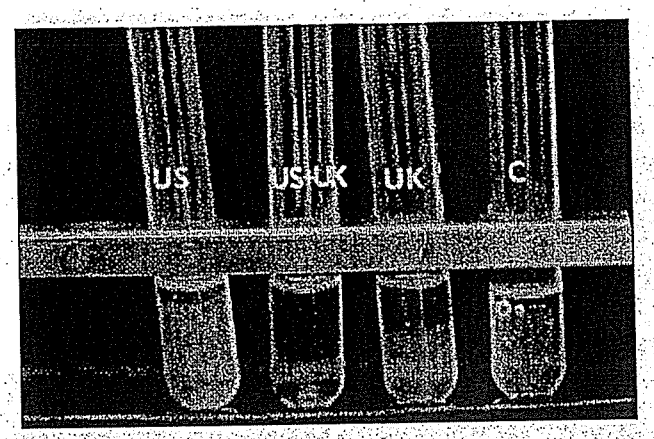

Fig. 2. Early experiments show evidence of ultrasound accelerated thrombolysis. Arificial thrombus (white area) was produced in test tubes. Each test tube was irradiated by ultrasound (50 kHz, 2 W/cm^2, 5 min) with or without thromobolytic drug (urokinase; UK). All test tubes were incubated (37°C) for 4 hours. Far right: control, no US, no UK; second from right: UK, no US; far left: US, no UK.

clots and lytic agents. The clots added with drug and treated with ultrasound showed acceleration of lysis, compared with the control. Tachibana *et al.* (1981) first introduced the concept of the therapeutic ultrasound device, of which this phenomenon could be used clinically for stroke patients (Fig. 3). Ultrasound could either be applied from outside the skull or with a miniature ultrasound emitting catheter from within. Catheters for mere drug delivery were just beginning to be used in coronary arteries for thrombolysis at that time.

One of the successful forms of DDS first introduced commercially in the 80s was the delivery of systemic drugs through the skin. The merit of transdermal delivery is that drugs can be administered systemically without interference of hepatic first pass metabolism and also at stabilized dose levels. Various types of drugs are currently available such as nicotine or estrogen patches that could be placed on the skin. However, these are among the few because of the low skin permeability to relatively large molecules such as proteins. This low permeability is mainly attributable to the stratum corneum, the outermost skin layer. The stratum corneum is effectively a 10–15 μm thick matrix of dehydrated, dead keratinocytes embedded in a lipid matrix. Once the drug crosses the stratum corneum, the next epidermal

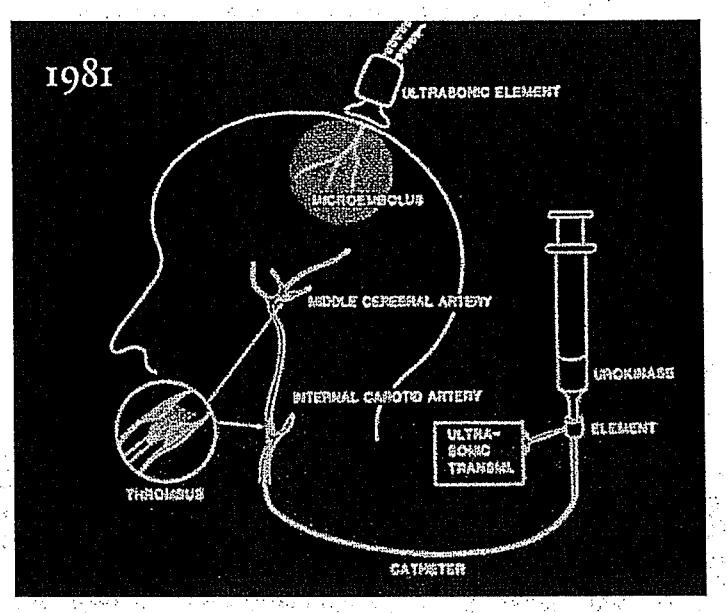

Fig. 3. Early concept drawing of stroke therapy by ultrasound (provided by Shunro Tachibana). Ultrasound was either applied extracorporeally through the skull or by means of miniature ultrasound catheter via the artery. Notice that the catheter also shows ultrasound being generated outside the body and propagating through the tube.

layer is less problematic to traverse, and consequently, the drug can reach the capillary bed to be absorbed. For protein and peptide drugs, the transdermal route has the potential to be an extremely efficient delivery site.

The clinically significant protein in the current delivery literature is that of insulin for controlling the blood glucose level in diabetic patients. Tachibana *et al.* (1991) and Kost *et al.* (1990) in the early 90s first introduced the use of ultrasound for the purpose of delivering insulin through the skin. Transdermal insulin delivery with low-frequency (<1 MHz) ultrasound condition was reported. Diabetic rat and rabbit experiments suggested a very rapid penetration of insulin through the skin, resulting in the reduction of blood glucose concentration. It has been postulated that ultrasound alters the stratum corneum of the skin, which functions as a barrier for most drugs. The disrupted skin surface may contribute to the increased absorption of drug by ultrasound. Acoustic cavitation, which is the

production and collapse of countless microbubbles induced by ultrasound, may contribute to increased drug penetration during ultrasound treatment of the skin. Theoretical and experimental results suggested that drug penetration occurred through cavitation-induced keratinocyte intercellular lipid bilayer disordering and depended on the chemical nature of the permeant. Scanning electron microscopic study of the surface of hairless mouse skin revealed that ultrasound energy disrupted the outermost layer of the stratum corneum and induced large, deep crater like clefts where the superficial capillaries became visible. It is suspected that insulin was transported through these artificial openings at the surface of the skin, which induced enhancement of drug absorption into the blood stream. Several companies are now developing small electronic "wearable" ultrasound devices attached to the arm or wrist which may become available in the near future (See Chap. VII).

