Local Delivery of Plasmid DNA Into Rat Carotid Artery Using Ultrasound

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Background—Although viral vector systems are efficient to transfect foreign genes into blood vessels, safety issues remain in relation to human gene therapy. In this study, we examined the feasibility of a novel nonviral vector system by using high-frequency, low-intensity ultrasound irradiation for transfection into blood vessels.

Methods and Results—Luciferase plasmid mixed with or without echo contrast microbubble (Optison) was transfected into cultured human vascular smooth muscle cells (VSMC) and endothelial cells (EC) with the use of ultrasound. Interestingly, luciferase activity was markedly increased in both cell types treated with Optison. We then transfected luciferase plasmid mixed with Optison by means of therapeutic ultrasound into rat artery. Two days after transfection, luciferase activity was significantly higher in carotid artery transfected with luciferase gene with Optison and ultrasound than with plasmid alone. In addition, we transfected an anti-oncogene (p53) plasmid into carotid artery after balloon injury as a model of gene therapy for restenosis. Two weeks after transfection, the intimal-to-medial area ratio in rats transfected with wild-type p53 plasmid complexed with Optison by means of ultrasound was significantly decreased as compared with control, accompanied by a significant increase in p53 protein. No apparent toxicity such as inflammation could be detected in blood vessels transfected with plasmid DNA with ultrasound and Optison.

Conclusions—Overall, we demonstrated that an ultrasound transfection method with Optison enhanced transfection efficiency of naked plasmid DNA into blood vessels without any apparent toxicity. Transfection of p53 plasmid with the use of this method should be useful for safe clinical gene therapy without a viral vector system. (Circulation. 2002;105:1233-1239.)

Key Words: gene therapy ■ restenosis ■ ultrasonics ■ vessels

Recent progress in molecular biology has led to the development of gene therapy, as a new treatment strategy for cardiovascular diseases. The targeted diseases range from single gene deficiency diseases to more complex diseases in adults such as vascular disease, including restenosis after angioplasty. Development of in vivo transfer techniques for genes whose product can correct a critical process into blood vessels may provide an approach to treating this disease. In vivo gene transfer into blood vessels by direct injection of “naked” DNA is extremely inefficient, resulting in gene transduction in <1% of cells in the area of DNA injection.1-5 Although the first human gene therapy to treat restenosis through the use of naked plasmid DNA has been started,6 apparently a more efficient gene transfer method is required to achieve therapeutic effects. Recently, many investigators have focused on the adenoviral gene transfer method.7-9 The adenoviral vector appears to be very efficient,9 but there are some theoretical disadvantages such as strong immunogenicity.10 In addition to efficiency, the safety of the gene transfer method is an important issue because infusion of adenovirus recently demonstrated deleterious side effects.11 This is especially important in the treatment of restenosis because only 30% to 40% of patients who undergo angioplasty have restenosis.12,13 Recently, we have developed an HVJ-liposome-mediated transfer method for in vivo gene transfer into blood vessels.14-16 Although this method is easy to manipulate and highly efficient and there is no limitation of the size of the vector DNA with little toxicity,16 its clinical utility such as large-scale production is still limited.

On the basis of these previous studies, we reasoned that the establishment of alternative efficient gene transfer approaches into blood vessels is necessary for the treatment of vascular diseases. In considering actual treatment for vascular diseases, it is desirable to modify non–virus-mediated plasmid DNA transfection. Innovations in plasmid DNA-based gene transfer should achieve high transfection efficiency.
without side effects. Thus, in this study, we modified the plasmid DNA gene transfer method for successful in vivo gene transfer into blood vessels as follows: (1) plasmid DNA alone, (2) ultrasound, and (3) ultrasound with microbubble material (Optison).

Methods

In Vitro Experiments

Analysis of Luciferase Activity

Human aortic endothelial cells and aortic vascular smooth muscle cells (VSMC) (passage 3) were obtained from Clonetics Corp. All the cells were used within passage 3 to 5. Cells were harvested at 1 day after transfection of luciferase gene with the use of “naked” plasmid alone or “naked” plasmid and ultrasound with or without Optison. Human wild-type p53 plasmid driven by the cytomegalo- virus promoter/enhancer was donated by Dr T. Yonemitsu (Kyushu University). The vector used as a control was cytomegalo-virus expression vector plasmid, which did not contain p53 cDNA. We obtained luciferase gene expression vector driven by SV 40 promoter from a commercial source (Promega Corp). Firefly luciferase activ- ity was measured with a luciferase assay system (PicaGene; Toyo-Inki). Plasmid DNA (20 μg) was added to the dishes. Cells were then treated with ultrasound for 1 minute (2.5 W/cm²) at 37°C (repeated 8 times for 1-minute irradiation). For enwrapping in Optison (human albumin microspheres; Mallinkrodt Inc.), Optison was added to plasmid solution, mixed, and kept for 30 seconds. After incubation, the medium was changed to fresh medium containing 0.5% serum. Before and immediately after transfection, we performed electron microscopic scanning of cells transfected with plasmid DNA by using ultrasound with Optison, as described elsewhere.

