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REVIEW

Sonoporation: Gene transfer using ultrasound

Minoru Tomizawa, Fuminobu Shinozaki, Yasufumi Motoyoshi, Takao Sugiyama, Shigenori Yamamoto, Makoto Sueishi

Minoru Tomizawa, Department of Gastroenterology, National Hospital Organization Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido City, Chiba 284-0003, Japan
Fuminobu Shinozaki, Department of Radiology, National Hospital Organization Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido City, Chiba 284-0003, Japan
Yasufumi Motoyoshi, Department of Neurology, National Hospital Organization Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido City, Chiba 284-0003, Japan
Takao Sugiyama, Makoto Sueishi, Department of Rheumatology, National Hospital Organization Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido City, Chiba 284-0003, Japan
Shigenori Yamamoto, Department of Pediatrics, National Hospital Organization Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido City, Chiba 284-0003, Japan
Author contributions: Tomizawa M wrote the manuscript; Shinozaki F described the figures; Motoyoshi Y, Yamamoto S, Sugiyama T and Sueishi M supervised the manuscript.
Correspondence to: Minoru Tomizawa, MD, PhD, Department of Gastroenterology, National Hospital Organization Shimoshizu Hospital, 934-5 Shikawatashi, Yotsukaido City, Chiba 284-0003, Japan. nihminor-cib@umin.ac.jp
Telephone: +81-43-4222511 Fax: +81-43-4213007
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Abstract

Genes can be transferred using viral or non-viral vectors. Non-viral methods that use plasmid DNA and short interference RNA (siRNA) have advantages, such as low immunogenicity and low likelihood of genomic integration in the host, when compared to viral methods. Non-viral methods have potential merit, but their gene transfer efficiency is not satisfactory. Therefore, new methods should be developed. Low-frequency ultrasound irradiation causes mechanical perturbation of the cell membrane, allowing the uptake of large molecules in the vicinity of the cavitation bubbles. The collapse of these bubbles generates small transient holes in the cell membrane and induces transient membrane permeabilization. This formation of small pores in the cell membrane using ultrasound allows the transfer of DNA/RNA into the cell. This phenomenon is known as sonoporation and is a gene delivery method that shows great promise as a potential new approach in gene therapy. Microbubbles lower the threshold of cavity formation. Complexes of therapeutic genes and microbubbles improve the transfer efficiency of genes. Diagnostic ultrasound is potentially a suitable sonoporator because it allows the real-time monitoring of irradiated fields.

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Key words: Gene therapy; Cavity; Microbubbles; Contrast agent; Diagnostic ultrasound

Core tip: Ultrasound causes cavitation bubbles to form cell membrane pores through which DNA/RNA are transferred. This phenomenon is known as sonoporation. Microbubbles lower the threshold of cavity formation. Sonoporation is less toxic and not associated with tumorigenicity as compared with retroviral and adenoviral vectors. Sonoporation does not require surgical procedure and enhances gene transfer with lipofection. Current limitations of sonoporation are low efficiency of gene transfer and damage of target cells are The use of complexes with chemicals and diagnostic ultrasound are promising approaches to overcome these limitations.

