Symposium

In vivo Gene Transfer for the Therapy of Vascular Diseases

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We have developed an efficient vector system for the delivery of genes into various organ cells. The vector, HVJ-liposome, consists of DNA-nuclear protein loaded liposomes and UV-inactivated HVJ (Sendai virus). We found that the HVJ-liposome was very effective for *in vivo* delivery of oligonucleotides. When FITC-labeled oligonucleotides were introduced into vascular smooth muscle cells *in vitro* and *in vivo*, fluorescence was accumulated in the nucleus in 5 min after the transfer and existed there for 3 days in cultured cells *in vitro* and for 2-3 weeks in vascular walls *in vivo*. Antisense oligonucleotides against basic fibroblast growth factor introduced into primary vascular smooth muscle cells by HVJ-liposomes reduced DNA synthesis of the cells responded to angiotensin II treatment to 40%, compared to the introduction of sense oligonucleotides. Next, we attempted to prevent the neointima formation of vascular walls after balloon injury by the introduction of antisense oligonucleotides against cell-cycle regulatory genes. Cotransfection of antisense cdc2 kinase and proliferating-cell nuclear antigen, or antisense cdc2 kinase and cyclin B1, or cdc2 kinase and cdk2 kinase oligonucleotides inhibited completely neointima formation at 2 weeks after angioplasty by a single intraluminal transfer of oligonucleotides.

Key words: HVJ-liposome, Gene therapy, Antisense oligonucleotides, Prevention of restenosis

Introduction

Abnormal cell growth *in vivo* induces various pathological changes in organs. Among them are tumors,

restenosis of blood vessels, glomerular sclerosis, lung fibrosis and so on. To date, no effective pharmacological treatment for preventing those diseases in humans has been reported. Here, we focused our efforts on the prevention of restenosis of blood vessels after angioplasty using a novel molecular delivery system.

First, effective *in vivo* gene transfer methods should be developed. Several problems have to be solved in transferring DNA into cells and obtaining the efficient gene expression. One of them is a problem of introducing DNA directly into the cytoplasm without degradation. We have studied direct introduction of macromolecules into the cytoplasm by HVJ (Hemagglutinating Virus of Japan, Sendai virus). DNA encapsulated in liposomes was successfully introduced into cells by making use of fusion activity of HVJ. Another barrier is efficient delivery of DNA into the nucleus even in non-dividing cells. We found that cointroduction of DNA with nuclear proteins facilitated nuclear migration of the DNA and enhanced its expression in animal organs^{1,2}. A method for delivering DNA with nuclear protein by HVJ-liposomes has been developed.

On the other hand, we have studied vascular remodeling using gene transfer. Multiple factors involved in the growth stimulation of vascular smooth muscle cells (VSMCs)^{3,4}. It appears unlikely that selective inhibition of a particular growth factor will completely inhibit the growth of VSMC. However, cell growth results from cell division which is regulated by cell cycle genes. Cyclins and cdks were isolated and the roles of those factors in the regulation of cell cycle have been identified⁵. We employed antisense ODNs to inhibit the expression of cell cycle genes. Cotransfection of cdc2 kinase and proliferating-cell nuclear antigen (PCNA) antisense ODNs completely inhibited the growth of VSMCs both *in vitro* and *in vivo*⁶. The combination of cdc2 kinase and cyclin B1 or cdc2 kinase and cdk2 kinase were also effective for the inhibition of neointima formation after balloon injury of rat carotid

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artery.

Materials and Methods

The preparation of HVJ-liposomes

HVJ-liposomes were prepared as described previously⁷⁾. Briefly, DNA and nuclear protein, HMG-1, were enclosed in liposomes consisting of phosphatidyl serine, phosphatidyl choline and cholesterol. The liposomes were fused with UV-inactivated HVJ and the resulting HVJ-liposomes were isolated through sucrose gradient centrifugation. For the delivery of oligonucleotides, cointroduction of nuclear protein was not always necessary.

Sequences of antisense ODNs

The sequences of ODNs against human basic fibroblast growth factor (b-FGF), mouse cdc2 kinase, rat PCNA were previously reported^{8,9)}. The sequence of human cdk2 antisense and rat cyclin B1 were as follows: antisense cdk2 kinase (5'-GAAGTTCTCCATGAAGCG-3') and antisense cyclin B1 (5'-GAGCGCCATGGCTCCTCC-3').

