ultrasound. Nussbaum (98) has reported that the scale of cavitation depends on the ultrasound characteristics; bubble growth is limited by low-intensity, high frequency, and pulsed ultrasound. Mitragotri *et al.* (82) confirmed this statement. They found that the cavitation threshold increases as the mode of ultrasound application changes from continuous to pulsed. Sun and Liu (120), however, suggested that cavitation is more likely to occur when pulsed ultrasound is used, provided that the ultrasound intensity during the pulses exceeds the threshold of cavitation occurrence and the duration of the pulses is long enough for the cavitation to develop.

– *Intensity*: The intensity I is directly dependent on the acoustic energy (E) emitted and the speed of sound (c) in the medium as expressed by the following expression (45, 48),

$$I = cE \qquad \text{Eq. 1}$$

Energy, E itself dependent on the density of the propagation medium ρ, on the total pressure p and on the speed of sound (equal to the sum of the atmospheric pressure and the pressure created by the ultrasound wave). Therefore, the emitted energy can be expressed as in the following equation (52),

$$E = p^2/\rho c^2 \qquad \text{Eq. 2}$$

The minimum ultrasound intensity required for the onset of cavitation, referred to as cavitation threshold or threshold intensity, increases rapidly with ultrasound frequency (71,72,82). The ultrasound power intensities usually employed for transdermal drug delivery lie between 0.1 and 3 W/cm^2 with low frequency application (20-100 kHz) and 0.1 and 10 W/cm^2 with high frequency application (1-3 MHz) (69). Below the threshold intensity no detectable enhancement has been observed. Once the intensity exceeds this threshold, the enhancement increases strongly with the intensity until another threshold intensity, referred to as the decoupling intensity is reached. Beyond this intensity, the enhancement does not increase with further increase in the intensity due to acoustic decoupling. The threshold intensity for porcine skin increased from about 0.11 W/cm^2 at 19.6 kHz to more than 2 W/cm^2 at 93.4 kHz. The origin of this substantial increase in the threshold intensity with frequency may be attributed to cavitation.

The effect of ultrasound (1 MHz) on transdermal absorption of indomethacin from an ointment was studied in rats by Miyazaki *et al.* (92). Ultrasound energy was supplied for between 5 and 20 min at a range of intensities (0.25, 0.5, 0.75 and 1 W/ cm^2), energy levels commonly used for therapeutic purposes. For evaluating skin penetration of indomethacin, the change of plasma concentration was measured. The pronounced effect of ultrasound on the transdermal absorption of indomethacin was observed at all ultrasound energy levels studied. The intensity and the time of application were found to play an important role in the transdermal sonophoretic delivery system of indomethacin; 0.75 W/cm^2 appeared to be the most effective intensity in improving the transdermal absorption of indomethacin, while the 10 min ultrasound treatment was the most effective. Although the highest penetration was observed at an intensity of 0.75 W/cm^2, 0.5 W/cm^2 was preferred because intensities of less than this for 10 min application did not result in any significant skin temperature rise nor did it have any destructive effect on rat skin. Progressively more skin damage was noted as the intensity and the time of application of ultrasound increased.

– *Cavitation nuclei*: As the sonophoresis enhancement is mediated through cavitation, i.e. the formation and collapse of gaseous bubbles, it is expected that by providing the nuclei for cavitation - externally, the efficacy of sonophoresis could be significantly enhanced. The occurrence of cavitation in water is facilitated by the presence of dissolved gas (32,113). Terahara *et al.* (133) have used two porous resins, Diaion® HP20 and Diaion HP2MG (2MG), as cavitation nuclei. Resultant cavitation effect was measured from the pitting of aluminum foil. It has been found that 2MG showed a higher efficacy in enhancing cavitation compared with Diaion HP20. 2MG was also effective in enhancing transdermal mannitol transport. These results confirm that the addition of cavitation nuclei such as porous resins further increases the effect of low frequency ultrasound on skin permeability.

In addition to the above parameters, sonophoretic enhancement also depends on transducer geometry as well as the distance between the transducer and the skin. Detailed dependence of enhancement on these parameters has not yet been studied (91).

Mechanisms behind sonophoresis

Driving force for increased skin permeability of transdermal drug delivery

– *Acoustic cavitational bubble collapse effects*: Passing ultrasound waves causes cavitation which is the growth and explosive collapse of the microscopic bubbles of a few μm in diameter. When the bubbles collapse quasi adiabatically, it results mainly in the following 3 effects:
a) Generation of extreme conditions: Adiabatically when the bubbles collapse or implode, it results in the concentration of energy or causes in the generation of a short-lived, localized high-energy spot. This energy concentration has been measured in terms of temperature and pressure, which are extreme on a microsecond timescale (124). An asymmetrically collapsing bubble next to a wall has been shown in Fig. 4 (Photo from ref. 26). The maximal diameter of the bubble is about 1 mm (Fig. 4).
b) Mechanical effect: The adiabatic collapse of bubbles also incites the mechanical effect, microstreaming. This is caused

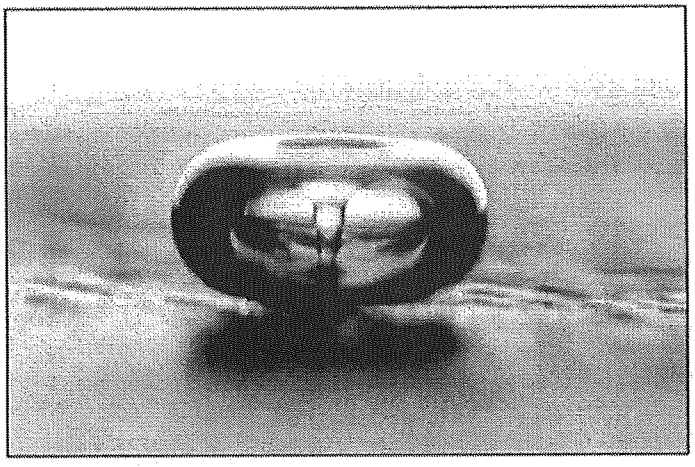

Fig. 4 An asymmetrically collapsing cavitation bubble

by the unidirectional movement of fluids along cell membranes. Oscillation of cavitation bubbles might also contribute to microstreaming. Microstreaming may alter cell membrane structure, function and permeability (138) or porosity (129). The potential clinical value of this microstreaming has not been explored much (58).

c) Thermal effects: The thermal effect of ultrasound on the skin results from the transfer and conversion of mechanical energy generated by the vibration of a piezoelectric crystal in the sonophoresis probe which prompts the absorption of ultrasound by the skin (9). Absorption by the skin of this energy causes a temperature increase, which is directly related to the intensity of the sound wave (43). Prediction of the actual temperature increase produced by a particular sonophoretic profile is difficult, however, without a precise knowledge of the acoustic absorption coefficients, and of the conduction and convection properties, of the tissues involved. Furthermore, experimentally, there have been few studies quantifying the matter by which it has been modified has also been represented (43). Recently, most investigations have focused upon the use of US at a frequency of about 20 kHz, at a 10% duty cycle (0.1 on, 0.9 off), for periods from minutes to a few hours (83,88). While the results obtained implicate cavitational effects as a principal mechanism, the role of the accompanying thermal effect has not been deduced. Given that skin permeability can increase significantly with temperature (for example, the absorption of estradiol doubled when the temperature was increased by 10°C) (82), and that phase transitions of the intercellular lipids of the SC can occur at temperatures close to physiological (37,101), it is clearly possible that thermal changes can contribute to sonophoretically-enhanced transdermal transport (78).

Excessive thermal effects, seen in particular with higher ultrasound intensities, may damage the tissue (30). Machet *et al.* (68) have also demonstrated that the thermal effect of ultrasound was the principal explanation for the increase in the diffusion rate of digoxin. The increased skin permeability thus could be due to the amalgamated effects of all the above mentioned. For example, the above said effects results in a modification or disorganization of the stratum corneum in terms of increased fluidity combined with enlargement or widening of the intercellular space, which paves the way for drug passage. In addition, the above effects on keratinocytes or corneocytes cause temporary or permanent holes in driving the drug and vehicle by convection (14).