In Vivo Experiments

Comparison of Transfection Efficiency Into Vascular Cells

Initially, we examined the transfection efficiency of naked luciferase gene plasmid by 3 different methods: plasmid DNA alone, plasmid DNA WITH ultrasound, and plasmid DNA complexed with Option with the use of ultrasound. As shown in Figure 1A, transfection of naked luciferase plasmid DNA exhibited lower transfection efficiency in human endo- thelial cells (125±31 ruminometer light units (RLU)/dish) at 1 day after transfection. Transfection of plasmid DNA by ultra- sound resulted in a significant increase in luciferase activity up to 70-fold (P<0.01). However, its transfection efficiency was still low. Therefore, we examined the transfection of naked plasmid DNA by means of ultrasound with Optison. Initially, we performed electron microscopic scanning of cells transfected with plasmid DNA by using ultrasound with Optison. As shown in Figure 1, A and B, immediately after transfection, both endothelial cells and VSMC exhibited small holes in the cell surface and eventually returned to a normal appearance within 24 hours. It is suspected that ultrasound irradiation with Optison causes transient holes in the cell surface, thereby resulting in rapid translocation of plasmid DNA from outside into the cytoplasm. Ultrasound alone appears to cause smaller holes as compared with coadministration of Optison. No holes were detected in untransfected cells or cells under Optison alone. There was no apparent cell toxicity in cells transfected with plasmid DNA by ultrasound with Optison. Indeed, there was no significant change in LDH activity at 24 hours after transfection (endo- thelial cells: untransfected, 0.0063±0.0012; ultrasound alone, 0.0020±0.0011; ultrasound+Optison, 0.0033±0.0010; abs- sorbance at OD 560 nm, NS, n=6). The combination of ultrasound with Optison exhibited a marked increase in luciferase activity >8000-fold in human endothelial cells as

Quantification of Proliferating Cell Nuclear Antigen–Positive Cells by Immunohistochemical Staining

Paraffin-embedded sections of carotid artery harvested 7 days after transfection were used for quantification of proliferating cell nuclear antigen (PCNA)-positive cells. Four sections of each vessel spaced at 0.4-mm intervals were measured by a computerized image analyzer system (Image Command 5098, Olympus). The number of PCNA-positive nuclei was counted in the neointima and media by the image analyzer system at ×400 magnification. The frequency of PCNA-positive cells was expressed as the ratio of the number of PCNA-positive nuclei to the total number of nuclei in the vessels. Samples were coded so that analysis was performed without knowl- edge of which treatment each individual vessel had received. The reproducibility of the results was assessed. Intraobserver variability was determined from triplicate measurements performed by one observer for all sections (2.4±0.3%).

Statistical Analysis

All values are expressed as mean±SEM. All experiments were performed at least 3 times. ANOVA with subsequent Duncan’s test was used to determine the significance of differences in multiple comparisons. Differences with values of P<0.05 were considered significant.
Optison also caused a marked increase in luciferase activity >7000-fold in human VSMC as compared with naked plasmid alone (P<0.01, Figure 1D), whereas transfection of plasmid DNA by ultrasound resulted in a significant increase in luciferase activity as compared with plasmid DNA alone (P<0.01). There was no apparent cell toxicity in cells transfected with plasmid DNA by means of ultrasound with Optison in human VSMC (VSMC: untransfected, 0.0127±0.0056; ultrasound alone, 0.0105±0.0031; ultrasound+Optison, 0.0102±0.0043; absorbance at OD 560 nm, NS, n=6). These results clearly demonstrate high transfection efficiency of non-viral gene transfer based on plasmid DNA into vascular cells.