Genes, which are carried on chromosomes, are the basic physical and functional units of heredity. Genes are specific sequences of bases that encode information on how to produce proteins, fundamental for life functions and majority of cellular structures. Gene therapy is a technique for correcting defective genes responsible for disease development. Researchers are currently developing several approaches for correcting faulty genes to cure various diseases. A normal gene may be inserted into a nonspecific location within the genome to replace a nonfunctional gene or an abnormal gene could be replaced for a normal gene through homologous recombination. Additionally, such methods for regulation (the degree to which a gene is turned on or off) of a particular gene could be altered. The major drawbacks of gene therapy are the short-lived nature of gene expression. The therapeutic DNA introduced into target cells must remain functional and the cells containing the therapeutic DNA must be long-lived and stable to completely cure the patient. The immune response prevents repeated gene therapy. The current system of using viruses as the carrier DNA to the target lesion presents a variety of potential problems to the patient, toxicity, immune and inflammatory responses, and gene control are among them. Certain tissues such as muscle have been reported to take up and express non-viral carried naked plasmid DNA *in vivo*. In general, however, the level of transfection after direct injection of naked plasmid DNA is variable and low. Greenleaf *et al.* (1998) first approached these problems by way of using non-viral plasmid DNA in combination with ultrasound. It was postulated

that ultrasound would initiate the delivery of DNA through the celluar membrane to within the cells and would not produce irreversible damage to the cells simultaneously. Bao *et al.* (1997) further added microbubbles to increase the rate of DNA transfer. Ward *et al.* (1999) and Tachibana *et al.* (1999) had earlier theorized that liquid microjets induced in the event of collapse of microbubbles could be the mechanism by which DNA easily penetrates the cell membrane. Scanning electron microscopy and high-speed video imaging technologies have recently revealed images of collapsing microbubbles and ruptured cell membrane surface that supports this theory. Unger (2004) introduced the concept of "tailored made" microbubbles and nanobubbles that target specific tissue lesions to deliver drugs and DNA.

Today, the concept of applying ultrasound as a means for altering the pharmacokinetics in various tissues and drug permeability through cell membranes, has expanded into a whole new field ranging from gene therapy to anti cancer drugs. A new generation of microbubbles and nano-sized bubbles are under development specifically for the purpose of "target and deliver" drug treatment with non-thermal ultrasound.

3. Stroke Therapy

The Stroke is the third most common cause of death in the United States, ranked only after heart disease and cancer. Approximately 700,000 new cases are reported in the US annually and ~160,000 Americans die each year from stroke. Stroke is caused by an interruption of the flow of blood to the brain (*i.e.*, an ischemic stroke) or the rupture of blood vessels in the brain (*i.e.*, a hemorrhagic stroke), which in turn causes brain cells in the affected area to die. The thrombolytic agent, tissue plasminogen activator (t-PA), is the most effective FDA approved drug to treat ischemic stroke; however, most stroke patients are not treated with t-PA because it must be administered within three hours of a stroke to be effective. Even if administrated with this drug, in some cases, there is no response to the drug due to reasons unknown. Large scale clinical trials such as the Pro-urokinase in Acute Cerebral Thromboembolism (PROACT) study (Del Zoppo *et al.*, 1998) have been conducted. In general, higher dosages of lytics increases the treatment success rate but have also resulted in higher incidence of

side effects such as unwanted bleeding in the brain and the digestive system.

It is well known that the thrombus structure resembles a fibrin net, with considerable space between the fibrin and red cells. Transport of fibrinolytic drugs into the thrombus is an important determination of the clot lysis rate. However, in the early stages of thrombolytic therapy, only a fraction of therapeutically administered plasmingogen activators can penetrate into the clots by passive diffusion. Investigators have not yet determined the exact mechanisms by which ultrasound accelerates fibrinolysis. To date, it is theorized that non-thermal ultrasound changes the pharmacokinetics of the surface or within the thrombus during fibrinolysis. There have been observations of ultrasound-induced heating in the these experiments, however, researchers have found that temperature increase made little contribution to the enhancement of fibrinolysis. Furthermore, ultrasound itself does not seem to activate the fibrinolytic cascade. Blinc (1993) demonstrated that ultrasound energy did not accelerate the hydrolysis of a peptide substrate by rt-PA, and the rate of plasmin degradation of fibrinogen was not increased. Acceleration of fibrinolysis by ultrasound also required the presence of a fibrin gel and was seen with clots of whole blood, plasma, and purified fibrin. Kimura *et al.* (1994) confirmed the increase of clot lysis and the fibrin degradation product, D-dimer, after ultrasound exposure plus re-PA. These data support the theory that the acceleration of fibrinolysis by ultrasound is primarily due to enhancement of drug transport within the clots through non-thermal ultrasound related mechanisms. The most likely mechanism in which ultrasound provokes drug movement into the thrombus is acoustic cavitation, which can be defined as the formation and collapse of bubbles in liquids. Acoustic cavitation can generate high streaming velocity in liquid and it in turn can assist drug diffusion, especially at locations where acoustic impedance differs. Tachibana *et al.* (1995) reported further acceleration of fibrinolysis by ultrasound in the presence of albumin microbubbles around the clots. These microbubbles were originally used as diagnostic echo contrast agents, however, when they were exposed to more intense ultrasound in this case, it was postulated that this material served as a source for cavitation, thus resulting in more fibrinolysis.