Contribution of pressure induced by ultrasound for drug permeation (non-thermal or non-cavitational effect)

Emission of the ultrasonic wave in the intercellular lipid accompanies a rise in the pressure. Considering the speed of sound in intercellular lipids at 1000 m/s (113), the pressure induced by the ultrasound was estimated as 5×10^{-6} bar with 1 W/cm^2 and 5×10^{-4} bar with 2 W/cm^2. Now, the question is whether this rise in pressure or the resultant pressure can cause the active drug ingredient to pass through mechanically. The relative contribution of this flow induced by the pressure, calculated for the diffusion of urea through a synthetic dialysis membrane represents 0.2% with 1 W/cm^2 and 2% with 100 W/cm^2. Thus, the pressure does not have a significant contribution to the increased percutaneous flow induced by ultrasound.

The non-thermal mechanical characteristics of ultrasound can also enhance drug diffusion by oscillating the cells at high speed, changing the resting potential of the cell membrane and potentially disrupting the cell membrane of some of the cells in the area (19). There may be some pushing and pulling of the cells with the propagation of the sound wave through heterogeneous tissues, but it is unlikely that radiation or streaming forces are sufficiently strong or

consistent enough to push drug molecules into the tissue.

From the ultrasonic parameters that have been discussed as above, if one identifies the dominant which induces sonophoresis to a greater extent, then, a better selection of ultrasound parameters and surrounding physicochemical conditions can be made. This will selectively enhance the favorable phenomena, thereby broadening the types of drugs that can be administered transdermally (82).

Supporting evidence on the role of ultrasonic cavitation effects for transdermal drug delivery

The role of cavitation in increasing percutaneous permeability due to ultrasound is well supported by a series of *in vitro* experiments: a) the importance of keeping dissolved gas in the medium to form nuclei of cavitation (82), and this gives indirect confirmation about the definite occurrence of cavitation as dissolved gases like entrapped air which contains both oxygen and nitrogen are present in stratum corneum, b) the possibility of permeating cell membranes *in vitro* is enhanced in the presence of artificial cavitation nuclei (39), c) demonstration of possible pores created by ultrasound on the skin surface (68), and within the SC (114,140), d) demonstration of multiple pits induced by bubble implosion on aluminium foil exposed to ultrasound and its correlation with intensity and skin conductivity (132). The possible occurrence and the consequences of cavitation in cells or tissues (22,100) and possible applications in therapy for destruction of cancers (47) and gene therapy (80) have also been studied. The promise of gene therapy lies in the potential to ameliorate or cure conditions that are resistant to conventional therapeutic approaches. Progress in vascular and all other fields of gene therapy has been hampered by concerns over the safety and practicality of recombinant viral vectors and the inefficiency of current non-viral transfection techniques. There is increasing evidence that exposure of eukaryotic cells to relatively modest ultrasound intensity, within the range emitted by diagnostic transducers, either alone or in combination with other non-viral techniques, can enhance transgene expression by up to several orders of magnitude over naked DNA alone. In combination with the flexibility and excellent clinical safety profile of therapeutic and diagnostic ultrasound, it has been suggested that the ultrasound-assisted gene delivery has great promise as a novel approach to improve the efficiency of many forms of non-viral gene delivery (96). Application of ultrasound for gene delivery to cells requires control of cavitation activity. Many studies have been performed using *in vitro* exposure systems, for which cavitation is virtually ubiquitous. *in vivo*, cavitation initiation and control is more difficult, but can be enhanced by cavitation nucleation agents, such as an ultrasound contrast agent. Sonoporation and ultrasonically enhanced gene delivery has been reported for a wide range of conditions including low frequency sonication (kilohertz frequencies), lithotripter shockwaves, HIFU (high intensity focused ultrasound), and even diagnostic ultrasound (megahertz frequencies). The use of ultrasound for non-viral gene delivery has been demonstrated for a robust array of *in vitro* and mammalian systems, which provides a fundamental basis and strong promise for development of new gene therapy methods for clinical medicine.

Indirect proof of cavitation was demonstrated in isolated epidermis *in vitro*, after incubating epidermis with fluorescein, resulting in bleaching of fluorescence probably due to the production of hydroxyl radicals generated by cavitation (82). The existence of dissolved gas deep in living tissue can allow the development of cavitation bubbles (42). Small cavities the size of a few microns, which could correspond to the size of cavitation bubbles on the surface of the stratum corneum, was shown *in vitro* using scanning electron microscopy (68). Scanning electron microscopy showed 1-3 mm holes on the surface of the stratum corneum after exposure to ultrasound (1.1 MHz, 1.5 W/cm^2). Such crater-like images of 5-15 mm were also reported in hairless mouse skin exposed to ultrasound *in vitro* (1 MHz, 4.3 W/cm^2) (44). Such images have also been shown when experimentally exposing aluminum foil to 20 kHz ultrasound (88,132) and the quantity of pits increased with intensity and reduction of the distance between the skin and the probe.

Yamashita *et al.* (141) have investigated the morphological changes induced in hairless mouse skin after ultrasound irradiation. The scanning electron microscopy examination of hairless mouse skin exposed to ultrasound demonstrated large craterlike pores of 100 μm diameter on the surface of the stratum corneum, corresponding to the size of the bubbles of cavitation, which has been shown in Fig. 5. No lesions were demonstrated after sonication using degassed water indicating that cavitation was the causative mechanism (Fig. 5).

Sonophoretic enhancement - Differences among drugs

After optimizing the cavitational parameters for an enhanced drug delivery, the question immediately comes into mind is whether all the drugs applied will be delivered to the same extent? Because, enhancements in the levels of drugs transported through the skin were only observed for particular drugs. This variation between drugs raised controversy about the use of sonophoresis for drug delivery. An explanation for the variation was recently offered based on the differences in physiochemical properties of drugs, for example, lipophilicity and molecular weight. Specifically, small lipophilic drugs, which rapidly diffuse through the skin under passive conditions, do not show enhanced transport after application of ultrasound (84).

The sonophoretic enhancement of transdermal drug transport has been quantitatively predicted based on the knowledge of two physiochemical properties of the drug:

passive skin permeability, P^P and octanol–water partition coefficient, $K_{o/w}$, using the following equation (84):

$$e \sim K_{o/w}^{0.75} / (4 \times 10^4) P^P \quad \text{Eq. 3}$$

where '*e*' is the relative sonophoretic transdermal transport enhancement defined as, [(sonophoretic permeability / passive permeability) - 1].

Based on this equation it can be inferred that this technology is most useful in transporting drugs of a high molecular weight and hydrophilic drugs. Experimental results indicate that the slower the diffusion of a permeant through the lipid bilayers of the SC, the more effective is ultrasound in enhancing its transport, i.e. the drugs passively diffusing through the skin at a slow rate are most enhanced by the application of ultrasound (82).

Whereas, recently Katz *et al.* (51) examined the speed of onset of cutaneous anesthesia by eutectic mixture of local anesthetics (EMLA) cream after brief (approximately 10-S) pretreatment of the underlying skin with low frequency (55 kHz) ultrasound, in human subjects. After ultrasound pretreatment and then 5, 10 or 15 min after EMLA cream application, pain scores and overall preference were statistically indistinguishable from EMLA cream application for 60 min (without ultrasound pretreatment). There were no significant adverse effects and found that low frequency ultrasound pretreatment appears to be safe and effective in producing rapid onset of EMLA cream in this model, with results as early as 5 min.

Role of cavitational effects on the stability of administered drugs

It is obvious and highly reasonable to expect the same cavitation and related effects which are responsible in increasing the skin permeability could have effect on the applied drug itself, for example degradation. Eventual degradation of drugs to ultrasound was studied *in vitro* and showed absence of degradation for oligodeoxynucleotides (74), insulin (12), fentanyl and caffeine (13). The persistence of biological activity of insulin and low molecular weight heparin *in vivo* is also an evidence for the absence of degradation (82,90).