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INTRODUCTION

Gene therapy is a promising approach to treat diseases

and is applicable to tissue engineering controlling differentiation of cells to form tissues[1,2]. Therapeutic genes are transferred using viral or non-viral vectors. Viral vectors are mainly retroviral vectors, adenoviral vectors and adeno-associated vectors. Retroviral vectors are the cause of tumorigenicity[3]. Adenoviral vectors provoke a severe systemic immune response[4]. Adeno-associated vectors do not cause major immune response, but they are not permissive to some types of cells[5]. On the other hand, plasmid DNA does not induce host immune responses because exogenous proteins such as viral capsid proteins are not produced[6]. Moreover, plasmid DNA rarely integrates with the host genome upon introduction into target cells and not associated with tumorigenicity[3,7]. Plasmid DNA is safe for gene transfer therapy, but its transfer efficiency is low. Methods should therefore be developed to improve transfection efficiency of plasmid DNA. Irradiation with low-output intensity ultrasound causes mechanical perturbation in the vicinity of cavitation bubbles. Collapse of the bubbles generates small transient holes in the cell membrane and induces cell membrane permeabilization. The membrane poration (cavitation) increases the efficiency of drug and gene delivery. This phenomenon, sonoporation, is a gene delivery technique that could potentially be used for gene therapy. Sonoporation, a method for targeted drug delivery and non-viral gene transfection, has new and advantageous possibilities. Sonoporation stimulates endocytosis of adno-associated virus and enhances efficiency of gene transfer[8,9]. However, no clinical trials using sonoporation have been reported to date because it is not yet a satisfactory technique for efficient and reliable gene transfer. In this chapter, we will review the potential application of sonoporation in gene therapy, with a focus on mircobubbles as a drug delivery agent. We will also discuss other non-viral delivery methods. Finally, we will outline the future direction of ultrasound-assisted gene delivery aiming at improvement of sonoporation and enhancement of gene expression for clinical applications.

NON-VIRAL GENE DELIVERY

In this section, we review non-viral gene delivery methods. Gene therapy requires the delivery of nucleic acid material to target tissues and its entry into target cells. Given that DNA and the cell membrane are both negatively charged, electrostatic forces result in the repulsion of DNA by the cell membrane. To overcome this limitation, a physical or chemical approach needs to be applied. Another restriction of gene transfer is the rapid degradation of DNA by nucleases in the plasma after systemic administration.

Electroporation

Electroporation is useful for gene transfer to primary cells. Primary cells are not introduced with genes with lipofection. Electroporation is the only method to introduce genes to cells, auch as normal human dermal fibroblasts[10]. Electroporation, in which high-voltage electrical currents create transient pores on the cell membranes, allows the transport of nucleic acid material into the cell[11]. Therapeutic genes are successfully transferred *in vivo*[12]. The electroporation method, however, has limitations such as short range of gene transfer, necessity of a surgical procedure, and tissue damage. Distance between electrodes normally requires 1 cm and is not suitable for a large area. Placement of internal electrodes requires a surgical procedure, and high voltage can damage tissue when applied. Therefore, less invasive methods are desirable.

Lipofection

Chemical non-viral vectors have been studied because they are generally considered safer than viral vectors. In addition to safety, liposomes can transfer larger genes with less toxicity and are relatively easy to prepare. Cationic lipids and polymers form complexes with negatively charged DNA. The complexes protect the DNA from nucleases and increase the effectiveness of transfection through the cell membrane. One major problem with lipofection is low efficiency of gene transfer. Transfection efficiency of lipofection is improved by condensing DNA with a double chain monovalent quaternary ammonium lipid[13]. Still genes are not introduced to target cells efficiently. More efficient methods have been waited.

Sonoporation

Sonoporation refers to the formation of small pores in cell membranes by using ultrasound for the transfer of nucleic acid materials (Figure 1). The biological effects of ultrasound are categorized as thermal and non-thermal. Non-thermal effects are composed of mechanical perturbation in the vicinity of bubbles. Cavitation bubbles cause membrane poration[14]. High speed camera images reveal that the cell membrane is fractionated, and cavitation bubbles are formed[15]. The cavitation bubbles induce cell death or permeability to allow the entry of a drug or genes into the cells. Sonoporation is similar to electroporation, wherein DNA is driven by an electrical force along the electric field. Sonoporation is mediated by passive diffusion. The transfer efficiency depends on ultrasound frequency and intensity[16]. The major advantages of sonoporation are its non-invasiveness and ability to transfer genes to internal organs without a surgical procedure[17]. Targeted gene transfer can be facilitated by ultrasound irradiation of selected tissues after systemic administration[18].

