1 and FLK-1, which activate intracellular tyrosine kinase. Neuropilin 1 (NP-1) is another receptor of VEGF, to which $VEGF_{165}$ binds (15). NP-1 and FLK-1 are key mediators of the PI3-K/Akt and MAPK kinase pathways, which result in neuronal development, protection, and neurite outgrowth (16). Interestingly, recent reports also showed a role of VEGF in improved cognition via hippocampal neurogenesis through FLK-1 (17).

VEGF and its receptors, FLT-1 and FLK-1, are quickly induced in both the ischemic core and peri-infarct region after middle cerebral artery occlusion (MCAo) in rats (1). VEGF121 and VEGF156 are mainly present in

**Table 1.** VEGF or HGF as potential therapeutic agents for neurological disorders

| Neurological disorders. Related articles | Cytokine | Reference |
|---|---|---|
| Cerebral ischemia | VEGF | 7-9 |
| | HGF | 10-13,33 |
| Parkinson disease | VEGF | 45 |
| | HGF | Unpublished data |
| Amyotrophic lateral sclerosis | VEGF | 46 |
| | HGF | 34,35 |
| Alzheimer disease | VEGF | 47 |
| | HGF | 36,37 |
| Hearing loss | HGF | 38 |

neurons, macrophages, and glial cells. Although expression is observed from 18 hrs to 14 days after MCAo in the peri-infarct region, the time course of its expression is dependent on the type of cells: the peak expression levels are 18 hrs - 1 day in macrophages, 2-7 days in neurons, and 2-10 days in glial cells (1). VEGF is also induced in endothelial cells in the peri-infarct and core region with the progression of angiogenesis (1). Another report also showed that immunoreactivity of VEGF, FLT-1, and FLK-1 is increased in the border zone of infarcts up to 3 days after transient or permanent MCAo (3), but the expression level differs between FLT-1 and FLK-1. FLT-1 is increased in neurons, glial cells, and endothelial cells, whereas FLK-1 is increased in glial cells and endothelial cells (3). Since VEGF colocalizes with Ang-2 and Tie-1, which exert their functions at later stages of vascular development, one of the roles of VEGF is considered to be acceleration of angiogenesis after cerebral ischemia (18). In parallel with neuroprotective and neurotrophic effects of VEGF in *in vitro* studies (19-21), post-ischemic expression of VEGF *in vivo* also plays a pivotal role in neuroprotection and neurite outgrowth.

Similar to VEGF's rise as a neuroprotective agent, HGF has been implicated as a therapeutic drug due to its pleiotropic effects. HGF is a well-known potent pleiotropic cytokine that exhibits mitogenic, motogenic, and morphogenic activity in a variety of cells (22-24). Both HGF and the c-Met/HGF receptor of membrane spanning tyrosine kinase are expressed in various regions of the brain (24). The functional coupling between HGF and c-Met enhances the survival of neurons in primary culture and induces neurite outgrowth in neuronal development in vitro (25). Recent studies suggest that HGF prevents apoptosis in cerebellar granular neurons via the phosphatidylinositol-3 (PI3)-kinase/Akt pathway (26,27). It is also reported that sympathetic neurons of the superior cervical ganglion coexpress bioactive HGF and its receptor, c-Met, both in vivo and in vitro (28). Exogenous HGF selectively promotes the growth of cultured sympathetic neurons; the magnitude of this growth effect is similar to that observed with exogenous nerve growth factor (NGF) (29). It is also reported that HGF cooperates with ciliary neurotrophic factor (CNTF) in promoting the survival and growth of parasympathetic and proprioceptive neurons (30). Given these unique properties, HGF is considered to be a novel neuroprotective agent. HGF is also involved in the development and maintenance of cortical neurons during differentiation, mitogenesis, neuritogenesis and neuronal survival during the development of the rat cerebral cortex (31). Interestingly, HGF promotes proliferation and neuronal differentiation of neural stem cells from mouse embryos (32). Due to these pleiotropic effects, HGF is believed to be a promising molecule to treat other neurological disorders, including cerebral infarction (Table 1) (10-13,33-38).

In the ischemic brain, the temporal profile of expression of HGF and c-Met is still controversial. Hayashi et al. reported that HGF is induced in neurons in the peri-infarct region from 3 to 24 hrs after permanent MCAo, but not in glial cells (4). However, recent reports showed that HGF-immunopositive cells are observed mainly in reactive astrocytes in the peri-infarct region from 4 days after occlusion, peaking at 14-28 days (6). Coincidentally, c-Met-immunopositive cells are also observed exclusively in reactive astrocytes in the peri-infarct region (6). In contrast, another recent report revealed that the production of HGF is decreased from 3 days after microsphere-embolization in rats, and gradually further reduced up to 7 days (5). These different results might be related to methodological differences, such as the type of ischemic model and the method of evaluation (i.e., immunohistochemistry, Western blotting, or RT-PCR). Although it is necessary to clarify the actions of HGF after cerebral ischemia in detail, HGF is also an important factor for neuroprotection and acceleration of angiogenesis, as presented below.