Role of cavitational effects on the skin

An increasing utility of ultrasound in medicine, in specific in the transdermal transport of various drugs as well as in transdermal extraction of various drugs have caused a much concern directed to the issues of ultrasound bioeffects and safety (58). The world federation for ultrasound in Medicine and Biology (WFUMB) (151) had issued several publications related to safety of ultrasound bioeffects, addressing specifically thermal bioeffects and non-thermal bioeffects in an attempt to reach an international consensus to adopt a policy on safety guidelines (58). The use of ultrasound as an aid to increasing skin permeability is based on its non-thermal bioeffects, mostly cavitation. In view of this much attention should be paid to the issue of ultrasound affecting the structure of the skin; is it a reversible change? What is the role of free radicals that are generated during the

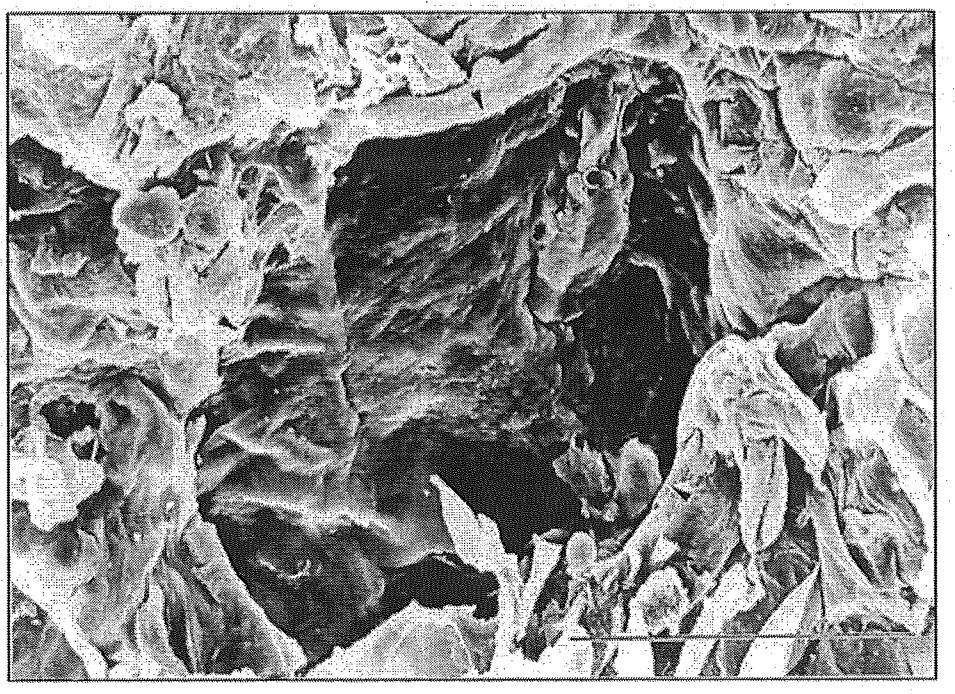

Fig. 5 Craterlike pores of hairless mouse skin after 5 min of ultrasound exposure (Bar 50 μm)

cavitation process within the skin (58)?

Studies have been carried out to determine the safety of low frequency (20 kHz) sonophoresis on human and rat skin by evaluating their structural modifications after ultrasound exposure. Skin samples were observed under optical and electron microscopy to detect any structural changes. The skin samples exposed to ultrasound intensities lower than 2.5 W/cm^2 showed no modification (15). However it was found that at intensities higher than 2.5 W/cm^2, it caused a slight and transient erythema whereas severe skin lesions (dermal and muscle necrosis) were observed 24 hr later (54,66,93).

The application of 20 kHz ultrasound at an intensity of 3 W/cm^2 was shown to enhance the transdermal transport of interstitial fluid across hairless rat skin (21). In this study, (H_2O)-H-3 was used as a tracer which was injected intravenously. A measurable amount of water (>1 ml) was extracted without producing any histologic evidence of injury, even after repeated exposures. Mitragotri *et al.* (83) have shown that the barrier properties of the skin can be modified using low frequency ultrasound (20 kHz) to enhance the efficiency of transdermal drug delivery. Improvement of as much as 1000-fold was achieved in the delivery of hydrophilic and/or large compounds without long-term damage to the barrier properties of the skin (81). Whereas, the effect of an ultrasound (1 MHz) intensities (0.25, 0.5, 0.75 and 1 W/cm^2), on transdermal absorption of indomethacin from an ointment studied in rats by Miyazaki *et al.* (92) confirms that 0.5 W/cm^2 is the preferred intensity. Although, an intensity of 0.75 W/cm^2 leads to the highest penetration but it results in an increase in skin temperature significantly and hence destructive effect on skin. Progressively more skin damage was also noted as the intensity and the time of application of ultrasound increased. Thus, to develop a useful tool based on ultrasound technology, further intensive research focusing on safety issues is required to evaluate limiting ultrasound parameters for safe exposure (58).

Ultrasound enhanced interstitial fluid extraction (for glucose monitoring)

Ultrasound permeation of the skin can also be used for glucose monitoring in a home setting. There are several studies on the transdermal extraction of interstitial fluid - enhanced by ultrasound which offers a potential minimally invasive method of obtaining a fluid sample for at-home blood glucose monitoring (21,23,55,112). Ultrasound application led to *in vitro* transdermal extraction of with permeabilities several orders of magnitude higher than those obtained with passive diffusion across skin. For example, passive skin permeability of glucose was about 0.0003 cm/hr, compared with 0.17 cm/hr after ultrasound application (an increase of 570-fold) (55). A device is currently being developed (150) that permeates skin (for up to 24 hr) and then uses a sensor/patch to continuously extract interstitial fluid and monitor blood glucose levels. The continuous non-invasive monitoring of blood glucose may significantly improve patient compliance to frequent glucose testing, which has been shown to reduce severe complications related to diabetes.

Novel portable ultrasound transdermal delivery system – Cymbal transducer design

Although a commercial sonicator has been an excellent device for demonstrating drug delivery, the major drawback so far in exploiting the commercial ultrasound device for non-invasive drug delivery is the large size and weight of the ultrasound device. In addition, they require power from a standard outlet with the converter (ultrasonic probe) approximately 20 cm in length and weighing almost a kilogram. For practical application related to portable and low-profile (smaller and light-weight) transdermal drug-delivery system, novel transducer design is the main criteria without compromising on frequency and intensity so that it operates very similar to commercial sonicator.

Recently, cymbal array (f = 20 kHz) design with a light-weight (<22 g), low-profile (37 x 37 x 7 mm^3) has been used to generate ultrasound and for transdermally enhancing the delivery of insulin (63). Advantage of using this cymbal array design is that the standard array covers a 37 x 37 mm^2 area, whereas the probe tip on a sonicator covers only 10 mm diameter (70). Additional advantage that has been demonstrated with this design was that with short ultrasound exposure time of 5 min, transdermal delivery of insulin reduced the glucose to a significant level. This gives an indication that ultrasound exposure times do not need to be long to deliver a clinically significant insulin dose to reduce a high blood glucose level. These results are further supported by smith *et al.* (119) who have used cymbal array for increasing the transport of insulin.

Synergistic effects of ultrasound and other enhancers

Sonophoresis has also been shown to operate in synergy with other enhancers of transdermal drug transport, including chemicals, electroporation and iontophoresis (85). Understanding the synergistic relationship that exists between various enhancers and selecting the right combination represents a large opportunity to develop potent and safe methods to enhance transdermal drug delivery. The effects of combination of enhancers including a) polyethylene glycol 200 dilaurate (PEG), b) isopropyl myristate (IM), c) glycerol trioleate (GT), d) 50% EtOH saturated with linoleic acid (LA/EtOH), and therapeutic ultrasound (1 MHz, 1.4 W/cm^2, continuous) on transdermal drug transport of corticosterone have been investigated. LA/EtOH was found to be the most effective of these enhancers, increasing the corticosterone flux from the saturated solutions by up to 13000-fold. Similar enhancements have been obtained with LA/EtOH, with and

without ultrasound for four other model drugs, dexamethasone, estradiol, lidocaine and testosterone (50).

Kost *et al.* (54) have found that the combination of electroporation with ultrasound produced a synergistic interaction and have suggested that this may be caused by ultrasound disorganising stratum corneum lipids to an extent where they were more susceptible to the effects of electroporation. The combination of low frequency ultrasound and iontophoresis also increased the flux of heparin across pig skin above that observed for each of the techniques alone (59). Despite these studies, however, the combination technologies will lag behind the development of individual technologies until safety and efficacy evaluations and validations *in vivo* and in human volunteers can be convincingly demonstrated (25).

