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Ultrasound-activated microbubbles for tendon gene transfer: *in vivo* efficiency and confocal microscopy real time intracellular investigations

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ABSTRACT

Ultrasound that is routinely used for imaging is now exploited for therapeutic applications including drug delivery or gene transfer. Today, ultrasound imaging is an established and confident technique for diagnosis. It is mainly based on the development of contrast imaging methods that aim to identify and display the echo from contrast agent as well as rejecting the echo from surrounding tissue offering thus a more resolutive detection. Ultrasound contrast agents or microbubbles (MB) are small gas bubbles encapsulated by a stabilizing shell, with a typical diameter of micron range. Ultrasound pulses are typically applied with a frequency near the resonance frequency of the gas bubble and the bubbles oscillations produce strong echoes from regions of perfused tissue [1-2]. Activation of microbubbles (MB) under specific ultrasound (US) beams induces a transient cell membrane permeabilization with a process known as sonoporation [3-4]. This work aims at evaluating the use of ultrasound and microbubbles for gene transfer in Achilles tendons.

EXPERIMENTAL METHODS

Microbubbles and ultrasound set-up: BR14, perfluorocarbon gas microbubbles were generously provided by Bracco (Bracco Research, Geneva, Switzerland). Ultrasound waves were generated from a transducer with 1 MHz frequency. The transducer was driven with an electrical signal generated by an arbitrary waveform generator and amplified with a power amplifier (ADECE, Artannes, France).

Animal studies: The animal study was carried out according to the guidelines of the French Ministery of Agriculture for animals experiments. Experiments were done on eight week old BalB/c mice. Mice were anesthetized by intraperitoneal injection of Ketamine (125 mg/kg Body Weight) and Xylazine (10 mg/kg Body Weight) solutions. Mice legs were depilated and Achilles tendons were transfected with plasmid DNA encoding luciferase (pLuc) as reporter gene. Luciferase activity of lysates was quantified with a luminometer after addition of luciferin (Luciferase Assay System, Promega) and expressed as Relative Light Units (RLU). Proteins content of tendons were quantified by BCA reaction.

In vivo bioluminescence imaging and quantification: Bioluminescence imaging was conducted using a cooled CCD camera (cooled to -80°C) mounted on a dark box chamber with a camera controller, a camera cooling system and a computer system for data acquisition and analysis (Hamamatsu Photonics). Signal intensity was quantified as the mean of grey level per second of time exposure within a region of Interest prescribed over the tendon. Levels of luminescence are indicated by pseudocolors on a scale of 0 to 255 units.

RESULTS AND DISCUSSION

Different ultrasound parameters were tested to determine optimal acoustic setting for tendon gene transfer. The transfection efficiency was evaluated by measuring the luciferase activity as read out. Our data clearly indicate that the transfection efficiency was dependent on acoustic pressure, time exposure, and microbubbles number. As observed in Figure 1A, optimal condition consisting of 1 MHz of frequency, 200 kPa, 40% duty cycle and 10 min of ultrasound exposure in the presence of 10^6 microbubbles gave the highest luciferase activity that is 100-fold compared to that obtained with pLuc injected alone (control). This efficiency was dependent on the

presence of microbubbles because no increase of the gene expression was found when pLuc was insonified at the same condition without BR14 bubbles.

The level of gene expression obtained with US waves at 400 kPa acoustic pressure was only 10-fold more than that of the control. Note that increasing the exposure time at this acoustic pressure induced a reduction of the luciferase activity that was due to a toxicity of this condition as shown by the histological analysis (Figure 1B).

The kinetic of gene expression was determined by *in vivo* bioluminescence imaging (Figure 2). The luciferase activity obtained in tendons treated with US at 200kPa for 10 min and MB was 10-fold higher than that obtained with tendons injected with pLuc alone. From 25 days, the level of luciferase activity in the latter tendons decreased as function of time and became very low 25 days post-treatment. All mice that have been insonated in the presence or absence of MB expressed a similar level of luciferase gene during the first 25 days. Then, the activity measured in tendons treated with US without MB dropped drastically and reached that of tendons injected with pLuc alone.

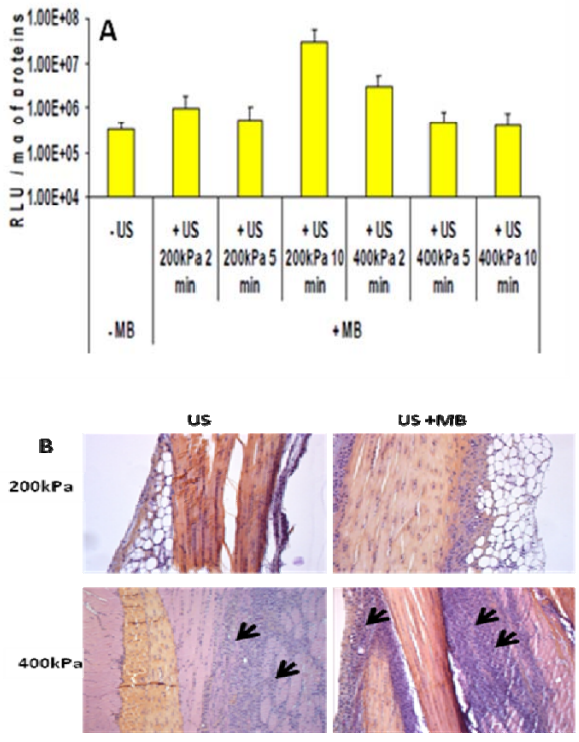

Figure 1: (A) Achilles tendons of mice were injected with 10 µg of pLuc gene in the presence of 10^6 BR14 bubbles. The insonification was performed at 1 MHz, at the indicated acoustic pressure and 40% duty cycle during 2, 5 or 10 minutes. Five days later, mice were sacrificed; their tendons were harvested and lysed. Values are means ± SED of data from 2 experiments with 3 mice per group. (B) Tendons were harvested, fixed in 10 % *p*-formaldehyde solution, paraffin-embedded, sliced in serial sections (4 µm), mounted on glass slides and HES counterstained. Arrows indicate inflammation area.