SONOPORATION

Emergence of sonoporation

Drug delivery with ultrasound was first reported by Tachibana *et al*[16]. The delivery of insulin on the skin surface when exposed to ultrasound energy in the range of 3000-5000 Pa or 5000-8000 Pa at 48 kHz for 5 min decreased blood glucose levels to 22.4% of the control

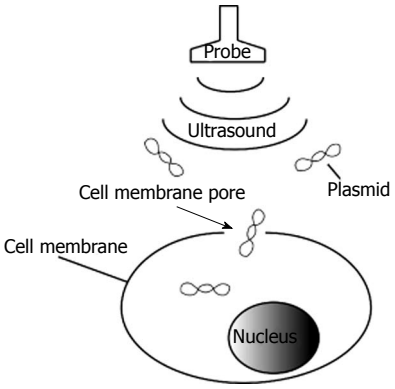


Figure 1 Formation of cell membrane pores after ultrasound irradiation. Nucleic acid such as plasmids enters the cells through the membrane pores that are formed with ultrasound.

in 120 min. It was postulated that insulin absorption increased with ultrasound vibration after intradermal injection. This report is interesting because it demonstrates the potential of ultrasound as a method to improve absorption of therapeutic materials. With ultrasound, chemotherapeutic agents are more efficiently absorbed in the mouse xenograft model of cancer[19].

In vitro experiments are simplified models to investigate the mechanisms of sonoporation *in vivo*. Fechheimer *et al*[20] transferred plasmid DNA encoding the G418 resistance gene to cultured mouse fibroblasts by sonoporation. Colonies were observed after G418 was added to the media. This was the first report demonstrating that plasmid DNA can be transferred to cells *in vitro* by using ultrasound. Kim *et al*[21] also reported that rat joint cells can be successfully transfected with plasmid DNA. Plasmid DNA encoding green fluorescent protein (GFP) was injected as a reporter of expression into the left ventricle of mice percutaneously[22]. Mice were irradiated with transthoracic ultrasound at 1 MHz for 1 min. Histological examination showed GFP expression in the subendocardial myocardium. Intraventricular co-injection of siRNA and GFP exhibited reduced expression of GFP in the coronary artery. These data indicate that plasmid DNA and siRNA can be introduced into cells *in vivo* by sonoporation.

Mechanism of sonoporation

Biophysical effects of ultrasound include cavitation, radiation pressure, and microstreaming[23]. Cavitation refers to the growth and collapse of microbubbles. Radiation pressure is the force in the irradiation field. Microstreaming is the shear forces that exist near the microbubbles. Formation of cavitation increases with a rising ultrasound intensity while the frequency decreases[24]. Mechanical index (MI) is defined as PRP/$\sqrt{F0}$ (PRP: peak rarefactional pressure; $\sqrt{F0}$: transmission center frequency)[25]. MI should be lower than 1.9 for clinical use of ultrasound. Forsberg *et al*[26] reported that cavitation is unlikely to occur at an MI of less than 0.7. Cavitation has been well investigated with ultrasound contrast agents because microbubbles lower the threshold of cavitation[27]. Zhou *et al*[28] developed a device to observe the behavior of a single bubble near the cell membrane. The motion of a single bubble was monitored with a high-speed camera. Cell membrane disruption was assessed by monitoring the transmembrane current. This study showed that a single microbubble expands and contracts with ultrasound irradiation. When the bubble collapses, the cell membrane is ruptured and a pore is generated. Changes in membrane permeability are directly correlated with the formation of pores. Carugo *et al*[29] reported that a standing wave is involved in sonoporation in the absence of microbubbles.