Ultrasound enhanced delivery through cornea

The successful results of ultrasound-enhanced transdermal drug delivery has also motivated on the investigation of the use of ultrasound to enhance drug delivery through the cornea (144). It has been found that the application of 20-kHz at intensity of 14 W/cm^2 resulted in a 4-fold increase in the corneal permeability of atenolol, carteolol, timolol and betaxolol drugs (143). Ultrasound application at medium frequencies (470-880 kHz) and intensities of 0.2-0.3 W/cm^2 has also been used for transcorneal drug delivery, to improve treatment of corneal inflammation, wounds, and retinal dystrophy (97,136).

SUMMARY AND OUTLOOK

Transdermal transport of drugs can be temporarily enhanced and controlled by exploiting the cavitational collapse effects of ultrasound. The most exciting results obtained from various experiments confirm that this method can give about 1000-fold better penetration compared to simple topical application. Sonophoresis is undergoing a renaissance and a significant amount of progress has been made towards this technologically attractive and more promising process. Thus, it has changed from a poorly understood magical treatment to a highly specialized mechanism, but, now that requires the utmost scientific accuracy to be effective which could lead to the development and availability of practical devices based on this technology. Also, the ultimate goal of sonophoresis is to enhance transdermal transport of a *broad variety of drugs*, including high molecular weight proteins which are again possible by the optimization of all the possible parameters of ultrasound and improved understanding of the cavitation events which are occurring on the skin. The recent FDA approval of the use of low frequency portable ultrasound device for skin permeabilisation and the development of low frequency low-profile transducers for sonophoresis has further given a strong hope on this field. Technological change, nonetheless, is transforming the landscape of ultrasound and we can believe that further breakthroughs are on the horizon.

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RESEARCH ARTICLE

An efficient gene transfer method mediated by ultrasound and microbubbles into the kidney

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Abstract

Background Safety issues are of paramount importance in clinical human gene therapy. From this point of view, it would be better to develop a novel non-viral efficient gene transfer method. Recently, it was reported that ultrasound exposure could induce cell membrane permeabilization and enhance gene expression.

Methods In this study, we examined the potential of ultrasound for gene transfer into the kidney. First, we transfected rat left kidney with luciferase plasmid mixed with microbubbles, Optison, to optimize the conditions (duration of ultrasound and concentration of Optison). Then, 4, 7, 14 and 21 days after gene transfer, luciferase activity was measured. Next, localization of gene expression was assessed by measuring luciferase activity and green fluorescent protein (GFP) expression. Expression of GFP plasmid was examined under a fluorescence microscope at 4 and 14 days after gene transfer. Finally, to examine the side effects of this gene transfer method, biochemical assays for aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN) and creatinine (Cre) were performed.

Results Optison and/or ultrasound significantly enhanced the efficiency of gene transfer and expression in the kidney. Especially, 70–80% of total glomeruli could be transfected. Also, a significant dose-dependent effect of Optison was observed as assessed by luciferase assay (Optison 25%: 12.5×10^5 relative light units (RLU)/g tissue; 50%: 31.3×10^5 RLU/g tissue; 100%: 57.9×10^5 RLU/g tissue). GFP expression could be observed in glomeruli, tubules and interstitial area. Results of blood tests did not change significantly after gene transfer.

Conclusions Overall, an ultrasound-mediated gene transfer method with Optison enhanced the efficiency of gene transfer and expression in the rat kidney. This novel non-viral method may be useful for gene therapy for renal disease.

Keywords gene therapy; kidney; ultrasound; microbubble

Introduction

Gene therapy through the delivery of genetic constructs is emerging as a revolutionary and promising form of therapy for the treatment of human diseases. For diseases involving the kidney, gene therapy to replace dysfunctional genes or to suppress the production of disease mediators represents a promising new therapeutic approach. Gene therapy was originally proposed

in the late 1980s as a treatment strategy for diseases caused by single gene defects, such as cystic fibrosis or adenosine deaminase deficiency (ADA) [1]. Because the molecular tools for genetic manipulation were available, effectiveness seemed guaranteed. However, there were problems with gene delivery, with failure to achieve therapeutic levels of transgene expression. With these setbacks came the realization that diseases are complex multigene phenomena, hat more basic research is required before moving to clinical trials, and that clinical trials of gene therapy must be more rigorously controlled.

Renal disease has been considered one of the target diseases for gene therapy since, even now, there is no satisfactory pharmacological treatment to prevent and cure the process leading to end-stage renal failure [2,3]. The rapid development of gene transfer technology provides an opportunity to study the biological effects of different genes in the kidney and to develop treatment for various inherited or acquired renal diseases [4–9]. Several strategies have been developed to deliver foreign genes into different segments of the nephron using viral or non-viral vectors as well as genetically modified renal cells [10–17]. The use of conventional liposomes and viral vectors for transfecting genes into the human kidney is limited in terms of safety and efficiency. Therefore, several modified approaches have been developed.

We have developed the hemagglutinating virus of Japan (HVJ)-liposome-mediated gene transfer method for *in vivo* gene transfer into the kidney [10–13]. Although this method is easy to manipulate and highly efficient, and there is no limitation to the size of the vector DNA and little toxicity [18,19], its clinical utility such as large-scale production is still limited. Several studies have shown that ultrasound, used either alone or in combination with ultrasound contrast agents, can increase cell membrane permeability to macromolecules such as plasmid DNA [20–24]. This phenomenon has been referred to as sonoporation [20]. Most sonoporation studies have been carried out on cultured cells [20–25] or tumors *in vivo* [26–29], or skeletal muscle [30–32]. Moreover, recently we have found that this approach is applicable to gene transfer into the artery [33]. However, to our knowledge, there has been no previous investigation of whether ultrasound is able to enhance plasmid-mediated gene transfer into the kidney. Although based on this background we have already published two papers [34,35], in those studies we did not optimize the condition for gene transfer. Thus, in this study, we tried to develop and optimize a successful *in vivo* gene transfer method into the kidney using ultrasound exposure with microbubble material (Optison). Therefore, the global objective of our study was to investigate the potential usefulness of ultrasound as a method for improving the efficiency of plasmid-mediated gene transfer into the kidney, utilizing luciferase and green fluorescent protein (GFP) plasmid.

Materials and methods

Plasmid DNA

Luciferase expression plasmid was obtained from Promega Corporation (Madison, WI, USA). In this plasmid, firefly luciferase cDNA is driven by the SV 40 promoter and enhancer. GFP plasmid was obtained from BD Biosciences Clontech (Palo Alto, CA, USA). This plasmid contains the CMV promoter. As a control, we used a control plasmid that contained neither luciferase nor GFP cDNA.

Gene transfer into kidney by ultrasound

Eight-week-old male Wistar rats weighing 150 g were purchased from Charles River Japan (Osaka, Japan). Plasmid was transfected into the kidney via the renal artery using an ultrasound-mediated system. The procedure for the ultrasound-based gene transfer technique includes: (1) mixing luciferase or GFP or the control plasmids with Optison (Mallinckrodt, St. Louis, MO, USA) in several v/v ratios and injecting the mixed solution containing 50 μg of plasmid in 0.5 ml into the left renal artery with temporary clipping of the renal artery and vein (<5 min); (2) applying the ultrasound transducer (Rich-Mar, Inola, OH, USA) directly onto one side of the left kidney with a continuous-wave output of 1 MHz ultrasound at 5% power output, for a total of 60 s at 30-s intervals; (3) turning over the kidney and treating the other side with ultrasound using the same procedure. The infusion cannula is then removed, blood flow to the renal artery restored by release of the ligatures, and the wound closed.

Luciferase activity assay

Rats were killed at 4, 7, 14 and 21 days after gene transfer. Kidney samples were rapidly frozen in liquid nitrogen and homogenized in lysis buffer. The tissue lysates were briefly centrifuged (3000 rpm, 10 min), and 20 μl of supernatant were mixed with 100 μl of luciferase assay reagents. Firefly luciferase activity was measured for 1 min using a luciferase assay system (PicaGene; Tokyo-Inki, Tokyo, Japan). The values for luciferase activity shown in this paper were adjusted by the tissue weight. So values are expressed as relative light units (RLU)/g tissue.

Examination of GFP expression

To detect expression of GFP in injected kidney, kidneys were halved on day 4 after gene transfer, placed in liquid nitrogen and embedded in OTC compound. Then the kidneys were sectioned on a cryostat (6-μm sections).