In vitro transfection

Rat aortic VSMCs (passage 4-8) were isolated and cultured. They were maintained in Waymouth's medium (GIBCO) with 5% calf serum. After confluence, cells were made quiescent by placing them for 48 hrs prior to the transfection in a defined serum free medium, as reported¹⁰⁾. Cells were washed with balanced salt solution (BSS: 137 mM NaCl, 5.4 mM KCl, 10 mM Tris-Cl pH 7.5), and 500 μ l of HVJ-liposomes (3 μ M encapsulated ODNs) was then added to the wells. The cells were incubated at 4°C for 5 min and then at 37°C for 30 min. Cells were maintained in fresh DSF or medium with 5% serum. To investigate the effect of antisense phosphorothioate ODNs on DNA synthesis rate, the cells were stimulated with angiotensin II (Ang II) and pulsed with ³H thymidine for 8 hrs period beginning 20 hrs after transfection and Ang II stimulation. When FITC labeled phosphorothioate ODN was

introduced into VSMCs, cells were fixed with 4% paraformaldehyde at various time points after the transfer and observed by fluorescence microscopy.

In vivo transfer of ODNs

A no. 2 French Fogarty catheter was used to induce vascular injury in male Sprague-Dawley rats (400-500 g). These rats were anesthsized and a cannula was inserted into the left common carotid artery via the external carotid artery. After vascular injury of the common carotid artery, the distal injured segment was transiently isolated by temporary ligatures. The HVJ-liposome complex was infused into the segment and incubated for 10 min at room temperature. After 10 min incubation, the infusion cannula was removed. Following the transfection, blood flow to the common carotid artery was restored by release of the ligatures. No adverse neurological or vascular effects were observed in any animal undergoing this procedure. At 2, 4, and 8 weeks after transfection, rats were killed, and vessels were perfused and fixed with 4% paraformaldehyde. Three individual sections from the middle of transfected segments were analysed. In addition, three sections from the middle section of the injured untransfected region were also analysed.

Results

In vivo gene transfer by HVJ-liposomes

In HVJ-liposome delivery system, DNA and nuclear protein were enclosed into liposomes, directly introduced into the cytoplasm and migrated into the nucleus. We succeeded in expressing exogenous genes in various organs by HVJ-liposome as shown in Table 1. HVJ-liposome is superior to other delivery system in the following points. 1) high transfection efficiency 2) short incubation time 3) ability to deliver oligonucleotides 4) no limitation of insert size 5) no requirement for cell division 6) no apparent toxicity 7) no apparent antigenicity. But, the transient expression of genes in tissues and inability for targeted delivery are limitations of HVJ-liposomes.

Table 1 Successful result by HVJ-liposomes.

Organ	Gene	Expression Time
Liver	SVT, Insulin, Renin, HBsAg	10 days
Kidney	SVT, TGF- β , PDGF	7 days
Artery	SVT, ACE, Renin,	14 days
Skeletal Muscle	Dystrophin	14 days
Testis	SVT, CAT	More than 4 months
Spleen	CAT	7 days
Heart	CAT	More than 14 days

Fig. 1 The preparation of HVJ-liposome. DNA and nuclear protein, HMG-1 were enclosed in liposomes consisting of phospholipids and cholesterol. The liposomes were fused with UV-inactivated HVJ to form HVJ-liposome. The resulting HVJ-liposome fused with cells at the cell surface and delivered the contents directly into the cytoplasm and the DNA-HMG-1 complex into the nucleus.

Delivery of ODN to VSMCs by HVJ-liposome

When FITC-ODN was introduced into VSMCs using HVJ-liposome, fluorescence was accumulated in the nucleus at 5 min after the transfer and the fluorescence was detected in the nucleus at least for 72 hrs (Fig. 1). In contrast, fluorescence was observed in the cellular components (probably in the endosome), not in the nucleus by direct transfer of FITC-ODN without HVJ-liposome and the fluorescence was not detected at 24 hrs after the transfer.

Effect of antisense b-FGF on DNA synthesis

Fig. 2 shows that antisense b-FGF (AS-FGF) had no effect on cellular DNA synthesis under basal conditions during 68–76 hrs when applied directly to VSMCs.

Directly applied AS-FGF had also no effect on angiotensin II (Adg II) stimulated DNA synthesis. When AS-FGF was delivered by HVJ-liposome, AS-FGF inhibited DNA synthesis either in unstimulated or Ang II treated conditions. The reduction of cellular DNA synthesis by AS-FGF was approximately 40% compared with that by sense-FGF. The concentration of AS-FGF required to reduce cellular DNA synthesis to 75% was about 0.1 μ M, 10 μ M and 20 μ M, by HVJ-liposome method, lipofection and direct transfer, respectively (data not shown). Therefore, the delivery of AS-ODN by HVJ-liposome was about 50 times more effective than lipofection and 100 times more effective than direct transfer.

DIRECT TRANSFER

HVJ TRANSFER

Fig. 2 Fluorescence microscopy of FITC labeled ODN using direct versus HVJ-liposome method. In direct transfer, VSMCs were incubated with 30 μ M of FITC labeled phosphorothioate antisense ODN (16 mer) (Clontech Inc. Palo Alto, CA) for 5 min on ice and for 30 min at 37 C. In HVJ transfer, 500 μ l of HVJ complex with FITC labeled ODN (3 μ M) was incubated for 5 min on ice and for 30 min at 37 C. Then, after changing to fresh media, the cells were incubated in a CO₂ incubator. Cells were fixed with 3% paraformaldehyde at 5, 30 min and 1, 3, 6, 12, 24, and 72 hrs. After mounting, cells were examined by fluorescent microscopy.

