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REVIEW

Advances in ultrasound-targeted microbubble-mediated gene therapy for liver fibrosis

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Abstract Hepatic fibrosis develops as a wound-healing scar in response to acute and chronic liver inflammation and can lead to cirrhosis in patients with chronic hepatitis B and C. The condition arises due to increased synthesis and reduced degradation of extracellular matrix (ECM) and is a common pathological sequela of chronic liver disease. Excessive deposition of ECM in the liver causes liver dysfunction, ascites, and eventually upper gastrointestinal bleeding as well as a series of complications. However, fibrosis can be reversed before developing into cirrhosis and has thus been the subject of extensive researches particularly at the gene level. Currently, therapeutic genes are imported into the damaged liver to delay or prevent the development of liver fibrosis by regulating the expression of exogenous genes. One technique of gene delivery uses ultrasound targeting of microbubbles combined with therapeutic genes where the time and intensity of the ultrasound can control the release process. Ultrasound irradiation of microbubbles in the vicinity of cells changes the permeability of the cell membrane by its cavitation effect and enhances gene transfection. In this paper, recent progress in the field is reviewed with emphasis on the following aspects: the types of ultrasound microbubbles, the construction of an ultrasound-mediated gene delivery system, the mechanism of ultrasound microbubble–mediated gene transfer and the application of ultrasound microbubbles in the treatment of liver fibrosis.

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1. Introduction

Liver fibrosis is caused by excessive deposition of fibrous tissue in the liver resulting from increased synthesis and decreased degradation of extracellular matrix (ECM). If the disease progresses, it can lead to liver cirrhosis, a condition in which the liver is severely scarred, its blood flow restricted and its ability to function impaired. Treatment to alleviate and reverse the process mainly involves the use of hepatocyte protective agents, immunotherapy, and hepatic stellate cell activation inhibitors. Patients infected with hepatitis B, C or other replicating viruses should also be treated with antiviral drugs. Although antifibrotic drugs are known to attenuate experimental liver fibrosis in animals, they remain to be tested in humans.

With the continuing development of gene therapy, it is now possible to import foreign genes into injured tissue or cells to treat disease. Based on our current understanding of the aetiology of hepatic fibrosis, the gene therapy approach includes inhibiting the activation of hepatic stellate cells (HSC), promoting the degradation of ECM, inhibiting inflammation and promoting the proliferation of liver cells. In particular, specific cellular factors can be regulated at the gene level to prevent the pathological progress of liver fibrosis. These factors include hepatocyte growth factor (HGF), matrix metalloproteinases (MMPs), epidermal growth factor (EGF), connective tissue growth factor (CTGF), inhibitors of MMP (TIMP), the augmenter of liver regeneration (ALR), transforming growth factor β (TGF-β), interleukin (IL), and keratinocyte growth factor (KGF).

Gene therapy mainly involves the use of viral and non-viral vectors. Viral vectors have high transfection efficiency and can be stably expressed. However, their use is restricted due to their immunogenicity and potential mutagenicity. Non-viral vectors, such as liposomes, are much safer in humans but limited by low transfection efficiency, short expression time and poor targeting. Accordingly, an important focus of gene therapy is to produce a safe and efficient non-viral gene delivery system with the potential to dramatically improve targeting and control release. Ultrasound-mediated release of genes encapsulated in microbubbles (so-called ultrasound microbubbles) is such a system that potentially allows controlled site-specific delivery of therapeutic agents with minimal off-target effects.