Tachibana *et al.* (1992) demonstrated *in vitro* that relatively low-intensity ultrasound irradiation of clots in the presence of lytic agents can

reduce the amount of drug required by a factor of ten, and shorten the lysis duration to one-fifth of the original time. This phenomenon has also been confirmed by other researchers (Francis, 1992; Blinc, 1993). Although the minimum ultrasound intensity needed to induce acceleration of fibrinolysis is currently under discussion, recent reports have shown that ultrasound of mechanical index (MI) ranging from 0.1 to 1.0 can produce enhanced fibrinolytic effects. Nonthermal effects of ultrasound contribute to the penetration of drugs into the thrombus. Experiments under conditions where cavitation is more easily produced have resulted in further enhancement of thrombolysis. Increased thrombolysis may also be associated to the unidirectional motion of a fluid or drug known as acoustic streaming, which originates within close range of the ultrasound transducers. Researchers have studied the driving force of acoustic streaming of microparticles theoretically and experimentally in various fluid conditions. Another possible explanation for the increased thrombolysis may be the temporary effect of ultrasound on the thrombus itself. It was suggested that bubble formation, growth and collapse causes reversible alteration in the fibrin structure that may result in an increased flow of the drug into the thrombus.

Clinical application of this new therapeutic ultrasound method for producing thrombosis has already started since 2001. Clinical trials in Europe and in the USA have been reported using miniature ultrasound transducers at the tip of catheters that approach the clots via arterial vessels (MicroLysUS infusion catheter, EKOS Corp, USA). The lytic drug, urokinase, is released at the distal end of the catheter during ultrasound irradiation (Figs. 4 and 5). The major goal of the catheters used in this study was to apply ultrasound at shorter distances with smaller ultrasound probes, and at the same time minimize damage to the surrounding normal tissues. Mahon (2003) presented early experience with the MicroLysUS infusion catheter for acute embolic stroke treatment in North America. This study was designed to demonstrate the safety of the device and to determine if ultrasound accelerates thrombolysis and improves clinical outcomes. Fourteen patients aged 40–77 years with anterior- or posterior-circulation occlusion, presented with cerebral ischemia 3–13 hours after symptom onset. Patients were treated with the catheter and simultaneous intraarterial thrombolysis. Procedural and clinical information, including time to lysis, degree of recanalization, NIHSS score, and modified Rankin Scale (mRS) score was recorded both before

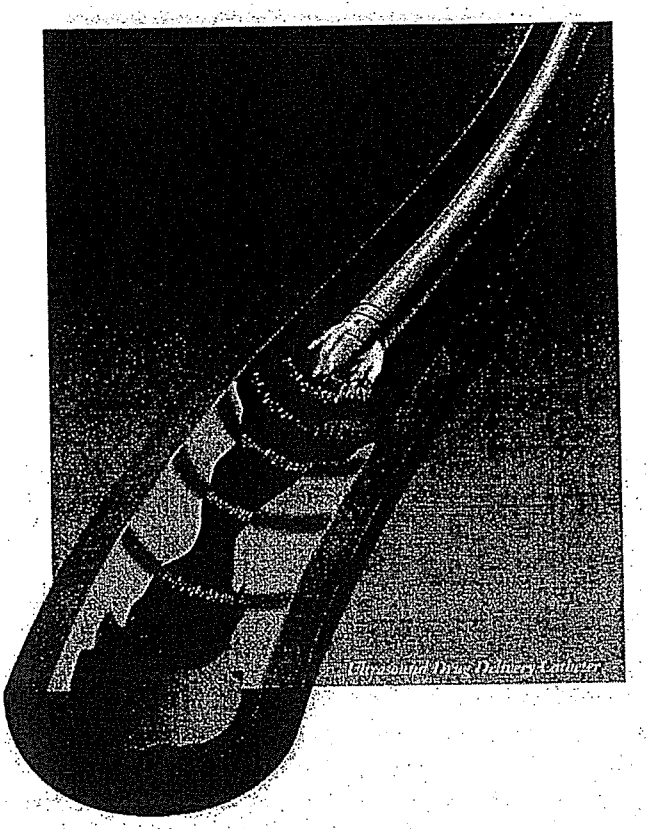

Fig. 4. Catheter type therapeutic ultrasound device that could be inserted into the artery. Miniature ultrasound generating element was attached to the very tip of catheter. Ultrasound and drug such as urokinase can be delivered at the same time near the target thrombus.

treatment and afterward. The numbers in the study were small, but the trends demonstrated that the rates of recanalization and neurologic outcomes in patients treated with the new ultrasound catheter were equivalent or slightly better than those in patients treated with standard microinfusion. The EKOS catheter for leg peripheral arterial thrombolysis was approved by the FDA in 2004 and will probably be the first drug/ultrasound combination product to be marketed in the cardiovascular field.