Fig. 3 Inhibitory effect of As-FGF on DNA synthesis (^3H thymidine incorporation) with (a) non-HVJ method or (b) HVJ method during 68–76 hrs after transfection. CONTROL: untreated VSMC, S-FGF: sense FGF transfected VSMC, AS-FGF: antisense FGF treated VSMC, BASAL: basal condition, Ang II: angiotensin II (10^{-6} M) stimulated condition, * $P < 0.05$, ** $P < 0.01$ compared to control group. Each group contains 4 samples. This experiment was repeated four times, and the figure shows the representative experiment.

Prevention of neointima formation in carotid artery by antisense ODNs

Since basic-FGF is one of the growth stimulants of VSMCs, other factors can stimulate the growth when AS-FGF is applied. Cell growth results from the cell division regulated by cell-cycle genes. Therefore we attempted to inhibit abnormal growth of VSMCs *in vivo* by suppressing cell-cycle regulatory proteins. When both AS-PCNA and AS-cdc2 kinase were employed, VSMC proliferation in response to serum stimulation was inhibited significantly *in vitro* (?). Similarly, the combination of AS-cdc2 kinase and AS-cyclin B1 or

Fig. 4 Long term suppression of neointima formation by AS-cdc2 kinase and AS-cdk2 kinase. As a control, sense ODNs for both molecules were used. $15\text{ }\mu\text{M}$ of ODNs were delivered into VSMCs of a balloon injured rat carotid artery by HVJ-liposome. At 2, 4, 8 weeks after transfection, rats were sacrificed and vessels were perfusion-fixed with 4% paraformaldehyde. Three individual sections from the middle of transfected segments were analyzed. * $P < 0.01$ vs. sense treatment.

AS-cdc2 kinase and AS-cdk2 kinase completely inhibited cellular DNA synthesis stimulated by the addition of serum. Given that neointima formation results from an initial acute phase of medial smooth muscle cell replication, we introduced both AS-PCNA and AS-cdc2 kinase or AS-cdc2 kinase and AS-cyclin B1 or AS-cdc2 kinase and AS-cdk2 kinase into smooth muscle cells of balloon injured rat carotid artery *in vivo*. As shown in Fig. 3, neointima formation was completely inhibited for two weeks after the transfer and the inhibitory effect was recognized up to eight weeks after a single transfection. However, sense ODNs were introduced, no inhibitory effect was observed.

Discussion

HVJ-liposome seems to be a powerful tool for *in vivo* analysis of gene function. Indeed, we succeeded in inducing various pathological changes in animal organs by introducing responsible genes using HVJ-liposome¹¹⁻¹³. This success results from high transfection efficiency, no apparent toxicity and no apparent antigenicity of the delivery system. In the aspect of gene therapy, antisense strategy by HVJ-liposome may be one of the most promising ways. There are no other methods to deliver oligonucleotides effectively. The findings that FITC-ODN delivered by HVJ-liposome rapidly accumulated in the nucleus (Fig. 2) and that the formation of mRNAs of PCNA and cdc2 kinase was inhibited by AS-ODNs against both genes (data not shown) indicated that AS-ODN played its inhibitory role in the nucleus. Since cointroduction of AS-ODN with RNase H raised the inhibitory effect about three times more than AS-ODN alone we suspected that AS-ODN may hybridize heterogeneous nuclear RNA in the nucleus and that the hybrid may be degraded by RNase H.

Besides the delivery system, the success of growth inhibition of VSMCs resulted from the employment of AS-ODN against cell-cycle regulators. However, several factors are responsible for the progression of each stage of cell-cycle. That may be the reason why the combination of different cell-cycle regulators were required for complete inhibition of cell growth. Actually, AS-ODN against a single regulator did not significantly inhibit cell growth.

The restenosis after angioplasty is a barrier to the therapy of myocardial infarction. No effective treatment has been reported for it. Since restenosis results from abnormal growth of VSMCs, we hypothesized that restenosis could be prevented by the blockade of genes regulating cell-cycle progression. Here we presented the evidence that complete inhibition of neointima formation could be achieved by a single administration of combined antisense ODN directed against cell cycle using HVJ-liposome. It appears that this antisense strategy is the most promising for the treatment of restenosis. To realize clinical use of the method, we will have to develop a catheter that will permit instillation/incubation of the HVJ-liposome complex and simultaneously maintain tissue perfusion. Moreover, we will have to remodel HVJ-liposome complex to simple and more homogeneous particle.

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