Ultrasound is a mechanical vibration with a frequency in excess of 20 kHz that can be used to drive delivery into tissues. It has the advantage of being non-invasive and can be focused into a beam with strong tissue penetration. Microbubbles are small bubbles (diameter < 50 μm) composed of a non-toxic, water-insoluble gas encapsulated with a thin layer of biocompatible material. They have been widely used as ultrasound contrast agents[1,2] but, in recent years, have been evaluated as a new type of drug delivery system. This is based on the fact that ultrasound irradiation of tissue in the presence of microbubbles leads to microbubble oscillation (known as stable cavitation) which generates fluid flow (microflow) in the vicinity of cell membranes sufficient to cause sonoporation and entry of extracellular molecules and particles into the cell. Overall, ultrasound targeted microbubbles have been used to deliver DNA, siRNA[3] and drugs[4] to liver, brain, kidney and other organs for treatment of cancer and cardiovascular disease. This review covers their potential use in the treatment of liver fibrosis.

2. Types of ultrasound microbubbles

Microbubbles have been extensively studied in combination with ultrasound because of their high transfection efficiency and low propensity to cause damage to cells and tissues. The microbubble shell and the bubble gas have a significant impact on drug loading and release and have been the subject of extensive research.

The microbubble shell not only determines the stability of microbubbles but is also important in preventing their identification by the reticuloendothelial system. Shell materials commonly used are albumin, lipid and polymer. Microbubbles with an albumin shell such as Optison were the first to be developed but are vulnerable to temperature-induced degeneration. Microbubbles with a lipid shell have better response to ultrasound (better acoustic properties) and are easy to link with targeting ligands but have relatively poor stability[5]. Microbubbles with a shell made of high molecular weight polymers such as polyethylene glycol (PEG), polylactic acid and poly(lactic-*co*-glycolic acid) (PLGA), have good stability and biocompatibility but poor acoustic properties. Their hydrophilic chains avoid identification by the reticuloendothelial system thereby prolonging their half-life *in vivo*[6].

Changes to the microbubble shell affect the flow characteristics and distribution of microbubbles *in vivo*[7] and thereby affect the transfection efficiency of the gene payload. Sirsi et al.[8] showed that cationic microbubbles in which the lipid shell was modified with polyethyleneimine (PEI) significantly prolonged the half-life *in vivo* due to reduced binding in the blood vessels. Similarly, modification to the microbubble surface using conjugating ligands can change the properties of the surface and the targeting of the microbubbles[9–12]. For example, hepatocytes are able to combine with galactose groups such that phospholipid-coated microbubbles ith surface galactose groups show improved targeting to liver[13].

Microbubbles were first produced containing air but they suffered from poor stability and short half-life. Subsequently fluorocarbon gases (C_3F_8, C_4F_{10} and C_5F_{12}) and sulfur hexafluoride were found to be superior. The former has low dispersion, low blood solubility and can be discharged through the lungs eliminating any potential toxicity. Sulfur hexafluoride microbubbles are not only significantly more stable in the systemic circulation but also have enhanced acoustic response[5].

Ultrasound microbubble delivery systems developed to date are shown in Table 1. Albunex and Optison have been approved by

Table 1 The development of ultrasound microbubbles.

Type	Representative drug	Characteristic
First generation	Echovist	Free gas (air or oxygen); without coating; poor stability; larger diameter, cannot enter pulmonary circulation
Second generation	Albunex, Levovist	Free gas (air or oxygen); microbubble shell (albumin, lipid and polymer); good stability; small size (<8 μm), can enter pulmonary circulation; short duration of enhancement (1–5 min)
Third generation	Sonovue, Optison	Fluorocarbon gas and microbubble shell (albumin, lipid and polymer); long duration of enhancement (15 min); stability can be further improved; low dispersion; low solubility

the US Food and Drug Administration (FDA) and Echovist and Levovist have been approved by the European Medicines Agency and applied in clinical treatment. The size of microbubbles affects their circulation half-life and ability to deliver genes to tissue. Tung et al.[14] demonstrated that the blood–brain barrier can be transiently opened using microbubbles with a diameter in the range 1–8 μm. The deposition of circulating fluorescent dextrans in the hippocampus could be achieved by stable cavitation of larger microbubbles (>4 μm), whereas smaller microbubbles (1–2 μm) required higher pressures.

