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Modulation of luciferase activity using high intensity focused ultrasound in combination with bioluminescence imaging, magnetic resonance imaging and histological analysis in muscle tissue ☆

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ABSTRACT

This study investigates the effect of high intensity focused ultrasound (HIFU) to muscle tissue transfected with a luciferase reporter gene under the control of a CMV-promoter. HIFU was applied to the transfected muscle tissue using a dual HIFU system. In a first group four different intensities (802 W/cm^2, 1401 W/cm^2, 2117 W/cm^2, 3067 W/cm^2) of continuous HIFU were applied 20 s every other week for four times. In a second group two different intensities (802 W/cm^2, 1401 W/cm^2) were applied 20 s every fourth day for 20 times. The luciferase activity was determined by bioluminescence imaging. The effect of HIFU to the muscle tissue was assessed by T1-weighted ± Gd-DTPA, T2-weighted and a diffusion-weighted STEAM sequence obtained on a 1.5-T GE-MRI scanner. Histology of the treated tissue was done at the end. In the first group the photon emission was at 3067.6 W/cm^2 $1.28 \times 10^7 \pm 3.1 \times 10^6$ photon/s (5.5 ± 1.2-fold), of 2157.9 W/cm^2 $8.1 \pm 2.7 \times 10^6$ photon/s (3.2 ± 1.1-fold), of 1401.9 W/cm^2 $9.3 \pm 1.3 \times 10^6$ photon/s (4.9 ± 0.4-fold) and of 802.0 W/cm^2 8.6x ± 1.2×10^6 photon/s (4.5 ± 0.6-fold) compared to baseline. In the second group the photon emission was at 1401.9 W/cm^2 and 802.0 W/cm^2 $14.1 \pm 3.6 \times 10^6$ photon/s (6.1 ± 1.5-fold), respectively, $5.1 \pm 4.7 \times 10^6$ photon/s (6.5 ± 2.0-fold). HIFU can enhance the luciferase activity controlled by a CMV-promoter.

1. Introduction

Despite the recognized potential of gene therapy for treating a broad array of human diseases, gene transfer efficiency and expression in human patients is still disappointing [1]. Different vectors based on DNA and RNA viruses as well as non-viral vectors are undergoing clinical trials as potential treatments for a range of diseases, including cancer, and for the development of DNA vaccines as an alternative to traditional antigen-based vaccines [2]. Attempts to control transgene expression involve the use of tissue-specific and inducible promoters [3,4]. A third type of promoter used widely in gene therapy strategies, in addition to tissue-specific and inducible is the constitutive promoter. The CMV promoter is one of the most commonly used constitutive promoters in eukaryotic expression vectors and it is used to induce a constant gene expression in nearly all mammalian cells [5]. Although, CMV promoters have a high rate of transgene expression, they are considered to be regulated only through vector dose, which is determined at the time of delivery, a strategy that is likely to result in widely varying levels of expression.

Other attempts to control and enhance transgene expression involve the use of external physical agents such as ionizing radiation and high intensity focused ultrasound (HIFU). Ionizing radiation may activate TNF-α gene expression under the control of promoter pEGR1 with some spatial and temporal control [6]. Irradiation of a non-immunogenic melanoma tumor transfected with an adenoviral vector coding for IL-12 under the control of the promoter of the human heat-shock protein 70B resulted in an increased expression of the transgene [7]. However, this method exposes the cells to ionizing radiation and may therefore not be suitable for human use.

Unlike ionizing radiation, HIFU could be used to control transgene expression without potentially harmful side effects. HIFU has been used clinically for thermal ablation of tumors [8,9] and is being evaluated for drug and gene delivery to tissue [10,11]. HIFU causes physical changes in tissues through both physical alteration, such as cavitation, and also energy deposition. HIFU can be used to induce localized heating in a tissue transfected with a transgene controlled by a heat inducible promoter, such as the

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promoter for the heat-shock proteins (HSP) which can activate gene expression in response to hyperthermia [12]. Previous studies have demonstrated the ability to control gene expression *in vivo* by local heat application, looking at the mRNA of the inducible endogenous HSP70 protein in rat leg muscle by MRI-guided focused ultrasound [13].

The *in vivo* use of HIFU used in conjunction with a strong constitutive promoter, like CMV, has not been studied. Therefore, in this study, we investigated the effect of HIFU on the expression of the luciferase reporter gene under the control of a CMV promoter in mouse muscle. The purpose of this study was to investigate the combination of a strong promoter under the influence of a focal energy source in order to see if the luciferase activity under the control of a CMV promoter can be influenced by HIFU.

2. Materials and methods

All animal experiments were performed in compliance with institutional animal care committee guidelines and with the approval of the animal care committee.

2.1. Plasmid injection into the muscle tissue

Thirty-five Balb3c mice (age 10–12 weeks) were anesthetized with continuous exposure to 1–2% isoflurane. The first set of experiments used 20 mice, the second set used 10 mice and to the positive control group 5 mice were assigned. The left flank of the mice was shaved and prepped with isopropyl alcohol. The CMV-Luc plasmid (20 μg diluted in 100 μl PBS, from Invitrogen, San Diego, CA) was injected into the flank muscle tissue of each mouse with a 27 gauge needle.