Ishibashi *et al.* (2002) have accepted the challenge of sonicating transcranially to accelerate thrombolysis. A noninvasive method was tested

Fig. 5. EKOS Corporation therapeutic device for strok therapy. A 2.5F (0.7 mm diameter) miniature catheter (MicroLysUS Infusion System) can be inserted deep inside the brain via the artery. Thrombolytic agents can be released at the tip of the catheter while applying ultrasound.

in an occlusion model of rabbit femoral artery, produced with thrombin after constriction of the artery led to stenotic flow and endothelial damage. After stable occlusion was confirmed, monteplase (mtPA) was administered intravenously, and ultrasound (490 kHz, 0.13 W/cm^2) was applied (TUS group). The ultrasound intensity was reduced from a higher value by passage of the ultrasound through a piece of temporal bone to simulate transcranial applications. The recanalization ratio in the TUS group was higher than that in the tPA group. Pfaffenberger *et al.* (2004) modified this experiment to determine if a 1.8-MHz commercial diagnostic ultrasound device would accelerate thrombolysis. Duplex-Doppler, continuous wave-Doppler, and pulsed wave (PW)-Doppler were compared on their impact on recombinant tissue plasminogen activator (rtPA) mediated thrombolysis. Blood clots were transtemporally sonicated in a human stroke model. Furthermore, ultrasound attenuation of 5 temporal bones of different thickness was determined. Results showed only PW-Doppler accelerated rtPA mediated thrombolysis significantly. Without attenuation by temporal bone, PW-Doppler plus rtPA showed a significant enhancement in relative clot weight loss. Measurements made when ultrasound was attenuated by

passage through temporal bone revealed decreases of the output intensity of over 85%, depending on temporal bone thickness. Ultrasound attenuation by the bone is a major limiting factor in the case of high frequency transcranial ultrasound application.

Clinical investigations were aggressively carried out by Alexandrov *et al.* (2004) with diagnostic transcranial ultrasound. The initial study included 40 acute stroke patients with occlusions of the middle cerebral artery (MCA), internal carotid artery or basilar artery, which revealed high rates of complete recanalization with dramatic clinical recovering, when continuous transcranial Doppler (TCD) monitoring was used during tissue plasminogen activator (tPA) infusion. Alexandrov *et al.* (2004) reported findings from a large phase II clinical trial entitled "Combined Lysis of Thrombus in Brain Ischemia using Transcranial Ultrasound and Systemic tPA (CLOTBUST). In this trial involving 126 patients, evidence was obtained for the existence of ultrasound-enhanced thrombolysis in the middle cerebral artery thrombus. The CLOTBUST aims were: (1) To compare recanalization and recovery in patients with standard IV tPA therapy and those receiving additional continuous targeted ultrasound monitoring. (2) Compare safety in the two groups. Patients had to meet standard tPA treatment criteria of symptom onset less than 3 hours prior to treatment and also show middle cerebral occlusion on TCD. The primary end point included clinical recovery or TCD recanalization at 2 hours with 24-hour and 3-month follow up. The safety end point was intracranial hemorrhage with clinical worsening. The final results reported revealed symptomatic intracerebral hemorrhage occurrence in three patients in the target group, and three in the control group. Complete recanalization or dramatic clinical recovery within two hours after the administration of a t-PA bolus occurred in 31 patients in the target group (49%), compared with 19 patients in the control group (30%). Twenty-four hours after treatment of the patients eligible for follow-up, 24 in the target group (44%) and 21 in the control group (40%) had dramatic clinical recovery. This large clinical trial concluded that continuous transcranial Doppler augmented t-PA-induced arterial recanalization, with a non significant trend toward an increased rate of recovery from stroke, as compared with placebo. More evaluation is needed to see if diagnostic level ultrasound intensity can truly penetrate the skull and accelerate thrombolysis.

To summarize, the differences between external and internal ultrasound applications are: (1) in external application, relatively higher energy and perhaps lower frequency ultrasound is needed at the surface of the body to sufficiently deliver energy to deeply located thrombus; (2) ultrasound must propagate through the skull, preventing sufficient ultrasound energy from reaching the target accurately; (3) for internal catheter ultrasound, the number of hospitals that can actually conduct this treatment within 3 to 6 hours after onset of stroke is limited. More clinical studies are needed to evaluate the medical significance of accelerated thrombolysis by ultrasound energy. However, this therapeutic ultrasound application for stroke seems to be the most promising among various ultrasound drug delivery clinical trials known to date.

4. Microbubbles

It is well known that microbodies of air or a gas, suspended in a liquid are exceptionally efficient ultrasound scatterers for echography; they are useful as ultrasonic contrast agents. For instance, injecting suspensions of gas microbubbles (in the range of 0.5 to 10 μm in diameter) in a carrier liquid into the bloodstream of living bodies, will strongly reinforce ultrasonic echography imaging, thus aiding the visualization of internal organs, for the detection of cardiovascular and other diseases. Coated microbubbles have the advantage of being stable in the body for a significant period of time, as the shells serve to protect the gases of the microbubbles from diffusion into the bloodstream. There has been a considerable degree of excitement in diagnostic ultrasound imaging regarding improvement, which come from the introduction of these echo contrast agents. Second-generation microbubbles contain perfluorocarbon gas rather than air, which results in an even longer life span of contrast agents within the circulatory system. This permits a longer window time for the echographers to observe patients. Recently, various *in vitro* and *in vivo* experiments have demonstrated that echo contrast agent microbubbles can be intentionally ruptured by diagnostic and therapeutic ultrasound. This acoustically induced destruction and collapse of the microbubbles produces a high amplitude response. Violent liquid jets and microstreaming can be produced during microbubble collapse. Researchers have hypothesized that

these microjets or microstreaming could be applied to promote diffusion of drugs into various tissues and lesions. Albumin microbubbles were first used in conjunction with ultrasound to further enhance the effects of thrombolytic agents (Tachibana, 1995).