Interestingly, the activity of tendons treated with US at 200kPa for 10 min and MB was stable and sustained till 100 days. A plasmid rescue assay was done from DNA extracted from these tendons and transformed *E. coli* colonies were obtained indicating that this long term expression was due to an episomal form of the pLuc.

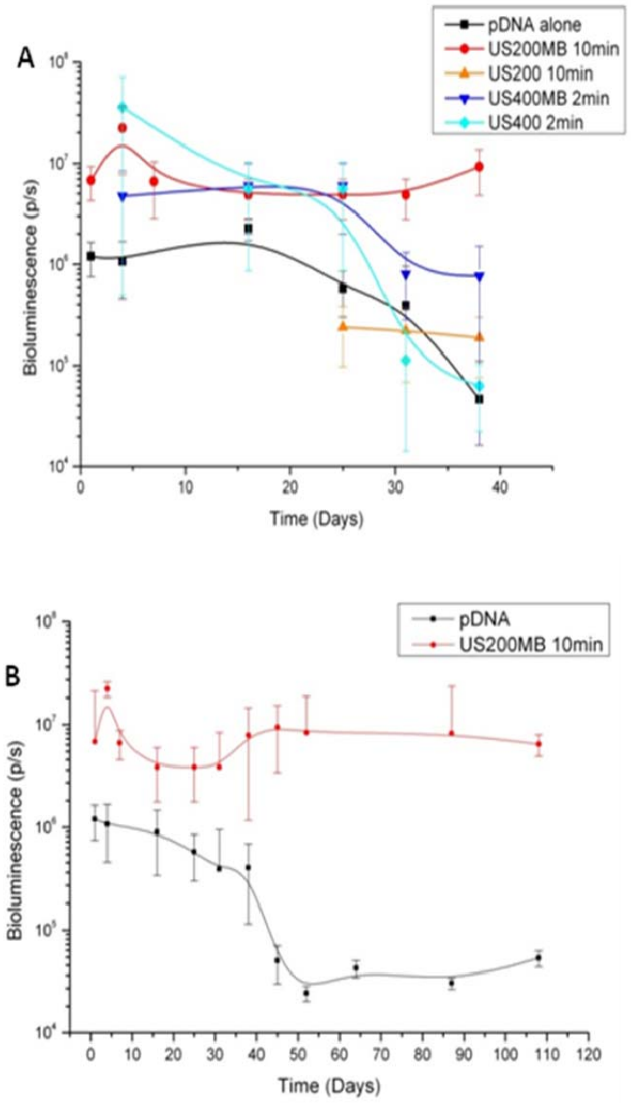

Figure 2: Bioluminescent signal relative quantification. Mice were transfected with 10 µg pLuc alone or pLuc mixed or not to $5x10^5$ BR14 followed by US exposure at 1 MHz, 200 kPa or 400kPa, 40% duty cycle. Bioluminescence corresponds to the luciferase activity were detected following luciferin injection in the tendon area. Bioluminescence measurement was done as described in Methods section.

A key to success of this technique lies in understanding mechanism governing MB and cell interactions. Several studies suggest that pore formation is less likely to be the dominant mechanism. Recently, it was suggested that besides transient pore formation, endocytosis mechanism might also be involved (5). We have started to perform real time confocal microscopy investigations on adherent cells.

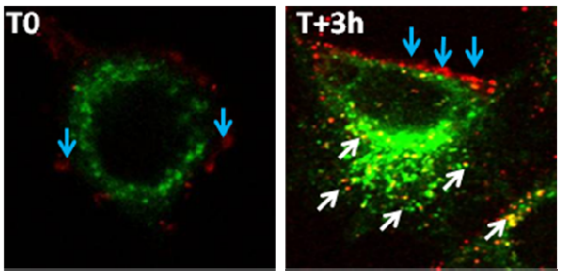

Figure 3: Confocal microscopy images obtained from live cells that stably express Rab7-GFP (green) insonated in the presence of MB and Cy3-labeled pLuc (red). Upon insonation (T0), pLuc was mainly localized at plasma membrane (blue arrow). Then, some moved inside the cytoplasm and reached rab7-eGFP endosomes

For this study, we used cell clones that stably express organelles markers fused with eGFP and red fluorescent labeled pLuc. Our observations confirm that pLuc pDNA was mainly localized as spots at plasma membrane straightaway following insonation. At later time, punctuate staining like vesicles progressively moved towards the nucleus area. After 3h post-insonation, pLuc (red) was clearly colocalized with rab-7 eGFP (green) as shown by the yellow staining (Figure 3). This suggests that pLuc reached rab7 positive organelles that correspond to late endosomes (6). Note that there are still some red spots stuck at the plasma membrane.

CONCLUSION

Overall, our data clearly demonstrate that the transfection efficiency was dependent on acoustic pressure, microbubbles' number and exposure time. Optimal ultrasound parameters are 1MHz, 200 kPa, 40% duty cycle and 10 minutes of exposure time in the presence of $5x10^5$ BR14 microbubbles. The long term expression of transgene obtained by ultrasound assisted microbubble makes it a suitable method for gene therapy in tendons. In vitro studies made on live cells indicate that plasmid joined the endocytosis pathway upon insonation. Most of them were still stuck at the plasma membrane. Specifying limitations of US assisted MB delivery will allow furthering improvement of the system by refining MB design.

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