## 4. TREATMENT OF EXPERIMENTAL CEREBRAL ISCHEMIA USING VEGF OR HGF

Based on accumulating evidence demonstrating VEGF and HGF as promising molecules for treating cerebral ischemia, as mentioned above, treatment using recombinant proteins of both growth factors was initially tried in rodents. First, intracortical infusion of VEGF was demonstrated to produce neovascularization in normal rats, but the newly formed vessels lack blood-brain barrier (BBB) phenotypic markers, such as GLUT-1 (39). A recent report showed that vessel density is dose-dependently increased by chronic VEGF165 infusions, but capillary permeability is increased when assessed using Evans blue and [(3)H]inulin (40). In fact, early postischemic (1 hour) administration of recombinant VEGF165 to ischemic rats significantly increases BBB leakage, resulting in hemorrhage (8). Also, a VEGF antagonist, mFlt (1-3)-IgG, which is a fusion protein, reduces ischemia/reperfusion-related brain edema and injury, implicating VEGF in the pathogenesis of stroke and related disorders (41).

In contrast, others have reported that there is no exacerbation of cerebral edema by VEGF. Hayashi et al. reported that application of $VFGF_{165}$ onto the surface of the brain after 90 minutes of transient MCAo shows a significant reduction of infarct volume and cerebral edema (7). Similarly, late (48 hrs) intravenous administration of $rhVEGF_{165}$ to ischemic rats also enhances angiogenesis in the ischemic penumbra and significantly improves neurological recovery (8). Interestingly, intracerebroventricular administration of VEGF on days 1-3 of reperfusion after transient MCAo reduces infarct size and enhances angiogenesis in the striatal ischemic penumbra and the delayed survival of regenerated neurons in the dentate gyrus and subventricular zone (9). The difference between these reports might be related to the route of administration, isoform of VEGF, or applied dose. The appropriate dose and administration route of VEGF need to be considered for optimal therapeutic outcome in ischemic injury.

Although the number of reports is small compared to those for VEGF, administration of recombinant HGF in cerebral ischemia was also shown to enhance neuroprotection and angiogenesis. First, Miyazawa et al. demonstrated that continuous preischemic (7 days before ischemia) or postischemic (after 6 hrs of ischemia) intrastriatal administration of human recombinant HGF (10 or 30 μg) prevents the delayed death of hippocampal neurons under both anesthetized and awake conditions in transient forebrain ischemia in Mongolian gerbils (10). Similarly, intraventricular administration of human recombinant HGF in a transient MCAo model prevents neuronal death and reduces the infarct volume in a dose-dependent manner (11). In rats treated with HGF, TUNEL-positive neurons are less abundant at the inner boundary of the infarct area, and Bcl-2 protein is markedly expressed in surviving neurons subjected to ischemia in the HGF group. The number of vascular laminae is significantly greater in the HGF group than in the vehicle group (11). These data suggest that recombinant HGF is effective for cerebral ischemia possibly by stimulating cell survival pathways associated with neuroprotection and angiogenesis.

Although VEGF and HGF are shown to protect against cerebral ischemia, their benefits are highly dependent upon administering a large amount of recombinant protein due to the short half-life of VEGF and HGF. For example, the alpha-phase of intravenous administration of rHGF is 3.2 minutes, while the beta-phase is 26.5 minutes (42). From this viewpoint, gene therapy may be superior to recombinant protein therapy, since continuous expression of a transgene over a long period could be achieved by gene therapy. It may also be preferable to deliver a lower dose over a period of several days or more by the actively expressed transgene in the brain, rather than a single or multiple bolus doses of recombinant protein, to avoid side effects. Moreover, when contemplating with clinical trials, the cost is too high to provide sufficient quantities of human quality grade recombinant protein, due in large part to the nearly prohibitive cost of scaling up from research grade to human quality recombinant protein.

With these limitations of recombinant protein therapy in mind, a concerted effort was made by many researchers to attempt gene transfer using viral vectors or non-viral vectors. Generally, viral vectors are efficient to transfer genes, but there are safety problems. Non-viral vectors, such as liposomes, are safer than viral vectors, but the efficiency is not so high. To take advantage of liposomes and viral vectors, we have developed HVJ-liposomes and HVJ-envelope methods.

## 5. HVJ-LIPOSOMES

The HVJ-liposome method utilizes a combination of liposomes and the fusion activity of proteins derived from the HVJ envelope (Figure 1) (14). In this delivery system, DNA is packaged in liposomes comprised of phospholipids and cholesterol.

To make HVJ-liposomes, in brief, phosphatidylserine, phosphatidylcholine and cholesterol are mixed in a weight ratio of 1:4.8:2. The lipid mixture is deposited on the sides of a flask by removal of tetrahydrofuran in a rotary evaporator. Dried lipid is hydrated in balanced salt solution with plasmid. Purified HVJ is inactivated by UV irradiation just before use. The liposome suspension is mixed with HVJ. Free HVJ is removed from the HVJ-liposomes by sucrose density gradient centrifugation. The top layer of the sucrose gradient is collected for use (13).