Porter later (1996) reported that intravenous perfluorocarbon-exposed sonicated dextrose albumin (PESDA) microbubbles in the presence of low frequency ultrasound can lyse very small clots without the help of lytic agents. They developed a method to declot full-size arteriovenous dialysis grafts in animals. In a trial, three declotting techniques were randomly applied during sonication: (1) direct injection of PESDA; (2) direct injection of saline; and (3) intravenous PESDA. Declotting was graded by cine-angiography score. Results showed high mean patency scores for direct PESDA and for IV PESDA, *vs* saline. The frequency and intensity of ultrasound were 1 MHz and 0.6 W/cm^2. Mizushige *et al.* (1999) reported comparison of different types of microbubble ultrasound contrast agent [sonicated albumin (A)-, SH-U508A (SH)- and dodecafluoropentane emulsion (DDFP)] for drug-mediated thrombolysis. A catheter-type transducer capable of US emission (*i.e.*, 10 MHz, spatial peak temporal average intensity 1.02 W/cm^2 and peak negative pressure 0.33 MPa) in the continuous-wave mode was employed during *in vitro* exposure of artificial white thrombi. Serial changes in acoustic properties monitored by echography showed greatest reduction of the thrombus in the DDFP, and indicated that in the sonicated albumin, microbubble was not significantly different from controls. The stability of the microbubbles was an important factor for the difference. Culp (2004) conducted a transcranial ultrasound experiment in swine (1 MHz, 2.0 W/cm^2) in combination with platelet-targeted microbubbles and obtained rapid opening of intracranial thrombotic occlusions. Based on these results, ImaRx Therapeutics, Inc. (Arizona, USA) recently announced initiation of a multicenter Phase II clinical trial with a 40-patient, randomized and blinded study. This will evaluate the safety and effectiveness of thrombolysis with nanosized bubbles and ultrasound for the treatment of acute ischemic stroke, without the use of lytic drugs. Results showing whether microbubble alone in the presence of ultrasound could breakup thrombus in the middle cereberal artery are anticipated from the trial. Other applications in a range of thrombus related conditions, including myocardial infarction, deep vein thrombosis and thrombi in dialysis grafts, are also under consideration.

Another emerging application, which cannot be ignored, comes from the possibility of using microbubbles to carry various drugs to target sites, and rupturing the microbubbles by localized ultrasound energy. At moderately high sound pressure amplitudes, the acoustic pressure waves can cause the shells of coated microbubbles to rupture, freeing the bubbles so that they behave as non coated microbubbles until they diffuse into the bloodstream. Drug-filled or drug-coated microspheres carrying a therapeutic compound may be targeted to specific tissues through the use of sonic energy, which is directed to the target area and causes the microspheres to rupture and release the therapeutic compound. Targeted drug delivery methods are particularly important where the toxicity of the drug is an issue. Specific drug delivery methods potentially serve to minimize toxic side effects, lower the required dosage amounts, and decrease costs for the patient. The most exciting application of this method is probably gene therapy. The methods and materials in the prior technology for the introduction of genetic materials to, *e.g.*, living cells, are limited and ineffective. Better means of delivery for therapeutics such as genetic materials are needed to treat a wide variety of diseases. Great strides have been made in characterizing genetic diseases and in understanding protein transcription, but relatively little progress has been made in delivering genetic material to cells for treatment. To date, several different mechanisms have been developed to deliver genetic material. These delivery mechanisms include techniques such as calcium phosphate precipitation and electroporation, and carriers such as cationic polymers and aqueous-filled liposomes. These methods have all been relatively ineffective *in vivo* and only of limited use for cell culture transfection. None of these methods potentiate local release, delivery and integration of genetic material to the target cell. A principal difficulty has been to deliver the genetic material from the extracellular space to the intracellular space or even to effectively localize genetic material at the surface of selected cell membranes. Viruses such as adenoviruses and retroviruses have been used as vectors to transfer genetic material to cells. However, it has also been difficult to develop a successfully targeted viral-mediated vector for the delivery of genetic material *in vivo*.

Instead of viral vectors as the carrier of genes to targeted locations, pure plasmid DNA can be attached either to the outside or inside of the microbubble capsule wall. Bubbles can be collapsed by extracorporeal ultrasound

Table 1. Comparison of delivery of plasmid DNA by sonoporation or electroporation vs viral vectors.

	Sonoporation (non-viral)	Electroporation (non-viral)	Viral vectors
Tissue invasiveness[1]	(−)	(+)	(−)
Mechanical or physical damage[2]	(−)	(+)	(−)
Extreme localization[3]	(++)	(+)	(−)
Systemic toxicity	(−)	(−)	(+)
Targeted timing of transfection[4]	(+)	(+)	(−)
Homogeneous transfection at target[5]	(−)	(+)	(+)
Monitoring location of DNA in tissue	DNA/microbubbles can be visualized with diagnostic US	(−)	(−)
Clinical experience	(−)	(−)	(+)
Technical difficulty in performing treatment[6]	(−)	(+)	(−)
Treatment for large volume targets	(+)	(−)	(+)
Cost performance	Cost of microbubbles is relative expensive at present	Electrodes are relatively expensive	Viral vectors are expensive to manufacture
Others	Microbubbles have to be combined with DNA		