3. Ultrasound microbubble–mediated gene delivery system

3.1. Construction

The aim of gene therapy using an ultrasound microbubble–mediated delivery system is to simultaneously inject microbubbles and genes into the systemic circulation and irradiate the target site with ultrasound to facilitate the entry of naked DNA or gene vectors into tissue. Methods for combining genes and microbubbles include forming a simple physical mixture, intercalating or connecting the gene to the microbubble surface, and binding of the gene to the microbubble surface through either electrostatic or chemical binding. These methods are described in detail in the remainder of this section.

3.1.1. Simple physical mixture

Microbubbles mixed with plasmids or naked DNA enhance their transfection efficiency by reducing their degradation by nucleic acid enzymes *in vivo*[15]. The most frequently used gene vectors are plasmids of bacteria which have the advantages of versatility and ease of production[16]. Kopechek et al.[17] successfully combined a cationic lipid microbubble with the gene of the signal transducer and activator of transcription, *STAT3*. After modifying the gene with ethylene glycol, they mixed the nucleic acid with the microbubble in phosphatidylcholine solution and tested the mixture in *in vitro* and *in vivo* experiments. The former showed that the microbubbles inhibited the STAT3 signaling pathway in SCC cells and the latter demonstrated that they inhibited tumor growth in mice.

3.1.2. Intercalating or connecting to the microbubble surface

Although highly convenient to prepare, simple mixtures do not reliably deliver genes and microbubbles to an *in vivo* target site simultaneously, thereby reducing the transfection rate especially in tissues with low blood supply such as muscle. Therefore, many novel approaches have been designed including some physical methods in which genes are wrapped into microbubbles or connected to their surface. In many of these methods, non-viral vectors and high molecular weight polymers have played important roles in prolonging circulation time and promoting gene transfer *in vivo*[18]. In particular, microbubbles connected to non-viral vectors have been shown to significantly increase transfection efficiency after injection into the body[19]. This approach has been applied to a combination of ultrasound microbubbles and cell-penetrating peptides. Thus Zhou et al.[20] combined cell-penetrating peptides and DNA encoding hepatocyte growth factor (*HGF*) in a liposome suspension and vibrated quickly in perfluoropropane gas to obtain microbubbles with both components on their surface. *In vivo* and *in vitro* experiments revealed that the microbubbles produced a satisfactory transfection rate.

3.1.3. Electrostatic binding

Because nucleic acids are negatively charged, they interact well with lipid-shell microbubbles carrying positive charges to form a stable gene delivery system[21–23]. Un et al.[12] developed microbubbles to which green fluorescent protein (GFP) and the gene of a fluorescent pigment enzyme were attached in this way. Modification of the microbubbles with mannitol enabled them to reach the target site specifically.

3.1.4. Covalent binding

Physical methods, such as intercalation and electrostatic adsorption, may affect the structure or stability of genes during microbubble preparation. In this case, specific affinity reaction or covalent connection can be employed. Sirsi et al.[8] modified the gene vector, branched PEI, with PEG and then thiolated it to allow covalent binding between the sulfhydryl group and maleimide on lipid-shelled microbubbles. They then introduced these PEI-microbubbles into tumors implanted in mouse kidney and examined the fluorescence of tumor tissue using *in vitro* ultrasonography. The results showed that the intensity of fluorescence was 10-fold higher in tumor tissue than in tissue without treatment. The authors concluded that this composite microbubble not only improved and controlled drug loading but was also very suitable for ultrasound-mediated tissue transfection.