**In Vivo Transfection of Plasmid Luciferase DNA Into Intact and Balloon-Injured Carotid Artery**

In vitro studies demonstrated that transfection of naked plasmid DNA by means of ultrasound with Optison had high luciferase activity in human endothelial cells and VSMC. Thus, we transfected luciferase plasmid DNA into rat intact carotid artery (Figure 2). Consistent with numerous previous reports,1–5 transfection of naked plasmid DNA alone failed to induce high luciferase activity (Figure 2B). Unexpectedly, transfection of naked plasmid DNA with ultrasound failed to increase luciferase activity (Figure 2B). In addition, Optison alone did not increase transfection efficiency (plasmid alone, 1205±220 RLU/g tissue; Optison alone, 1350±220 RLU/g tissue; NS). Interestingly, transfection of naked plasmid DNA by means of ultrasound with Optison resulted in a marked increase in luciferase activity >1000-fold as compared with plasmid DNA alone at 2 days after transfection (P<0.01, Figure 2B).

Regarding gene therapy to treat restenosis, it should be tested with the use of balloon-injured blood vessels. Of importance, extremely high transfection efficiency of plasmid DNA transfection by means of ultrasound with Optison was confirmed in balloon-injured blood vessels (Figure 2C). In contrast, transfection with either plasmid DNA alone or ultrasound alone did not exhibit high luciferase activity. To increase transfection efficiency, we compared various ratios of Optison with plasmid DNA. As shown in Figure 2D, 25% Optison resulted in the highest luciferase activity in balloon-injured blood vessels (P<0.01).

We then examined the inhibitory effects of transfection of wild-type p53 plasmid DNA into balloon-injured blood vessels. As shown in Figure 3, a marked increase in p53 protein was only observed in blood vessels transfected with p53 plasmid DNA by means of ultrasound with Optison at 5 days after transfection (P<0.01), whereas no significant increase in p53 protein could be detected in blood vessels transfected with p53 plasmid DNA by means of ultrasound without Optison. Accompanied by a significant increase in vascular p53 protein, transfection of p53 DNA by means of ultrasound with Optison resulted in significant inhibition of the ratio of neointimal-to-medial area as compared with transfection of control vector by means of ultrasound with Optison at 2 weeks after transfection (P<0.01; Figure 4, A and B). In contrast, transfection of p53 plasmid DNA by means of ultrasound without Optison into balloon-injured blood vessels...
failed to inhibit neointimal formation (Figure 4B). Inhibition of neointimal formation by p53 plasmid DNA transfection by means of ultrasound with Optison was associated with a significant decrease in DNA synthesis in neointimal cells at 1 week after transfection, as assessed by PCNA-positive staining (\(P<0.01\), Figure 5, A and B), whereas many PCNA-positive cells could be detected in the neointimal area of blood vessels transfected with p53 DNA by means of ultrasound without Optison. No difference was observed between blood vessels transfected with control vector by means of ultrasound with Optison and those transfected with p53 DNA by means of ultrasound without Optison (data not shown).

**Discussion**

One important disease potentially amenable to gene therapy is restenosis after angioplasty, because the long-term effectiveness of this procedure is still limited by the development of restenosis in >40% of patients.\(^{12,13}\) Neointimal formation after angioplasty involves a complex interaction between multiple growth factors that promote VSMC proliferation and migration.\(^ {23}\) Therefore, we and others successfully reported the inhibition of neointimal formation by agents targeting abnormal VSMC growth with the use of a virus-based vector.\(^ {16,24–26}\) Although the therapeutic strategy to prevent restenosis is realistic, a major problem is the vector system to deliver therapeutic genes. In considering the treatment of restenosis, it is important to transfect therapeutic genes into patients in whom restenosis will not develop, because only 30% to 40% of patients have restenosis after angioplasty.\(^ {12,13}\)

Thus, the safety of the vector system should be carefully evaluated. From this viewpoint, a viral vector may not be ideal. Most of the preclinical studies of the treatment of restenosis have used intraluminal injection of adenoviral vector because of its high transfection efficiency. However, the potential toxicity of adenovirus, such as strong immunogenicity, is well known. Of particular importance, deleterious side effects of adenovirus were reported in 1999.\(^ {11}\) Therefore, it is critical to develop a novel safe gene transfer method with high efficiency.