The efficiency of HVJ-liposomes has been reported in various tissues (14). The transfection efficiency of the HVJ-liposome vector is much higher than that of cationic liposomes (14), without any pathogenicity (14). Using this HVJ-liposome, gene transfer into the brain is also achieved in rodents and primates, as discussed below.

### 5.1. Gene transfer into brain using HVJ-liposome method

Using the HVJ-liposome method, widespread gene expression is observed in the cerebral cortex, hippocampus, and choroid plexus by transfer of the β-galactosidase gene into the cisterna magna in gerbils (13), rats (12), and primates (43). Also, the overexpression of VEGF or HGF in the cerebrospinal fluid (CSF) is achieved by transfer of the VEGF or HGF gene using this method (12,13). Based on these preliminary data, we transferred the VEGF gene and/or HGF gene into two cerebral ischemic models: a chronic cerebral hypoperfusion model in rats and a transient forebrain ischemic model in gerbils.

### 5.2. Gene transfer into rat cerebral hypoperfusion model

To clarify whether HGF or VEGF gene transfer could induce angiogenesis in cerebral hypoperfusion states, we transferred these genes into the CSF after bilateral carotid artery occlusion in rats (12). We observed that decreased cerebral blood flow (CBF) in untransfected rats immediately, 7 and 14 days after occlusion. CBF was markedly decreased immediately after occlusion of the bilateral carotid arteries, and thereafter gradually increased over time. However, CBF was still significantly lower 7 days after occlusion as compared to pre-treatment level.

In contrast, transfection of the HGF or VEGF gene (30 min before ligation) significantly prevented the reduction in CBF induced by occlusion of the carotid arteries as compared to CBF 7 days after transfection in rats that received the control vector. Interestingly, a combination of recombinant HGF and HGF gene transfer resulted in a greater increase in CBF 7 days after occlusion as compared to HGF gene transfection alone. Also, administration of the human HGF or VEGF gene immediately (5 min) after occlusion of the carotid arteries significantly improved the decrease in CBF as compared to CBF 1 week after occlusion in rats transfected with control vector. Similarly, transfection of the human VEGF gene also significantly attenuated the reduction in CBF induced by occlusion of the carotid arteries as compared to control vector. The number of blood vessels was significantly increased in rats transfected with the HGF gene. These results suggest that angiogenesis induced by HGF or VEGF gene transfer using HVJ-liposomes either before or after artery occlusion improves chronic cerebral hypoperfusion.

### 5.3. Gene transfer into transient forebrain ischemic model in gerbils

We also examined the effects of over-expression of HGF in delayed neuronal death induced by transient occlusion (5 minutes) and reperfusion of the carotid arteries in gerbils (13). In this model, the CA1 pyramidal cells progressed to death within 7 days after transient forebrain ischemia (delayed neuronal death). However, HGF gene transfer 5 minutes after reperfusion prevented the delayed neuronal death more effectively than did single administration of human recombinant HGF (30 μg). TUNEL-positive CA1 cells were less abundant in rats treated with the HGF gene. These results suggest that neuroprotection is likely mediated by anti-apoptic effects of HGF, and HGF gene transfer is more superior than the single administration of recombinant protein in promoting therapeutic benefits.

## 6. PERSPECTIVES

The HVJ-liposome method is one of the promising approaches to transfer genes into the brain. However, the major factor limiting the use this vector in the clinical setting is its tedious and complicated procedure in manufacturing HVJ-liposome vesicles. In addition, long-term storage could not be possible. To overcome these problems, we have recently developed a second generation of HVJ vector, the so-called HVJ-envelope vector (Figure 1) (44). This was constructed by incorporating plasmid DNA into inactivated HVJ particles (44). The advantages of the HVJ-envelope vector as compared to the HVJ-liposome vector are: 1) it is easy to make, 2) the production does not take much time (less than 90 minutes), and 3) the HVJ-envelope vector can be stored for a long period (at least 6 months). It has already been demonstrated that some reporter genes and oligonucleotides could be transfected into various tissues and cells without complications (44). The efficiency of HVJ-envelope carrying the HGF gene was previously demonstrated by intracisternal injection in a rat MCAo model (33) and hearing impairment model (38). However, transfected cells are limited to meningeal cells and are not expressed in the cerebral parenchyma when injected into the cisterna magna, which is different from the distribution of HVJ-liposomes. From this viewpoint, gene transfer of a secretory protein, such as VEGF or HGF, seems possible with this technique, since the protein secreted from the meningeal cells is likely to reach the brain via the CSF. Nonetheless, for optimal transfer of a gene into the cerebral parenchyma, it is necessary to further improve the vector.

In summary, HVJ-liposomes and HVJ-envelope vector seem to be the “next generation” vectors for gene therapy in ischemic stroke, though they have some limitations at present. For clinical use, additional manipulations in the design of these vectors with improved efficiency, but less immunogenicity are indicated.

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**Key Words:** Brain, Nervous system, Treatment, Therapeutics, Gene Therapy, HVJ-liposome, HVJ-envelope, Cerebral Ischemia, HGF, VEGF, Review

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