Sections were examined for GFP using fluorescence microscopy with a fluorescein filter set.

Preparation of HVJ-liposomes

HVJ-liposomes were prepared in an identical fashion to that previously described [10–13,36,37]. Phosphatidylserine, phosphatidylcholine, and cholesterol were mixed in a weight ratio of 1 : 4.8 : 2 [10–13,36,37]. The lipid mixture (10 mg) was deposited on the sides of a flask by removal of tetrahydrofuran in a rotary evaporator. Dried lipid was hydrated in 200 μl balanced salt solution (BSS; 137 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl, pH 7.6) containing 50 μg of luciferase or GFP or the control plasmid. Liposomes were prepared by shaking and sonication. Purified HVJ (Z strain) was inactivated by UV irradiation (110 erg/nm^2/s) for 3 min just before use. The liposome suspension (0.5 ml, containing 10 mg lipids) was mixed with HVJ (30 000 hemagglutinating units) in a total volume of 4 ml BSS. The mixture was incubated at 4 °C for 5 min and then for 30 min with gentle shaking at 37 °C. Free HVJ was removed from the HVJ-liposomes by sucrose density gradient centrifugation. The top layer of the sucrose gradient was collected for use [10–13,36,37].

Collection of glomeruli

Glomeruli were isolated from the outer renal cortex of gene-transfected rats by means of a sieving technique (passing the cortical pulp through calibrated sieves of 180, 125 and 63 μm, respectively, as described previously [38]).

Histological analysis

Animals were killed under injection of sodium pentobarbital (50 mg/kg i.p.) on day 14 after gene transfer. The kidneys were fixed in 4% paraformaldehyde after perfusion with phosphate-buffered saline (PBS), and 5-μm-thick paraffin sections were stained with hematoxylin and eosin (HE). All HE-stained sections were evaluated by three investigators with no knowledge of experimental treatments. To assess the histological damage we followed the method described previously [39].

Measurement of plasma parameters

To examine the side effects of administration of microbubbles combined with ultrasound exposure, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN) and creatinine (Cre) were measured after the rats had been killed. These levels were determined using a commercially available assay kit (Sigma Chemical Co., St. Louis, MO, USA) with a modification of the assay protocol suggested by the supplier.

RT-PCR analysis

Using reverse transcriptase polymerase chain reaction (RT-PCR) analysis on isolated glomeruli and the remaining parts of the kidney, we further examined whether expression of the transfected reporter plasmid could be observed in the glomerulus and the remaining parts of the kidney on day 21 after gene transfer. Total RNA (glomeruli and remaining parts) extracted from gene-transfected kidneys was subjected to RT-PCR analysis using two primers specific for luciferase DNA, GCC TGA AGT CTC TGA TTA AGT and ACA CCT GCG TCG AAG T, which yield 96-bp fragments [40]. The cycles were 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min. After 35 cycles, the PCR product was analyzed as well as samples amplified using GAPDH primers, which served as the internal control. GAPDH primers were purchased from Clontech Inc. (Palo Alto, CA, USA).

Statistical analysis

All values are expressed as mean ± SEM. Analysis of variance with a subsequent Bonferroni/Dunnet's test was employed to determine the significance of differences in multiple comparisons. Values of $p < 0.05$ were considered statistically significant.

Results

A series of preliminary studies were performed in normal rats in which the left kidney was perfused with luciferase plasmid or control plasmid, and Optison mixture accompanied by ultrasound exposure. First, we tried to determine the optimal duration of ultrasound exposure. Fifty micrograms of luciferase reporter plasmid were dissolved in 0.5 ml saline containing echocardiographic contrast agent, Optison, at two different concentrations (25 and 50%, v/v; n = 4 for each concentration) and infused into the left kidney through the renal artery. The kidney was then exposed to ultrasound for 1 or 2 min as detailed 'Materials and methods'. Luciferase activity assay was performed on day 4 after gene transfer. As shown in Figure 1a, there were no significant differences between exposure times at both concentrations of Optison (25 and 50%, v/v). From this result, we decided to use 1 min for exposure of ultrasound. Of course, no luciferase activity was observed in kidneys transfected with control plasmid (data not shown). The next step was to determine the optimal concentration of Optison. Several different concentrations of Optison (0, 5, 10, 25, 50 and 100%, v/v) with ultrasound exposure for 1 min were used, and luciferase activity was examined on day 4 after gene transfer. In this experiment, a dose-dependent effect of Optison was observed, as shown in Figure 1b. However, at higher concentrations of Optison, such as 50 or 100% (v/v), histological damage on day 14 after gene transfer could be seen in the