[1]Sonoporation and viral vectors treatment is basically minimally invasive, whereas electrodes have to be invasively inserted into the target tissue area in the case of electroporation.
[2]Although ultrasound can induce damage to the tissue at high intensities, outcome could be obtained with non-harmful levels. Electroporation could result in burns near the electrodes.
[3]Gene transfection by sonoporation or electroporation can be targeted to an extremely localized area either by local injection of DNA or focused energy.
[4]Gene transfection could be obtained in a matter of seconds with sonoporation or electroporation whereas viral vectors requires hours or days.
[5]Viral vectors and electroporation gene transfection occurs diffusively within tissues whereas some papers suggest "cobble stone" like transfection in the case of sonoporation.
[6]Custom made electrodes are required for each treatment for electroporation whereas ultrasound probes are reusable and could be applied for various situations.

or by intravascular ultrasound catheter, permitting the DNA to penetrate directly into the tissue and cells. Greenleaf *et al.* (1997) demonstrated an increase in the transfection rate of DNA in the presence of albumin microbubbles *in vitro*. Unger *et al.* (1997). demonstrated similar results with microbubble liposomes. Porter *et al.* (2001) succeeded in reducing restenosis by antisense to the c-myc protooncogene bound to perflurocarbon microbubbles in pigs. Ultrasound may become a new, effective and safe means for introducing genetic material into the target cells of tissues. Although the exact mechanism is still unknown, it is believed that microspheres, upon rupture, create a local increase in membrane fluidity, thereby enhancing cellular uptake of the therapeutic compound (Ogawa *et al.*, 2001). There have been reports that differences in the gene transfer rate depended on the type of microbubbles similar to the results from the thrombolysis (Tachibana *et al.*, 2003). However, it is clear that gene delivery phenomenon occurs at a far smaller scale, thus further investigation is needed to understand the exact mechanism involved in ultrasound microbubble gene transfer. Recent observation of the collapse of microbubbles by the newly developed high speed video microscope Brandalis-128 system, which has an average speed of 13 million frames per second, has produced massive information on the dynamic behavior of ultrasound insonified encapsulated microbubbles (Postema, 2004). Understanding more on the physics involved in the event of microjet in the following few years will perhaps solve how genes actually penetrate the cell membrane (Marmottant *et al.*, 2003, 2004).

5. Regenerative Medicine

Today, the concept of applying ultrasound as a means to alter the pharmacokinetics of drugs in various tissue and cell membrane permeability to these drugs has expanded into a whole new field beyond just drug delivery. Application of ultrasound for regenerative medicine is one of them. One example is the delivery of genes into cells. Certain tissues such as muscle have been reported to take up and express naked plasmid DNA *in vivo*. In general, however, the level of transfection after direct injection of naked plasmid DNA is variable and low. Different methods have been devised to improve the transfection efficiency. The use of certain viral vectors leads to more efficient transfection, compared with naked

plasmid DNA. However, serious concerns have been voiced regarding the use of viral vectors, especially when clinical trials are involved. Instead of viral vectors as the carrier of genes to targeted locations, pure plasmid DNA can be attached either to the outside or inside of the microbubble capsule wall. Bubbles can be collapsed by extracorporeal ultrasound or by intravascular ultrasound catheter, permitting the DNA to penetrate directly into the tissue and cells. Ultrasound may become a new, effective and safe means for introducing genetic material into the target cells of tissues.

Ultrasound can induce cell-membrane porosity (Tachibana *et al.*, 1999), and enhance the delivery of naked plasmid DNA into cells *in vitro* (Fig. 6). Moreover, recent studies have shown enhanced permeability of naked plasmid DNA into tumors *in vivo* (Manome *et al.*, 2000). Li *et al.* (2003) recently made a comparison of gene transfection using various microbubbles available in the market. Ogawa *et al.* (2002) also made comparison with different dissolved gases and found changes in the extent of gene transfection. Although the exact mechanism is still unknown, it is believed that microspheres, upon rupture, create a local increase in membrane fluidity, thereby enhancing cellular uptake of the therapeutic compound. "Sonoporation" as this phenomenon is frequently named, is a new means to overcome limitations of the other gene transduction methods. Different genes for various purposes are now under intensive investigation for possible use in regenerative medicine. These could be for angiogenesis, antiangiogenesis, apoptosis, bone generation and other future treatments methods.

Anti-sense oligodeoxynucleotides (AS-ODNs) have been recognized as a new generation of putative therapeutic agents, Miura *et al.* (2003) established a delivery technique that could transfect AS-ODNs, which are designed for endothelin type B receptor (ETB), into cultured human coronary endothelial cells (HCECs) by exposure to ultrasound in the presence of echo contrast microbubbles. Taniyama (2002) have successfully transfected genes (HGF) for angiogenesis by ultrasound/microbubbles in skeletal muscles. This could lead to a cure for critical limb ischemia. In addition, Taniyama *et al.* (2003) transfected an anti-oncogene (p53) plasmid into carotid artery after balloon injury, as a model of gene therapy for restenosis. Bone morphogenetic proteins (BMPs) are morphogens implicated both in embryonic and regenerative odontogenic differentiation. Gene