Microbubbles

The presence of microbubbles reduces the threshold of cavitation. Microbubble contrast agents are spheres filled with gas and stabilized with shells. Their size ranges between 1 and 10 μm[30]. They are small enough to circulate in blood vessels, but do not exit from the vessels. The contrast agents scatter ultrasound stronger than the surrounding blood and tissue. Thus, they are used as contrast agents for daily clinical practice. Tachibana *et al*[31] reported that albumin microbubbles (Albumex) accelerate thrombolysis by ultrasound. This was the first report that microbubbles improve the effects of ultrasound in tissues for purposes other than diagnostic imaging. Transfection of plasmid DNA into rat joint cells was improved in the presence of Albumex[21]. This report paved the way for the utilization of ultrasound contrast agents in sonoporation. Microbubbles expand and contract in response to compression and rarefaction of ultrasound (Figure 2). The microbubbles collapse at the high-pressure phase, emitting shock waves that perturb the cell membrane and increase permeability. Qiu *et al*[32] reported that pores on the cell membrane, generated by sonoporation with microbubbles, ranged from 100 nm to 1.25 μm in size. Their experiments used 1 MHz ultrasound at low acoustic pressures from 0.05 to 0.3 MPa. The pores generated with sonoporation were examined by scanning electron microscopy. The size of the pores enlarged with increased acoustic pressure or longer treatment. They concluded that the pores formed with shear stress. Liquid microjet, visualized with a high-speed camera in cultured cells exposed to single-shot short-pulsed ultrasound has been demonstrated[33]. Contrast agents are shells containing gas. Ultrasound scatters on the surface of contrast agents, and are visible as high echo on the display of diagnostic ultrasound. Physical and biological characteristics of contrast agents are basically the same as those of microbubbles. When a contrast agent (Levovist) was added to the media, the jet caused cell membrane damage. Interestingly, the cell membrane repair process suggests that the Ca^{2+}-independent and Ca^{2+}-trigger mechanisms are involved in rapid resealing. Positively charged microbubbles are more efficient in gene transfer than neutral ones[34]. It is hypothesized that positively charged microbubbles are more close to the cell membrane to enable more efficient gene transfer. Plasmids are

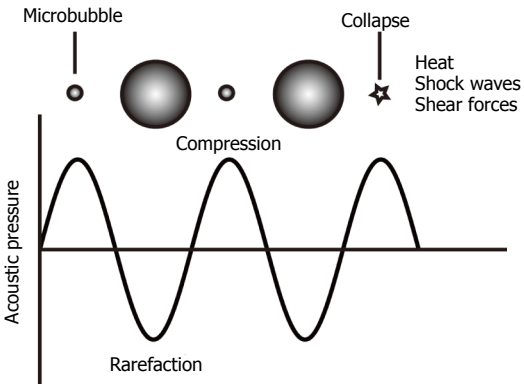


Figure 2 Microbubble response to an ultrasonic pressure wave. Microbubbles expand and contract when exposed to ultrasound at rarefaction and compression, respectively. At high pressure, microbubbles collapse and a shock wave is emitted.

introduced into cells *in vitro* and *in vivo* with sonoporation with microbubbles more efficiently than liposome that is commonly used for transfection[35,36].

Clinical trials

To date, no clinical trials have been reported using sonoporation. There are several reasons for this, including low transfer efficiency and difficulty in monitoring irradiated fields using sonoporators.

Limitations

Sonoporation harbors complicated aspects. Gene transfer efficiency is low with sonoporation. 37.5-50 μg of plasmid should be applied to a single rat with sonoporation *in vivo*[34]. This limitation is one of the reasons that sonoporation has not been applied clinically as mentioned above. Another limitation is cell damage caused with sonoporation. Miller *et al*[37] irradiated cells with 2.25 MHz continuous ultrasound for 1 min. When an ultrasound contrast agent (Definity, a perflutren lipid microsphere injectable suspension; Bristol Myers Squibb Medical Imaging, N. Billerica, MA) was added to the culture media, the irradiated cells underwent apoptosis. Enzymatic activity and mitochondrial membrane are changed after sonoporation[38]. Stresses to endoplasmic reticulum and mitochondria trigger apoptosis[39,40]. Sonoporation delays DNA synthesis to arrest cell cycle[41]. It should be noted that sonoporation itself may cause apoptosis when it is applied to cancer with therapeutic genes[37].