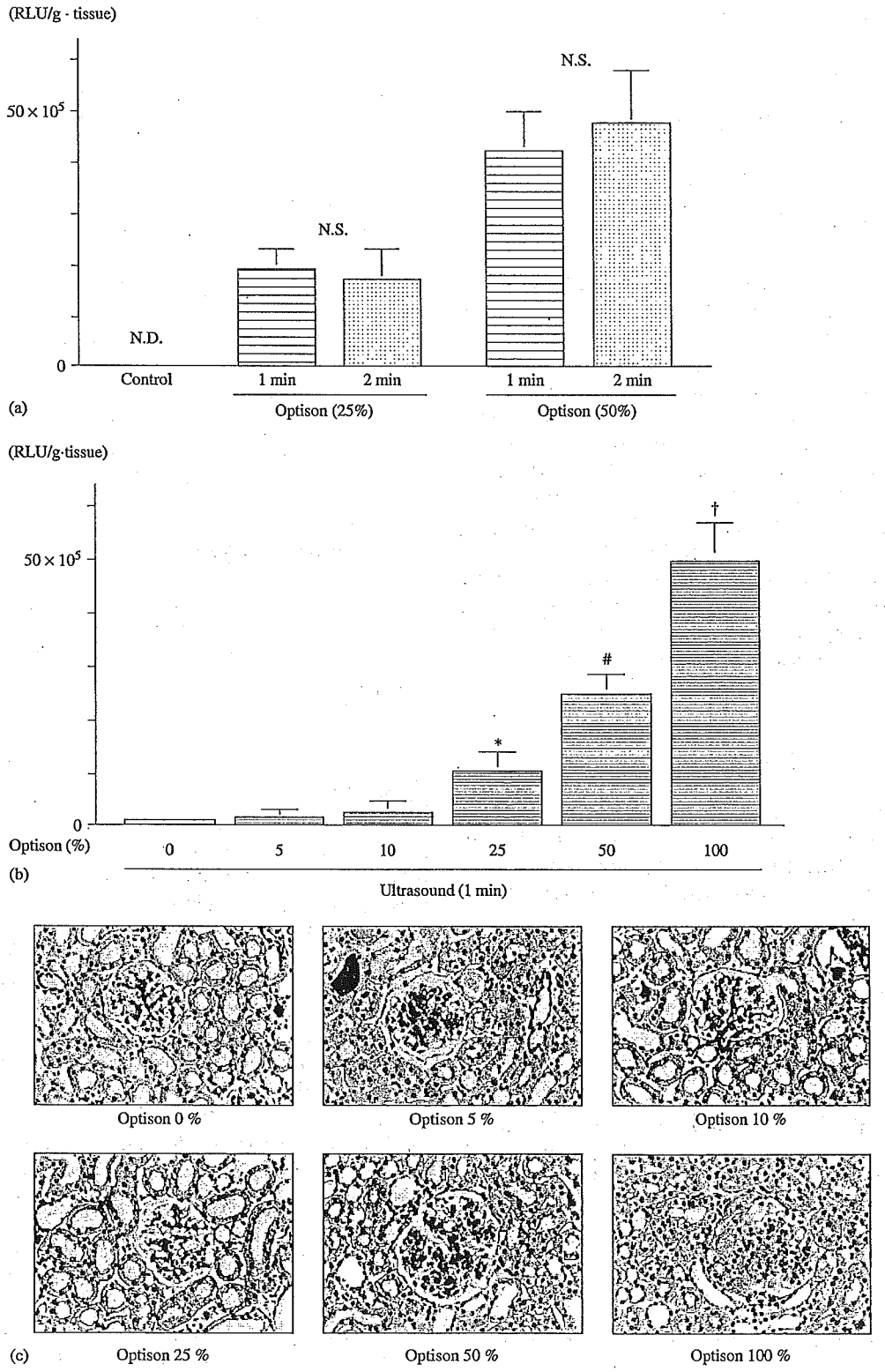

Figure 1. (a) Luciferase activity in kidney on day 4 after gene transfer to examine the effect of ultrasound exposure time. 1 min and 2 min indicate the ultrasound exposure time. Control = kidneys with no gene transfer. N.S. = not significant. RLU = relative light units. Concentrations of Optison are expressed as v/v. n = 5 per group. Data are calculated from five independent experiments. The total amount of transfected plasmid per one kidney was 50 μg. (b) Luciferase activity in kidney on day 4 after gene transfer to examine the effect of Optison concentration. In this experiment, ultrasound exposure was 1 min. Concentrations of Optison are expressed as v/v. $*p < 0.01$ vs. Optison 0%, and $\#p < 0.01$ vs. Optison 25% and 50%. RLU = relative light units. n = 5 per group. Data are calculated from five independent experiments. The total amount of transfected plasmid per one kidney was 50 μg. (c) Representative photograph of kidneys on day 14 after gene transfer. In this experiment, ultrasound exposure was 1 min. Concentrations of Optison are expressed as v/v. n = 5 per group. The total amount of transfected plasmid per one kidney was 50 μg

Table 1. Blood test data (AST, ALT, BUN and Cre) on day 4 after gene transfer. In this experiment, ultrasound exposure was 1 min. 0%, 5%, 10%, 25%, 50% and 100% are the concentrations of Optison expressed as v/v. Values indicate mean ± SE. There were no significant differences between each group. n = 6 per group. Data are calculated from five independent experiments. The total amount of transfected plasmid per one kidney was 50 μg

Optison	0%	5%	10%	25%	50%	100%
AST (IU/l)	142 ± 19	207 ± 28	184 ± 19	192 ± 21	203 ± 24	186 ± 21
ALT (IU/l)	59 ± 8	68 ± 9	57 ± 7	62 ± 9	69 ± 7	57 ± 8
BUN (mg/dl)	12.1 ± 1.3	14.2 ± 1.9	11.7 ± 2.0	14.8 ± 1.5	13.9 ± 1.7	13.2 ± 1.5
Cre (mg/dl)	0.2 ± 0.03	0.3 ± 0.05	0.4 ± 0.02	0.3 ± 0.05	0.3 ± 0.02	0.4 ± 0.03

kidneys, even though plasma parameters of hepatic and renal function did not change, as shown in Figure 1c and Table 1. Representative photographs of kidneys transfected with luciferase plasmid mixed with Optison at the concentrations of 50 and 100% (v/v) clearly showed glomerular damage, as shown in Figure 1c. Also, quantitative analysis for histological damage in glomeruli supported this fact. Significant damage, such as matrix expansion and increase in glomerular diameter, was observed in kidneys infused with 50 and 100% (v/v) Optison (data not shown). Moreover, although we do not show here the data from control kidneys transfected with control plasmid, the same extent of damage could be seen in the sections. From these results we can easily speculate that renal damage seen in rats might be due to the high concentration of Optison. These data led us to decide to use a concentration of Optison of 25% (v/v) which showed an apparently significant effect on gene expression of the luciferase reporter gene.

Then, we examined how long the expression would last. Rats whose kidneys were transfected with luciferase plasmid with 25% Optison (v/v) accompanied by ultrasound exposure for 1 min were sacrificed on days 7, 14 and 21 days after gene transfer. As shown in Figure 2, the peak gene expression was seen on day 7; however, significant gene expression in the kidney lasted until at least day 21 after gene transfer. Moreover, there were no significant changes in plasma parameters during the observation period. The data of plasma parameters (AST, ALT, BUN, Cre) at 0 (Pre), 7, 14 and 21 days after gene transfer are shown in Table 2. As shown here, the gene transfer method mediated by ultrasound and Optison (less than 25%, v/v) was safe and efficient. We did not detect luciferase activity from kidneys transfected with control plasmid at each time point (data not shown).

The next question was the site where gene expression occurred. We isolated the glomeruli and performed a luciferase assay using isolated glomeruli and the remaining parts of the kidney. Interestingly, both glomeruli and the remaining parts of the kidney showed reporter gene expression at least until day 21 after gene transfer, as shown in Figure 3a. To enhance this result, we performed RT-PCR for luciferase in the same samples. As shown in Figure 3b, RNA expression could be detected on day 21 after gene transfer. From these data, we confirmed that gene expression induced by gene transfer mediated by Optison and ultrasound exposure was detected in both glomeruli and the remaining parts

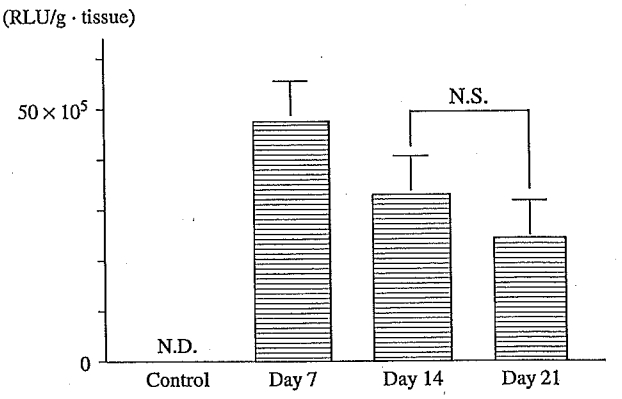

Figure 2. Luciferase activity in kidney after gene transfer. Assays were performed on days 7, 14 and 21 after gene transfer. In this experiment, ultrasound exposure was 1 min and the concentration of Optison was 25% (v/v). Control means kidney before gene transfer. N.D. = not detected. N.S. = not significant. n = 6 per group. Data are calculated from five independent experiments. The total amount of transfected plasmid per one kidney was 50 μg

Table 2. Blood test data (AST, ALT, BUN and Cre) after gene transfer. In this experiment, ultrasound exposure was 1 min and the concentration of Optison was 25% (v/v). Assays were performed on days 7, 14 and 21 after gene transfer and before gene transfer. Values indicate mean ± SE. There were no significant differences between each group. n = 6 per group. Data are calculated from five independent experiments. The total amount of transfected plasmid per one kidney was 50 μg

	Pre	Day 7	Day 14	Day 21
AST (IU/l)	142 ± 19	207 ± 28	184 ± 19	192 ± 21
ALT (IU/l)	59 ± 8	68 ± 9	57 ± 7	62 ± 9
BUN (mg/dl)	12.1 ± 1.3	14.2 ± 1.9	11.7 ± 2.0	14.8 ± 1.5
Cre (mg/dl)	0.2 ± 0.03	0.3 ± 0.05	0.4 ± 0.02	0.3 ± 0.05

of the kidney as assessed by RT-PCR and protein assay. In this RT-PCR assay we showed the positive control as indicated as P in Figure 3b. RNA was extracted from the fibroblasts and luciferase plasmid was over-expressed (Promega Corporation).

Moreover, to visualize the expression of the transfected gene, we examined GFP plasmid expression transfected by this new technique with the same approach as shown above. On days 4 and 14 after gene transfer, frozen sections of the kidneys were observed by fluorescence microscopy. As shown in Figure 4, in both glomeruli and the remaining parts of the kidney such as tubules or interstitial tissue, fluorescence was detected, suggesting