3.2. Mechanism of ultrasound microbubble–mediated gene delivery

The diameter of acoustically responsive microbubbles is generally in the range 1–10 μm. The smaller microbubbles easily enter blood capillaries whereas the larger ones readily overflow into the surrounding tissue. Therefore, the mechanism of ultrasound microbubble–mediated gene delivery depends on the effect of ultrasound-mediated cavitation of the microbubbles on the vascular wall and vascular endothelial cells. Cavitation can increase vascular permeability and facilitate entry of accompanying genes or promote entry of the microbubbles into tissue cells in the ultrasound irradiated area. Currently, it is believed that cavitation modulates vascular permeability mainly through sonoporation, reducing the integrity of vascular endothelial cells and stimulating absorption by internal cells (Fig. 1).

Sonoporation is the process by which microbubbles oscillate and rupture in an acoustic cavitation field to form microflows, jets and shock waves which disrupt the cell wall of the surrounding tissue and plasma membrane to produce reversible or irreversible holes (or pores). The process has been considered to be the main

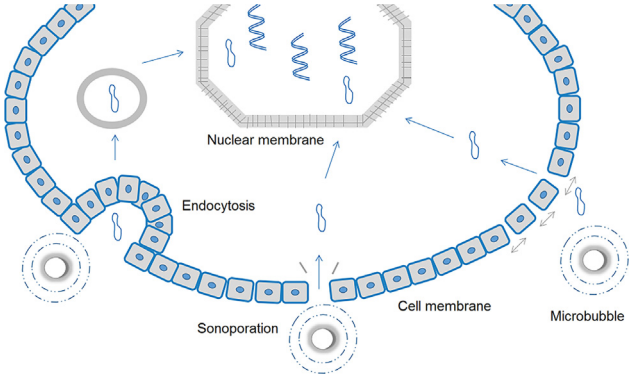

Figure 1 Mechanisms of ultrasound microbubble–mediated gene delivery.

mechanism of drug or gene delivery[24]. In studying the absorption of nanoparticles by cells using scanning electron microscopy, optical microscopy and electrophysiological phenomena, the small holes proved to be transitory[25,26]. Modulation of the integrity of vascular endothelial cells is the result of transient changes in the volume of blood vessel endothelial cells and opening of cell gap junctions due to the oscillation of microbubbles. This results in the overflow of microbubbles from blood vessels into the surrounding tissue. Chen et al.[27] observed the effect of cavitation of microbubbles on blood vessels using high speed photography and confirmed the importance of the expansion of microbubble volume on their interaction with blood vessels. Stimulation of absorption by internal cells is also the result of oscillating microbubbles on cell membranes. In this case, cavitation leads to changes in the membrane potential of cells which subsequently affects intracellular pathways and stimulates endocytosis[26]. Meijering et al.[28] showed that the endocytotic activity of cultured cells increased in the presence of low-intensity ultrasound and microbubbles.

3.3. *Ultrasound microbubble–mediated gene transfection in vitro*

Hepatocyte growth factor (HGF) is a cell growth factor with antifibrotic activity through inducing apoptosis of activated hepatic stellate cells, reducing excessive collagen deposition and stimulating the regeneration of liver cells. It also regulates the inflammatory response and the synthesis of collagen and is a common cytokine for gene therapy of liver fibrosis[29]. Li et al.[30] prepared lipid microbubble–cationic nanoliposomes (LMB-CNLP) carrying HGF and studied their influence on the hepatic stellate cell line HSC-T6. After 24 h of transfection, cells treated with LMB-CNLP showed greater green fluorescence than other groups indicating they were more successfully transfected with HGF than other cells. Control HSC-T6 cells were polygonous while cells treated with LMB and LMB-CNLP were quasi-circular due to changes in their biological activity subsequent to the synthesis of HGF protein after transfection. The authors concluded that LMB-CNLP overcame the general disadvantages of carrying a limited amount of gene and inadequate targeting to increase the transfection of HGF under ultrasound and promote apoptosis of HSC-T6 cells, thus providing a foundation for gene therapy of liver fibrosis.