2.2. Animals and experimental setup

The Balb3c mice were anesthetized with intraperitoneal Nembutal (58 mg/kg) to achieve deep anesthesia. The mouse body was placed in a plastic tube having a window for positioning and treatment of the muscle side. No movement of the mouse should occur during the HIFU treatment. The plastic tube with the mouse body was put into a deionized, degassed water bath to provide a proper ultrasound coupling. The water was heated up before the treatment in order to maintain body temperature. The transducer of the dual ultrasound system (imaging 6 MHz/therapeutic 1 MHz) (Focus Surgery; Indianapolis, IN) was positioned in the same water bath. The targeting of the muscle was done by ultrasound imaging of the muscle in the vertical and horizontal plane.

In total 65 mice were used for this study. In the first set of experiments 40 mice were used. Twenty mice got the injection of the plasmid and 20 mice as negative control group did not get the plasmid. In the second set of experiments 20 mice were used; 10 received the plasmid injection and 10 mice used as negative control did not get the plasmid injection. To the positive control group five animals were assigned. These animals got the injection of the plasmid into the muscle tissue not receiving HIFU application. The animals assigned to the negative group received the same HIFU intensities like the animals with the plasmid injection. To each HIFU intensity 5 mice were assigned.

2.3. Focused ultrasound system

A modified Sonoblate® system (Focus Surgery; Indianapolis, IN) was used for mechanistic studies. The system contains both imaging and therapy components in a single spherical and concave transducer. The therapy portion of the transducer has a frequency (f_0) of 1.0 MHz, an aperture diameter of 50 mm, focal length of 40 mm, a maximum total acoustical power of 120 W, and a maximum focal intensity in water of 8000 W/cm^2. Focal area was 1.5 mm^2, and electrical impedance magnitude at f_0 of 81 Ω. The imaging portion of the transducer has f_0 of 6.0 MHz, bandwidth of 80%, aperture diameter of 8 mm. The sinusoidal wave signal was amplified by a 50 dB radiofrequency power amplifier (Agilent 33120A15 MHz Function/Arbitrary Waveform Generator) coupled to the transducer device (50 Ω impedance). HIFU application was performed seven days after the injection of the plasmid into the muscle tissue. In order to ensure the application of HIFU at the identical position, the skin of the flank where the plasmid got injected was marked with a permanent marker. Before each HIFU application the marker was targeted with a pointer of the HIFU system. In the first set of experiments four different HIFU intensities in a continuous wave mode (802 W/cm^2, 1401 W/cm^2, 2117 W/cm^2, 3067 W/cm^2) were applied for 20 s every 14 days so that each mouse received in total four times the same HIFU intensity. In a second set of experiments two different intensities in a continuous wave mode (802 W/cm^2, 1401 W/cm^2) were applied for 20 s every fourth day so that each mouse received in total twenty times the same HIFU intensity. The HIFU parameters lead in a total acoustical power between 11.95 and 45.71 W and a pressure between 49.04 and 95.93 kPa.

2.4. Bioluminescence imaging

Bioluminescence imaging (BLI) was performed with a highly sensitive, cooled CCD camera mounted in a light-tight specimen box (IVIS®, Xenogen, Alameda, CA) using protocols similar to those described previously [14]. Imaging and quantification of signals were controlled by the acquisition and analysis software Living Image® (Xenogen, Alameda, CA). For *in vivo* imaging, animals were given the substrate D-luciferin (Biosynth Staad, Switzerland) by intraperitoneal injection at 150 mg/kg in PBS, and anesthetized (1–3% isoflurane). Mice were then placed onto the warmed stage inside the light-tight camera box with continuous exposure to 1–2% isoflurane. To obtain a baseline reading, mice were imaged at 7, 3, and 1 day before the application of HIFU. In the first experimental set mice were imaged 10 h, 1, 4, 7, 10, 13 days after application of HIFU. In the second experimental set mice were imaged every other day after the application of HIFU for 40 days. The data acquisition time was 30 s. In previous experiments it was shown that this imaging time gave the best results. Generally, 2–3 mice were imaged at a time. The low levels of light emitted from the bioluminescent muscle cells were detected by the IVIS® 100 camera (Xenogen, Alameda, CA) system, integrated, digitized and displayed. Regions of interest from displayed images were designated around the muscle area and quantified as total photon counts or photon/s using Living Image® software (Xenogen, Alameda, CA).

2.5. MR imaging

In order to evaluated tissue changes due to different HIFU intensities MR imaging was performed 9 h after HIFU application of those animals included in the first experimental set. In the second experimental set no MR imaging was done. MR imaging was performed under continuous exposure to 1–2% isoflurane. A clinical 1.5-T GE Signa MR Scanner (GE Medical Systems, Milwaukee, WI) was used with a custom designed quadrature high-pass birdcage coil tuned to 64 MHz for signal reception. To obtain accurate and reproducible images the mouse was placed prone and fixed in wrapped tissue. The body temperature was maintained throughout the MRI studies with a warm blanket. The following scan protocol was carried out in the axial plane with a FOV of 6, 256 × 192 pixels, slice thickness of 2.0 mm, and two acquisitions: (A) pre-contrast T1-weighted SE (TR/TE 400/15 ms), (B) T2-weighted FSE ((TR/TE