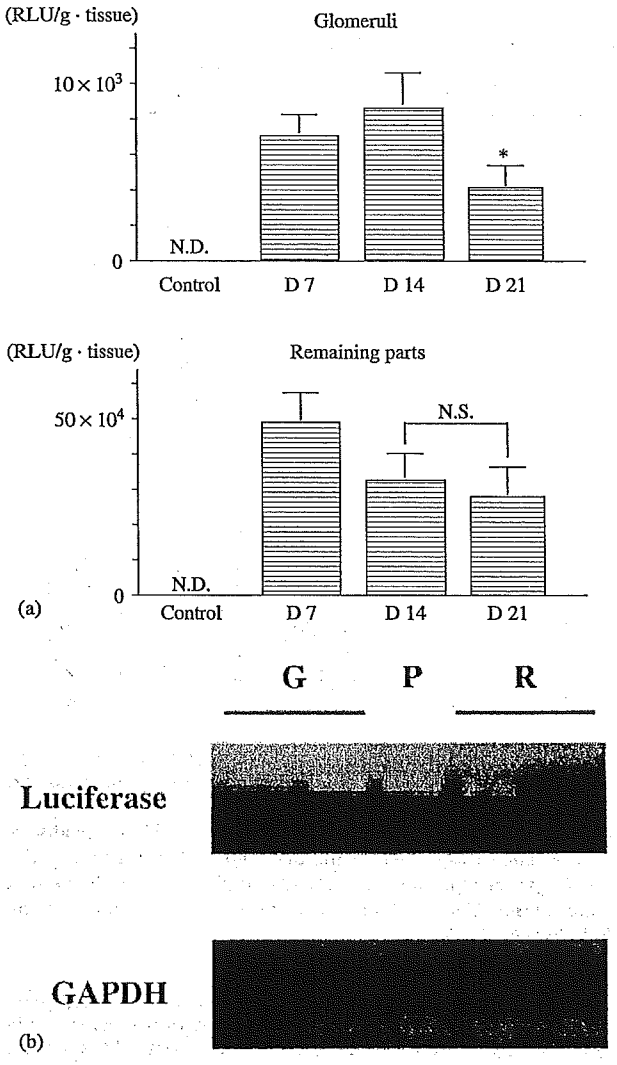

Figure 3. (a) Luciferase activity in kidney (glomeruli and remaining parts of kidney (interstitial tissue and tubules)) on days 7, 14 and 21 after gene transfer. In this experiment, ultrasound exposure was 1 min and the concentration of Optison was 25% (v/v). Isolation of glomeruli was performed as described in 'Materials and methods'. Control = control kidney which had no gene transfer. D7, D14 and D21 = days 7, 14 and 21 after gene transfer, respectively. *$p < 0.01$ vs. D14. N.S. = not significant. n = 6 per group. Data are calculated from five independent experiments. The total amount of transfected plasmid per one kidney was 50 μg. (b) Representative photograph of RT-PCR performed on RNA samples extracted on day 21 after gene transfer. In this experiment, ultrasound exposure was 1 min and the concentration of Optison was 25% (v/v). G = glomeruli, P = positive control and R = remaining parts of the kidney. In lanes G and R there two different bands from different samples, respectively. The total amount of transfected plasmid per one kidney was 50 μg

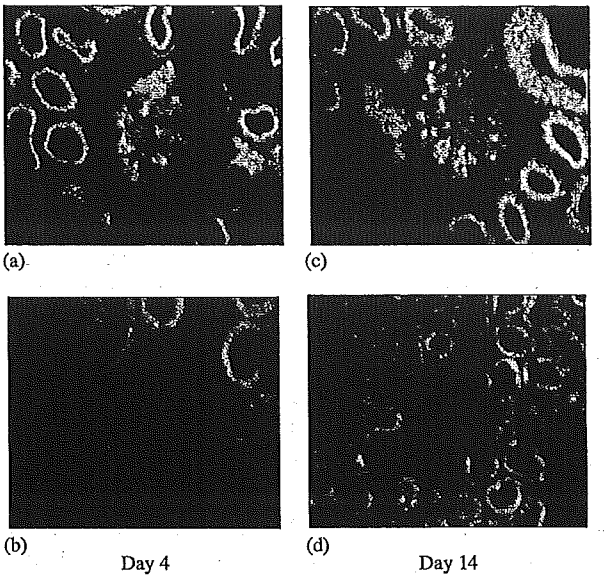

Figure 4. Representative photographs of kidney transfected with GFP plasmid on day 14 after gene transfer. In this experiment, ultrasound exposure was 1 min and the concentration of Optison was 25% (v/v). Kidney samples were collected on days 4 and 14 after gene transfer. Photographs of (A) left kidney transfected with GFP plasmid on day 4; (B) right kidney of control which had no gene transfer on day 4; (C) left kidney transfected with GFP plasmid on day 14; and (D) right kidney of control which had no gene transfer on day 14. The total amount of transfected plasmid per one kidney was 50 μg

that the transfected GFP plasmid was expressed in these areas. In control kidneys transfected with control plasmid on days 4 and 14 no fluorescence could be observed (Figures 4B and 4D). There seemed to be no significant changes between the distribution pattern of fluorescence in kidneys on days 4 and 14. This fact is almost consistent with results of luciferase activity.

Finally, we compared the efficiency of gene transfer by this new method with that of the HVJ-liposome method, which we previously reported as an efficient gene transfer method to the kidney [10–13]. The HVJ-liposome method is based on liposomes with inactivated virus. From this point of view, the HVJ-liposome method has many hurdles before application in clinical trials. We must develop an efficient and safe gene transfer method aimed at application in human trials. As shown in Figure 5, ultrasound (1 min) itself enhanced gene expression. However, compared with the HVJ-liposome method, the level of gene expression just with ultrasound was still significantly less (ultrasound: 1.37 ± 0.14 0215 10^4 RLU/g tissue; HVJ-liposome: $2.69 \pm 0.28 \times 10^4$ RLU/g tissue). On the other hand, the combination of Optison (25%, v/v) and ultrasound exposure (1 min) significantly enhanced gene expression compared with the HVJ-liposome method (Optison + ultrasound: $17.45 \pm 1.82 \times 10^4$ RLU/g tissue; HVJ-liposome: $2.69 \pm 0.28 \times 10^4$ RLU/g tissue, $p < 0.01$).

Discussion

Gene therapy is now moving from experimental studies to clinical applications. It could be applicable not only as therapy for inherited diseases, but also as new treatments for acquired diseases. However, one of the limiting steps in gene therapy is the gene transfer

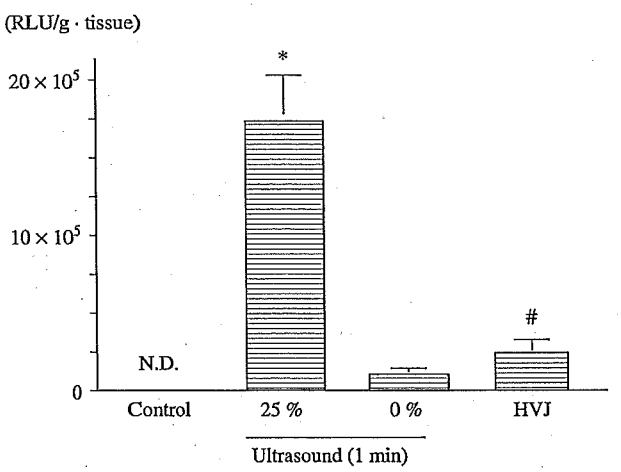

Figure 5. Comparison of gene expression with the HVJ-liposome method. To perform this experiment, luciferase plasmid was used. Luciferase activity assay was performed on day 4 after gene transfer using the whole kidney. The concentration of Optison was 25% (v/v). Control = kidney with no gene transfer. 1 min and 0 min indicate the ultrasound exposure time, respectively. HVJ = kidney transfected with luciferase plasmid by HVJ-liposome method. N.D. = not detected. *$p < 0.01$ vs. 0 min and HVJ, respectively. #$p < 0.01$ vs. 0 min. n = 6 per group. Data are calculated from five independent experiments. The total amount of transfected plasmid per one kidney was 50 μg

method. Several strategies have been developed to deliver foreign genes into different segments of the nephron, using viral and non-viral vectors as well as genetically modified renal cells [10,17,41–45]. Generally, gene expression by non-viral vectors is transient and rather weak, while genetically modified renal cells trapped in the kidney could potentially induce additional biological effects. On the other hand, genetically modified cell vectors are not practical. Although viral vectors such as adenoviral vectors, retroviral vectors or adeno-associated viral vectors yield higher gene expression, these vectors have unfavorable effects on the host body such as immunosuppression or oncogenesis [46,47]. In the present study, we established an easy, safe and efficient gene transfer method mediated by ultrasound and microbubbles. GFP expression, which indicates successful transfection of foreign genes, could be observed in more than 70–80% of total glomeruli and most tubular cells in the kidney treated with the ultrasound and Optison-mediated transfer method. In contrast, the ratio of glomeruli transfected with the reporter gene to total glomeruli was at most 30% with the HVJ-liposome method [10,42]. Although in the present study we did not compare the efficiency of this method with that of another method, judging from the previously published data, transfection efficiencies of other methods may not be as high as that of this novel transfer method, even though this was evaluated from the values published already. This method should be quite impressive for future clinical applications and have potential as a new treatment strategy.