FUTURE PROSPECTS

Microbubbles are expected to improve gene transfer at lower MI for the safety of patients; therefore, microbubbles may be suitable for sonoporation in human trials. Microbubbles are destroyed more efficiently with decreased pulse frequency and increased acoustic pressure and pulse length[42]. Care should be taken when applying *in vitro* data to *in vivo* studies because the effects of MI on microbubble destruction might not be similar[43]. Standing wave may be another option to improve sonoporation. Real-time monitoring of the irradiated field is desirable to introduce therapeutic genes to target tissues. Diagnostic ultrasound could therefore be used as a sonoporator.

Cationic microbubbles

Wang *et al*[27] analyzed the protection of plasmid DNA xenograft tumors in mouse hind limb after intravenous administration of a complex of plasmid DNA (luciferase) with cationic or neutral microbubbles. Luciferase activity was 3.8-fold stronger in complexes with cationic microbubbles than in those with neutral microbubbles. Factors influencing the transfection efficiency were analyzed, and the superiority of cationic microbubbles was more evident at lower doses of microbubbles and plasmid DNA[34]. This strategy appears suitable for this application because nucleic acids are negatively charged. It is expected that plasmid DNA or siRNA will interact and form better complexes with cationic microbubbles than with neutral microbubbles.

Liposomal bubbles

It is difficult to modify surfaces of microbubbles with functional molecules for targeting. On the other hand, liposomes are easy to modify for targeting. Suzuki *et al*[44] developed polyethylene glycol-modified liposomes containing perfluoropropane, a contrast agent for ultrasound imaging. These so-called "bubble liposomes", significantly improved the transfection efficiency of plasmid DNA encoding luciferase into cultured cells. The luciferase reporter plasmid was injected into the femoral artery of mice. Two days after ultrasound irradiation, a signal was detected along the artery. These data suggest that the bubble liposome is a candidate for gene delivery both *in vitro* and *in vivo*. Bubble liposomes were transferred with the interleukin-12 gene in xenograft ovarian cancer mice, and the tumor sizes were reduced[45]. These animal experiments demonstrate that bubble liposomes may be applicable to clinical studies.

Diagnostic ultrasound

Sonoporators are typically used for sonoporation. One of the problems with sonoporators is that the irradiation field cannot be monitored. Diagnostic ultrasound is widely used in daily clinical practice. Diagnostic ultrasound is equipped with a display for image diagnosis. If diagnostic ultrasound could be utilized for sonoporation, therapeutic genes can be accurately introduced into the target fields. Miller and Quddus transfected cultured cells with plasmid DNA using diagnostic ultrasound[46]. They used a 3.5 MHz curved linear transducer of diagnostic ultrasound. A contrast agent of ultrasound (Optison) was added to the media. Although Optison may improve transfection efficiency, even without contrast agents, plasmid DNA was successfully introduced into cultured cells by using diagnostic ultrasound[47]. SiRNA of frizzled (Fz)-9, a receptor of the Wnt signaling pathway, transferred into cultured cells using diagnostic ultrasound sup-

presses cell proliferation[48]. SiRNA of Fz-9 transferred into cultured cells using lipofection also suppresses cell proliferation[49]. These 2 papers indicate that transfection efficiency of siRNA with diagnostic ultrasound is comparable with that of lipofection. Wang *et al*[27] investigated cell permeability of Evans Blue *in vivo* with diagnostic ultrasound. They observed the dye in xenograft hepatoma. Interestingly, they reported that efficiency of Evans Blue transfer was affected by MI sonication duration and dye dose. These data clearly demonstrate that ultrasound causes sonoporation, a biological process that is a promising new approach for the delivery of DNA/RNA for gene therapy.

CONCLUSION

Sonoporation is able to introduce plasmids to cells. Sonoporation is less toxic method of gene transfer as compared with retro viral vectors and adenoviral vectors because plasmids hardly causes immune response and are not associated with tumorigenicity. Sonoporation does not require surgical procedure and enhances gene transfer with lipofection. Current limitations of sonoporation are low efficiency of gene transfer and damage of target cells are the use of complexes with chemicals and diagnostic ultrasound are promising approaches to overcome these limitations.

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