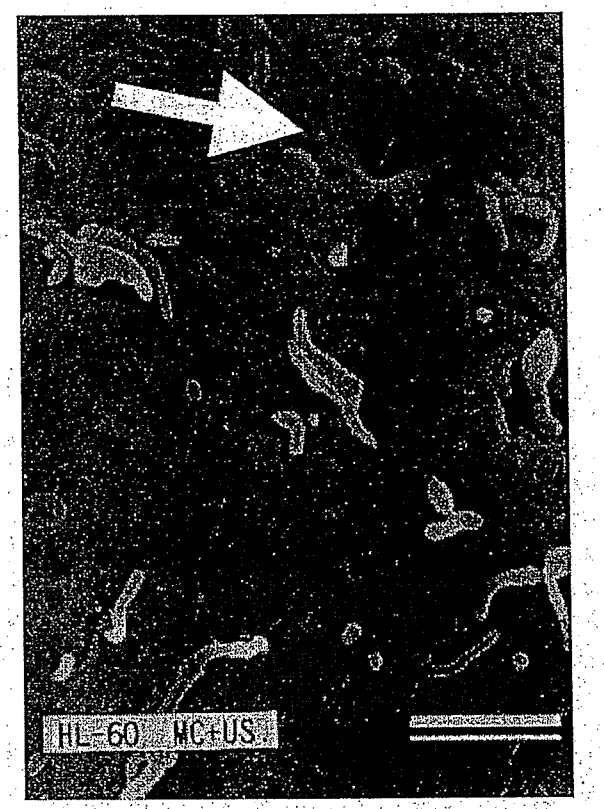

Fig. 6. Scanning electron microscopic image of the surface of cell membrane partially ruptured by microbubble collapse (scale 0.1 μm). The arrow shows where possible disruption of the cell membrane, probably in the event of micro jet impact. Drugs or DNA could easily be delivered into the cells through these openings (cell type: HL-60; 50 kHz; 3 W/cm^2, 2 minutes).

therapy has the potential to improve induction of reparative dentin formation or potent bioactive pulp capping. Nakashima (2003) optimized the gene transfer of Growth/differentiation factor 11 (Gdf11)/Bmp11 plasmid DNA into dental pulp stem cells by sonoporation, *in vivo*. Dental pulp tissue treated with plasmid pEGFP or CMV-LacZ in 5–10% Optison® and irradiated by ultrasound (1 MHz, 0.5 W/cm^2, 30 sec) showed significant efficiency of gene transfer and high level of protein production selectively

in the insonated region, within 300 μm under the amputated site of the pulp tissue. The Gdf11 cDNA plasmid transferred into dental pulp tissue by sonoporation *in vitro*, induced the expression of Dentin sialoprotein (Dsp), a differentiation marker for odontoblasts. The transfection of Gdf11 by sonoporation stimulated the large amount of reparative dentin formation on the amputated dental pulp in canine teeth *in vivo*. These results suggest the possible use of BMPs, employing ultrasound-mediated gene therapy for endodontic dental treatments. It is estimated that genes for regeneration tissues could become a realistic mode of treatment in future. Other genes that have potential function for therapy have been reported to increase tranfection rate by ultrasound and microbubbles (Taniyama *et al.*, 2004, 2005). Manome *et al.* (2005) demonstrated that the use of ultrasound (210.4 kHz, 5.0 W/cm^2, 5 seconds) most effectively transfected a plasmid DNA into culture slices of mouse brain (147-fold increase compared to controls). The effect was reinforced by combination with echo contrast agent, Levovist. Intracranially injected DNA with Levovist also enhanced gene transfection in newborn mice.

6. Breakthrough in Develpmental Research

An unexpected breakthrough in technology was recently reported in a publication on developmental research, using chick and mouse embryo. It is surprising that such a non-invasive method as sonoporation for injecting various genes into cells, can lead to the discovery of understanding the function of genes in the early period of development. Ohta *et al.* (2003) recently succeeded in delivering functional gene into chick embryo. This study is probably the first of a series of discoveries in understanding the early development stages of animals using ultrasound. The gene transduction technique is a useful method to study gene functions that underlie especially in vertebrate embryogenesis. Gene transduction technique was reported using microbubble-enhanced sonoporation to achieve ectopic and transient gene expression for several embryonic organs including embryonic chick limb bud mesenchymes (Fig. 7). The technique has the advantage of (1) relatively simple gene transduction procedures, and (2) efficient exogenous gene transduction and expression with lower damages to embryos. Green fluorescent protein (GFP) or LacZ was

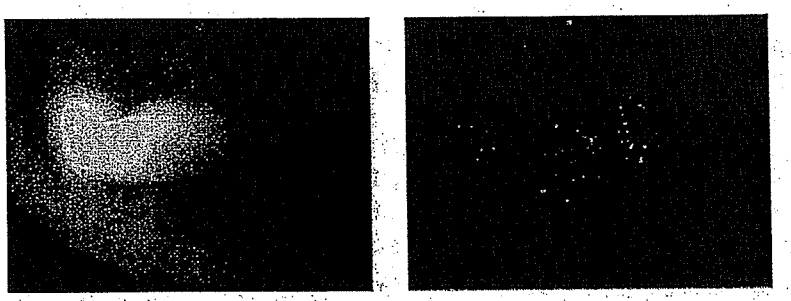

Fig. 7. Limb bud ectoderm of mouse embryo. Induction of GFP was obtained by ultrasound and microbubble collapse. Left photo shows gross view of the limb. Fluorecent microscope was used to visualize localized transfection of GFP genes. Green glow spots were observed around the limb skin (US: 1 MHz, 2 W/cm^2, 1 minute).

misexpressed in limb bud mesenchymes by sonoporation, with the introduced expression transiently detected in the injected sites. Most of the transduced chick embryos survived without showing significant embryonic abnormalities or cell death after sonoporation. To demonstrate its efficacy for assessing the effect of transient gene transduction, the *Shh* (*sonic hedgehog*) was transduced into the developing chick limb bud. The transduced limb bud displayed limb malformations, including partial digit duplication.