As stated earlier, hepatic fibrosis can lead to cirrhosis and ultimately to hepatocellular carcinoma. One way to slow disease progression involves using the antisense oligodeoxynucleotide of c-myc (c-myc ASODN) to inhibit cell proliferation by interfering with the transcription of the c-myc gene. Jing et al.[13] provided proof-of-concept of this potential therapy by showing that the proliferation of hepatocellular carcinoma cells was inhibited by transfection of c-myc ASODN. It was found that the transfection rate of c-myc ASODN and suppression level of the c-myc gene were higher using microbubbles modified with biotinylated galactosylated poly-L-lysine and SonoVue. It was also found that liver cells with asialoglycoprotein receptors (ASGP-Rs) on their surface which specifically recognize and combine with galactose groups on the ends of the glycoproteins were specifically targeted and gave enhanced gene transfection efficiency.

Neuroepithelial transforming protein 1 (NET-1) is a member of the NET-x family which is associated with signal transduction, cell adhesion, migration, proliferation and differentiation. Chen et al.[31] found that NET-1 was highly expressed in hepatocellular carcinoma (HCC) tissues but poorly expressed in peripheral tissues of HCC. Therefore, it is possible to use NET-1 siRNA transfection to reduce the development of HCC. Han et al.[32] constructed ultrasound microbubbles carrying NET-1 siRNA (labeled fluorescent) and injected them into human HCC cells (HepG2). After irradiation with ultrasound for 48 and 72 h, proliferation of the cells was inhibited by 32% and 54%, respectively. Expression of mRNA and protein was significantly reduced as shown by RT-PCR and Western blot. This was the first time that gene therapy of NET-1 siRNA combined with ultrasound microbubble technology achieved satisfactory results. It provides an excellent platform for future research *in vivo*.

3.4. *Ultrasound microbubble–mediated gene therapy for hepatic fibrosis in rats*

HGF can regulate the synthesis of ECM and the inflammatory response to inhibit the activation of HSC and prevent the development of liver fibrosis. In research by Wang et al.[33] to evaluate the efficacy of ultrasound microbubble delivery of HGF, a total of 40 Wistar rats were randomly divided into 5 groups, *viz* a model group (MA), pure plasmid group (HGF), plasmid+microbubble group (HGF+MB), ultrasound+plasmid group (HGF+US) and ultrasound microbubble + plasmid group (HGF+US/MB). The results showed that, after treatment, the expression of HGF in the HGF+US/MB group was significantly higher than in the other groups. In addition, livers removed from rats after sacrifice revealed that those from the HGF+US/MB group had complete lobule structures and, on HE staining, showed only small amounts of fibrous septum. This was in contrast to other groups where the proliferation of fibro-connective tissue was obvious especially in the MA group where fibrous septum and lobule structures were disordered. Interestingly, in those rats which developed liver fibrosis, the degree of liver damage and expression of type I collagen was greatest and lowest respectively in the HGF+US/MB group.

Zhang et al.[34] showed that biotinylated ultrasound microbubbles plus a biotinylated cationic nanoliposome composite (Bio-MB+Bio-CNLP) could provide effective gene transfer. Jiang et al.[35] also used histopathology to show that *HGF* gene therapy using ultrasound microbubbles reduced the degree of development of liver fibrosis. In particular, the expressions of type I collagen, collagen type III and α-smooth muscle actin (α-SMA) measured by immunohistochemical methods were significantly decreased in the ultrasound microbubble+plasmid group. When HGF and TGF-β were transferred into rats with hepatic fibrosis, the severity of hepatic fibrosis was reduced, the liver function was improved and the regeneration of liver cells was promoted[36].