In contrast to the efficiency of this new method, overexposure to ultrasound, which may cause irreversible tissue injury, should be considered as a critical issue, and it is important to establish the optimal conditions for this method. In the present study, we demonstrated that ultrasound exposure did not change gene expression up to 2 min. Because longer ultrasound exposure may cause organ damage, we did not examine exposure times longer than 2 min. Moreover, injection of plasmid DNA into muscle is known to induce mild inflammatory damage, which has been linked to the presence of proinflammatory unmethylated CpG motifs within prokaryotic DNA [48,49]. However, we did not observe even a mild degree of inflammation in the kidney after gene transfer. Of note, ultrasound itself was not associated with any increase in damage or inflammation in the kidney as long as the exposure time was less than 1 min (data not shown).

In addition to ultrasound exposure, the use of Optison at higher concentrations (50 or 100%, v/v) led to severe injury as assessed histologically. Accordingly, we applied ultrasound exposure for 1 min, and Optison at a concentration of 25%. Thus, we decided the optimal conditions for this newly developed gene transfer method into the kidney, which should be of great value for the clinical application of this method. It has been previously reported that ultrasound exposure increased the efficiency of gene transfer into cultured cells, and its mechanism of action is thought to be increased cell membrane porosity [20–25]. Consistent with previous studies, the present study demonstrated that ultrasound exposure increased reporter gene expression in the kidney, however, the level was not as high as reported. We therefore modified the procedure using microbubbles, Optison, to increase the reporter gene expression, as previously reported [30,33]. This concept is based on the previous observation that microbubbles, that contain gas bodies filled with perfluoropropane, lowered the threshold for the production of acoustic cavitation and enhanced the sonoporation of cultured cells with ultrasound exposure. Of interest, other echo contrast agents such as Levovist, which contains air-based gas bodies with a different stabilization strategy, and Hexabrix, which does not contain gas bodies, did not enhance gene expression (data not shown). Optison contains about 5×10^8/ml gas bodies, 2–4.5 μm in diameter, which are filled with perfluoropropane and stabilized by a solid shell of heat-denatured human albumin [50,51]. Although further studies are needed to investigate the exact molecular mechanism by which Optison enhances transfection efficiency, the molecular structure of gas-filled microspheres and their contents may be implicated in the mechanisms of action.

Not all viral vectors, for example, adeno-associated viral vectors, are able to accommodate the full-length target gene to be transfected. An alternative approach is to use a non-viral vector such as plasmid DNA, which is less toxic, cheaper, easier to prepare, and able to accommodate the full-length target cDNA. However, the major drawback of the naked DNA approach up to now has been the

very low gene transfer efficiency compared with viral vectors. The use of ultrasound as an adjuvant measure to enhance plasmid DNA delivery has a number of advantageous features, which should increase the overall prospects for therapeutic application of naked DNA in the kidney. It is true that electroporation-mediated gene transfer resulted in rather high gene expression [52–54]. However, in contrast to electroporation, ultrasound is a non-painful and well-established tool in clinical medicine. The non-invasive nature of ultrasound and the absence of neutralizing antibodies against plasmid DNA also raise the possibility that treatment could be easily repeated on a relatively frequent basis. Lastly, ultrasound-mediated destruction of intravascularly injected microbubbles has been used to induce microvessel breaches that are large enough to permit extravasation of macromolecules, including plasmid DNA [55]. Therefore, ultrasound could be a powerful adjunct to intravascular delivery of plasmid DNA into the kidney. Further studies will be required to investigate the full therapeutic potential of ultrasound-mediated approaches to gene delivery in the setting of kidney disease.

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Molecular Delivery into a Lipid Bilayer with a Single Shock Waves Using Molecular Dynamic Simulation

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Abstract. Cell permeabilization by shock waves may have application in gene therapy and anticancer drug delivery. In the present study we performed direct molecular dynamic (MD) simulation of the interaction of a single shock wave with a cell membrane to investigate the mechanism of the cell permeabilization. The shock wave was characterized by an impulse that was expressed with a velocity determined by the change in the momentum. The cell membrane was designed as a dipalmitoylphosphatidylcholine (DPPC) lipid bilayer placed between two layers of water molecules. The MD simulation determined the relationship between water penetration into the bilayer, the order parameter, the fluidity of each lipid molecule, and the intensity of impulse. These structural changes in the bilayer may be an important factor in the use of shock waves to produce transient membrane permeability.

INTRODUCTION

Cell permeabilization using shock waves may be a promising way of introducing macromolecules and small polar molecules into the cytoplasm, and may have applications in gene therapy and anticancer drug delivery. The pressure profile of a shock wave indicates its energy content, and shock propagation in tissue is associated with cellular displacement, leading to the development of cell deformation. Shock waves are nonlinear, finite-amplitude waves, and the flow induced behind the shock waves cannot be ignored. The duration of the particle motion is the order of the pulse duration, dt, of the shock wave, and the displacement, d, of the particle is about the order of $d = u_p \times dt$, where u_p is the induced speed, which is inversely proportional to the density of the particle. A rough estimate of tissue displacement obtained with a single shock wave generated by a clinical lithotripter is calculated to be 1-20 μm, using pressure data obtained in water [1]. This value is similar to that measured in rabbit liver resulting from a shock wave produced by detonation of an explosive micropellet (7-10 μm) [2]. Kodama et al.[3] reported that the shock wave impulse (defined as the integral of pressure with duration) is an important factor governing the temporary permeability increase in cell membranes necessary for delivering macromolecules into

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cells. The detailed mechanism of the transient membrane permeability increase is still unclear. In present study we conducted molecular dynamics (MD) simulations of the interaction of the shock wave impulse with a lipid bilayer to investigate the mechanism. In addition, we studied the structural change of the bilayer and subsequent characteristic delivery of water molecules into the bilayer.

METHOD

A cell membrane was designed as a 32 dipalmitoylphosphatidylcholine (DPPC) lipid bilayer, placed between two 1200 water layers in the calculation box. This box was a cuboid whose longitudinal axis was set to the z-axis perpendicular to the xy plane. The stable liquid-crystal phase bilayer was calculated for several tens of nano seconds with a constant temperature of 323K and pressure of 1 bar with periodic boundary conditions. The detailed calculation conditions are presented elsewhere [4]. The velocity and positions of molecules in the system were used as initial conditions for applying shock wave.

A single shock wave was applied downwards to a part of the upper water layer. The shock wave was characterized by an impulse that was expressed with a velocity V_2 determined by the change in the momentum in the upper water layer. The V_2 was calculated as

$$V_2 = \frac{I_P}{M} A \ , \tag{1}$$

where M (kg) is the mass of the water molecules in the upper layer, $A(m^2)$ is the area of the x-y plane in the calculation box, and I_P is the shock wave impulse from 1.6 to 16 mPa s (pressure times time) which corresponds to 0.1 to1.0 mPa s /lipid[5].

The simulations were performed using the AMBER 7 set of programmes [6].

RESULTS AND DISUCUSSION

Figure 1 shows the time evoloution of the structural change in the bilayer with an impulse of 0.7mPa· s. Water molecules in the stable state rarely penetrate into the hydrophobic region of the bilayer (Fig.1a). However, when the impulse was applied to the water region, a wave propagated into the bilayer, followed by movement of water molecules into the bilayer, and structural change occurred (Fig.1b-d). Table 1 shows the impulse intensity dependence of the number of water molecules delivered into the bilayer with the impulse, the averaged order parameter, and the lateral mass center velocity of all DPPC lipids. The number of delivered water molecules increased with increasing impulse intensity. This trend was in qualitatively good agreement with previously obtained experimental results. The averaged order parameter decreased with increasing impulse intensity and this meant the alkyl chain structure became disordered. Further, increase in the impulse intensity led to an increase in V_2, which means that each DPPC lipid moves faster than those in the stable state with increasing impulse intensity. Therefore, we concluded that the impulse might increase not only the penetratalon of water molecules into the bilayer, but also the disorder of the alkyol chain, and the fluidity of the lipid, which might be related to transient membrane permeability.