Recent research at our lab (unpublished data) has suggested a revolutionary technique in "ultra site specific" drug delivery into honeybee brain. A high molecular weight Rhodamine labeled dextran was selectively injected into several neurons of the brain tissue, without irreversible damage to the cells (microbubble: BR14, Bracco: 750 kHz, 2.0 W/cm^2, SonoPore KTAC-3000). The object was to visualize the axon of the cells in the brain in order to evaluate the neural network involved in basic behavioral functions, such as language or communication among insects. We were able to identify single nerve cells in the brain by using 3-dimensionally reconstructed images by confocal laser microscopy (Fig. 8). This ultra-localized "injection" technology of various substances into single cells, still in the infant stage, may help in understanding the neural network and mechanism of the function of the brain and could be applied for research in artificial intelligence and robot technology in future (Fig. 9).

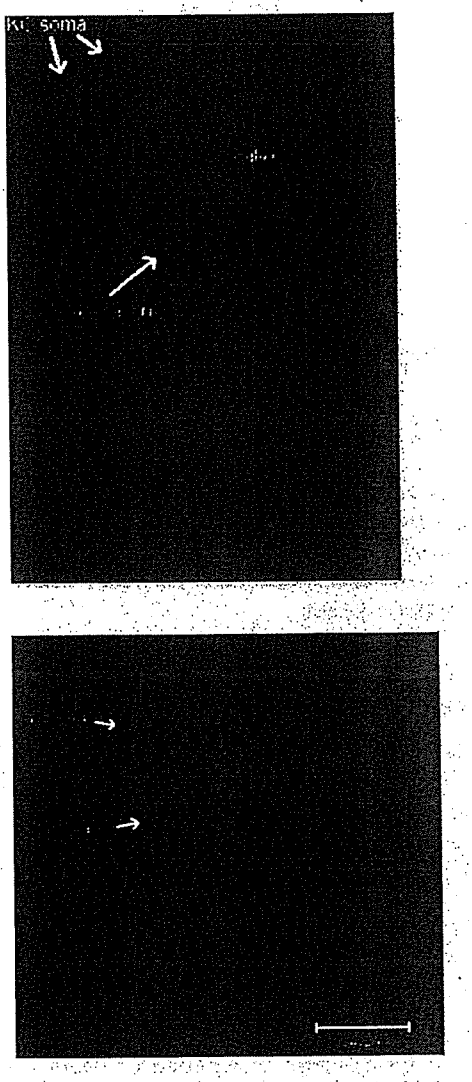

Fig. 8. Staining of single nerve fibers in Honeybee brain by ultrasound (tetramethylrhodamine-dextran staining). Optison microbubbles were attached to the surface of the brain and irradiated by ultrasound (1 MHz, 2 W/cm^2, 1 minute) in the presence of the stain. Sliced cross section view of the brain shows staining at the surface of the bee brain (above arrows) as well as the axons in the deep layers.

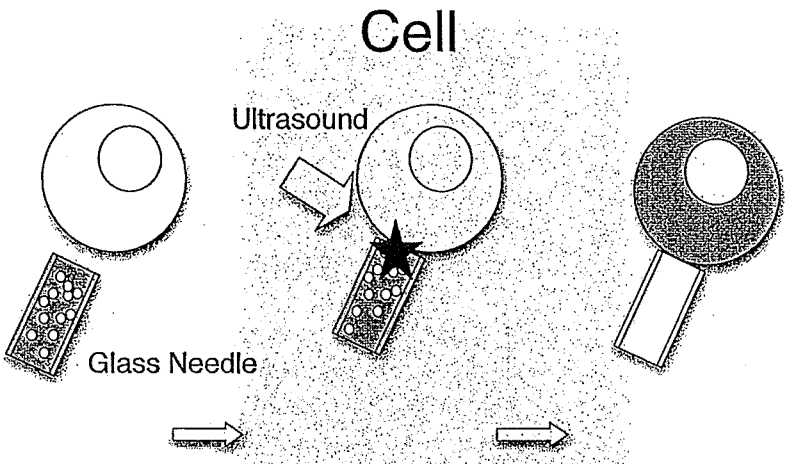

Fig. 9. Illustraion of possible injection of drug into a single living cell by "ultra" targeted sonoporation method without irreversible damage. Microbubble and the target drug can be carried at high concentrations by a micro glass needle (far left). Ultrasound could be irradiated at a certain intensity of duration (center). Drug can penetrate the cell membrane without irreversible damage to the cell.

7. Sonodynamic Therapy

As physical methods used for the treatment of leukemia and solid cancers, compared with radiotherapy and light irradiation, ultrasonic irradiation is superior in that the applied energy can be focused solely on the cancer tissues to be treated with little effect upon normal tissues, and is better than light irradiation in terms of the degree of penetration. Umemura *et al.* (1990) pioneered the development of non-thermal ultrasound to activate a group of chemicals that were originally used as light activated chemicals for cancer therapy. This new ultrasound therapy has been termed as sonodynamic therapy. Ultrasonic waves are known to cause chemical actions if cavitation occurs; for example, irradiation of water can cause a reaction to generate hydrogen peroxide. It was found that certain drugs, upon ultrasonic irradiation, create active oxygen such as superoxide radicals and singlet oxygen, effectively destroying cancer tissues (Miyoshi *et al.*, 1995). The agents themselves have no anti tumor activity and are very low in toxicity, exhibiting anti tumor activity only by the chemical action caused