Increase in ECM through increased synthesis or reduced degradation leads to its deposition in the liver and eventually to liver fibrosis. ECM is produced mainly in HSC whose activation, proliferation and apoptosis are subject to regulation by the tissue inhibitor of matrix metalloproteinase 1 (TIMP-1) which inhibits the activity of MMPs and hinders the degradation of ECM. Therefore, inhibiting *TIMP-1* gene expression in liver tissues promotes MMPs to degrade collagen and potentially prevent hepatic fibrosis. Tian et al.[37,38] constructed an eukaryotic expression vector of TIMP-1 siRNA which could fixed-point knock-out the *TIMP-1* gene in HSC-T6 cells. Combined with microbubbles, the transfection rate and targeting of TIMP-1 siRNA in rats increased and the activity of MMPs was improved. The results showed that the contents of TIMP-1, hyaluronic acid (HA),

laminin protein (LN), type I collagen and type III collagen in rats in the TIMP-1 siRNA+US/MB group were significantly lower than in other groups while MMP-1 expression was increased significantly. *In situ* hybridization, immunohistochemical staining and Masson staining showed that the degree of fibrosis was reduced, and the disordered lobular structure conspicuously improved in the TIMP-1 siRNA + US/MB group compared with the control group.

Transforming growth factor β1 (TGF-β1) is the strongest cytokine promoting liver fibrosis. It can regulate the conversion of HSCs directly or indirectly but may exert serious side effects if completely blocked due to the complexity of its effects. Connective tissue growth factor (CTGF) is the downstream mediator of TGF-β1 and acts to promote hepatic fibrosis directly or jointly as a cofactor of TGF-β1. However, it has been shown that using siRNA to inhibit the expression of CTGF can be used as a new model of gene therapy for liver fibrosis. Studies have also shown that artificial microRNA is more effective and less toxic to cells compared with siRNA and shRNA[39]. A new type of ultrasound microbubble carrying artificial microRNA was prepared by Yang et al.[40]. In their study, targeted ultrasound microbubbles were constructed by coupling microRNA with a cationic lipid. The results showed that ultrasound microbubble–mediated microRNA could reach rat liver effectively to reduce hepatic fibrosis and significantly reduce the protein expression of TGF and *CTGF* mRNA. The expression of type I collagen and α-SMA were also reduced indicating the treatment has the effect of mitigating liver fibrosis.

Even when liver fibrosis has developed into liver cancer, gene therapy with ultrasound-mediated microbubbles can still achieve good results. Suicide gene therapy has been widely used in treating liver cancer with good prognosis. Treatment with herpes l simplex virus-thymidine kinase (HSV-TK) is the most widely used such therapy. The *tk* gene can be expressed in tumor cells to produce TK protein and interfere with DNA synthesis in cell division leading to cell death[41]. After administering a combination of ultrasound microbubbles and HSV-*tk* gene plasmid to the tissues of rats bearing liver cancer, TK protein expression was greatest and tumor inhibition significantly higher in the HSV-TK+US/MB group than in other groups[42]. This again has the potential to provide a new method of gene therapy for the treatment of liver cancer.

4. Conclusions

Generally, ultrasound-targeted microbubble-mediated gene therapy can improve gene transfection efficiency in liver tissue to inhibit or reverse the progression of liver fibrosis and prevent cirrhosis. It can also significantly improve the clinical symptoms due to its high efficacy, specific targeting and low immunogenicity. However, the technology still faces many challenges. These include: (1) constructing gene vectors with good safety, high efficiency and tissue specificity; (2) effectively combining microbubbles with target genes; (3) understanding the relationship between the dosage of ultrasound, the concentration of microbubbles, and the extent of tissue damage; (4) achieving stable expression of target genes after transcription; and (5) optimizing ultrasound parameters (frequency, sound pressure and interaction time), the type of microbubble and its concentration.

Ultrasound targeted gene therapy is a new, safe and efficient gene transfer technology which is different from the commonly used viral vectors and liposomes. It can promote the site-specific transfection and expression of naked plasmid DNA in the cell with no immune response and no mutation of the virus vectors. Ultrasound microbubble–mediated gene delivery has great potential in gene therapy generally and in the treatment of liver fibrosis in particular.

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