In this study, we used a plasmid DNA-based gene transfer method, since the plasmid DNA transfer method...
appears to be the safest. To increase the transfection efficiency, we modified ultrasound-mediated plasmid DNA transfection because ultrasound induced cell-membrane porosity. Indeed, the transfection efficiency of plasmid DNA was increased by ultrasound in cultured cells. Consistent with previous reports, the present study revealed a marked increase in transfection efficiency by ultrasound in cultured vascular cells. Nevertheless, the increased efficiency was still not enough. Thus, we further modified ultrasound-mediated plasmid DNA transfection by using an echo contrast microbubble. This idea is based on the previous observation that the presence of an echo contrast agent such as albumin microbubbles, which lowered the threshold of acoustic cavitation production, induced further acceleration of thrombolysis by ultrasound energy. Of importance, plasmid DNA transfection by means of ultrasound was markedly improved by the echo contrast microbubble Optison. The increase in transfection efficiency might be due to transient holes in the cellular membrane produced by the spreading of bubbles (Figure 1). Unexpectedly, other echo contrast microbubbles did not enhance the transfection efficiency (plasmid alone, 12040 RLU/10⁶ cells; sonicated Hexabrix, 12600±4500 RLU/10⁶ cells; Levovist, 10550±3790 RLU/10⁶ cells; NS versus plasmid alone). Optison is an ultrasound contrast agent consisting of gas-filled microspheres surrounded by a solid shell of heat-denatured human albumin, different from other contrast agents. Further studies are necessary to investigate the exact molecular mechanisms by which Optison enhanced transfection efficiency. In addition, we optimized ultrasound-Optison-mediated plasmid DNA transfer into blood vessels in vivo. Intraluminal incubation within blood vessels for a short

Figure 3. Western blotting of p53 protein in rat balloon-injured blood vessels transfected with naked control or p53 plasmid DNA by means of ultrasound and Optison 5 days after transfection. A, Typical example of Western blotting of p53 protein. B, Effects of transfection of p53 plasmid DNA by means of ultrasound and Optison on p53 protein. 1: Control indicates naked control plasmid DNA; 2: p53 indicates naked p53 plasmid DNA; 3: US+Optison indicates naked plasmid DNA by means of ultrasound and Optison. **P<0.01 vs control.


Figure 5. Effect of transfection of naked p53 plasmid DNA on DNA synthesis in rat balloon injury model 1 week after transfection, assessed by PCNA staining. A, Representative cross sections of PCNA staining (×200). B, Effect of transfection of naked p53 plasmid DNA on ratio of PCNA-positive cells to total cells in rat balloon injury model 1 week after transfection. Control+US+Optison indicates naked control plasmid DNA by means of ultrasound and Optison; p53+US+Optison, naked p53 plasmid DNA by means of ultrasound and Optison.
time, such as 2 minutes, appears to be sufficient to transfect genes.

To examine the feasibility of gene therapy, we chose the wild-type p53 gene because overexpression of the p53 gene has been reported to inhibit neointimal formation. As expected, p53 protein was significantly increased in blood vessels transfected with p53 plasmid DNA by means of ultrasound with Optison. Accompanied by a significant increase in p53 protein, neointimal formation was significantly inhibited in blood vessels transfected with p53 plasmid DNA by means of ultrasound with Optison. The specificity of the inhibition of neointimal formation by overexpression of human p53 gene transfer by means of ultrasound with Optison into balloon-injured vessels is supported by several lines of evidence: (1) No significant inhibition of neointimal formation was achieved by transfection of control vector by means of ultrasound with Optison. (2) Ultrasound alone or ultrasound with Optison did not affect neointimal formation. (3) Transfection of p53 plasmid DNA by means of ultrasound without Optison also did not inhibit neointimal formation. (4) Inhibition of DNA synthesis as assessed by PCNA staining was observed in blood vessels transfected with p53 plasmid DNA by means of ultrasound with Optison. It is noteworthy that no apparent injury was found in blood vessels transfected with plasmid DNA by means of ultrasound. These data clearly demonstrate the clinical utility of a therapeutic strategy based on plasmid DNA-mediated transfer. What is the clinical relevance of a highly efficient gene transfer method based on plasmid DNA by means of ultrasound with Optison? First, it is possible to decrease the amount of plasmid DNA, thereby decreasing the potential side effects and cost. Second, it is possible to achieve high transfection efficiency without a viral vector. Avoiding a viral gene transfer method such as the use of an adenovirus may increase the safety of gene therapy and extend its application to a wide variety of targeted diseases. Third, further modification of delivery tools such as a catheter with ultrasound may expand the utility of the present modification to transfection into blood vessels, ovary, or other organs. Fourth, as we expected, an in vivo study demonstrated no apparent toxicity in rats.

Overall, the present studies demonstrated a novel non-viral, safe, efficient gene transfer method into cultured vascular cells and blood vessels. A novel therapeutic strategy with the use of p53 plasmid DNA with Optison by means of ultrasound may be useful to inhibit restenosis in clinical practice without a viral vector. In addition, transfection of other therapeutic genes by ultrasound-Optison-mediated plasmid DNA delivery is likely to create new therapeutic options in other diseases such as cancer, inflammatory diseases, and cardiovascular disease such as coronary heart disease.

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