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Selective Clinical Ultrasound Signals Mediate Differential Gene Transfer and Expression in Two Human Prostate Cancer Cell Lines: LnCap and PC-3

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Low intensity ultrasound signals, similar to that employed in clinical therapy, are found to mediate differential gene transfer and expression of the Green Fluorescence Protein (GFP) reporter in two human prostate cancer cell lines, LnCap and PC-3. Cell suspensions in the presence or in the absence of GFP (44.5nM) were treated at 37°C under a standing wave condition. Cells were exposed to either continuous wave, 932.7kHz ultrasound, or to several independent bursts, each burst comprising a 20% duty cycle (932.7kHz) sine wave. The burst "repetition" frequency was varied from 10Hz to 10kHz in several different experiments and each treatment received a net identical ultrasound energy exposure. Transient GFP expression levels in viable cells were monitored by flow cytometry. The findings revealed a strong ultrasound tone-burst frequency dependence on the transfection efficiencies. Interestingly, the ultrasound signal parameters which are routinely employed in clinical therapy did not yield any statistically significant enhancement in transfection efficiency relative to their sham counterparts. © 1997 Academic Press

Rapid and efficient transfer of therapeutic gene(s) of choice into benign or malignant tumors is a critical first step in genetically engineered therapy. A few common non-viral / physical *in-vitro* techniques have been reported to mediate gene transfer and transgene expression with varying degree of efficiency. Some common physical methods employed for transfection of mammalian cells in culture include electroporation, particle bombardment mediated gene transfer, calcium phosphate precipitation, and liposome mediated gene transfer (1-4). Recent studies have demonstrated that application of low frequency ultrasound with a needle-tip sonicator (commonly utilized in the laboratory for cleaning and sterilization of equipment) can yield gene transfer and expression in viable HeLa and plant cells (5,6). These studies employed high peak ultrasonic power levels (~100W) and low frequencies (20kHz-50kHz), namely, parameters which were surmised to be conducive to the production of acoustic cavitation. However, the transfection efficiency of the reporter genes under these conditions were poor, typically ranging from 0.1% to 1%.

The objective of the current study was to identify low intensity ultrasound signals similar to those currently used in clinical therapy, which could facilitate rapid gene transfer and expression of a non-viral reporter vector expressing Green Fluorescence Protein (GFP) in hormone sensitive and insensitive human prostate cancer cell lines LnCaps and PC-3, respectively. A novel feature of this study is that the net ultrasound energy of exposure was maintained constant throughout all treatment conditions. The potential role of low intensity ($0.33W/cm^2$) ultrasound, operating either in a continuous wave (CW) mode or in a tone burst mode (within a burst repetition frequency (RF) range $10Hz \leqslant RF \leqslant 10kHz$) was evaluated in facilitating rapid gene transfer and expression of the GFP plasmid. These findings reveal a strong ultrasound tone-burst frequency dependence on transfection efficiencies.

MATERIALS AND METHODS

Transducer and set up. An unfocused ultrasound transducer (the sound source) with an active element of 3.25 cm in diameter ($8.3cm^2$ surface area) was employed to expose 5ml cell suspensions, in RPMI growth medium with 10% fetal bovine serum, to low intensity ($0.33W/cm^2$) standing wave ultrasound burst signals, in the absence or presence of a fixed plasmid concentration [GFP]=1.0μg/ml (44.5nM), in commercially available 5cm × 5cm T-25 polystyrene tissue culture flasks. The transducer was completely submerged in a rectangular heated water bath containing 4 liters of distilled water, the transmitting medium maintained at 37 ± 0.2°C and mounted

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with its radiating face parallel to the base of the tank. A special platform unit with a 4 cm diameter hole for unobstructed ultrasound beam transmission was constructed to position the T-25 flasks. The entire platform and 5 mm under side portion of the T-25 flask, was immersed in the heated water bath. The underside surface of the T-25 flask was positioned parallel with the transducer aperture, thus enabling ultrasound transmission through its under surface.

Transducer calibration. The unfocused 932.7kHz plane wave transducer was characterized with a broad frequency band (0.5 - 8.0 Mhz) 0.5 mm diameter hydrophone (NTR, Washington, USA) at the identical location from the radiating face of the transducer to the position where the sample chamber was positioned, on the central axis (1.0 cm) in the near-field. The transfection chambers utilized in this study were sterile tissue culture flasks T-25 comprising 1.1 mm thick polystyrene walls with a 25 cm^2 surface area on its major faces. Only 2% of the ultrasound beam was found to be attenuated, as measured with the hydrophone under the above mentioned near field transmission mode condition. The average acoustic intensity (spatial average temporal average) was determined by the acoustic power measured by a radiometer (without allowing beam spreading), divided by the surface of the transducer's aperture.

Transducer operation. A Hewlett Packard function generator / arbitrary waveform generator (HP 33120A) capable of operating in the continuous wave (CW) mode and also in the tone-burst mode (with sine wave carrier frequency of 932.7kHz), was employed. The multi-function generator's low amplitude sinusoidally varying voltage signal (0.265 V peak) was amplified (~316 times) with an Electronic Navigational Instrument power amplifier (100 electrical Watts) to drive the transducer element at the desired calibrated intensity (spatial average temporal average intensity) level of I_{SATA} = 0.33W/cm^2.

Cell treatment conditions. LnCap and PC-3 cell lines were obtained from American Type Culture Collection (Rockville, MD) and grown as monolayers under CO_2 at 37°C and maintained in T-175 flasks in RPMI 1640 with 10% of fetal bovine serum. Ultrasound facilitated gene transfer and expression studies were performed at cell passage 35 for both cell lines. Prior to treatment, 75 - 80% confluent cells were trypsinized, and brought into a low concentration suspension of 3×10^4 cells/ml in their original growth medium. The low cell suspension concentration was selected to avoid/minimize cell-cell interaction mediated cell death. The suspension stock was divided into two identical groups: group A and group B. Group A served as the control group, i.e., without plasmid. The Green Fluorescence Protein gene (GFP) was utilized as the transgene reporter. The 4.7 kilobase GFP plasmid, driven by human cytomegalovirus CMV immediate early promoter, was purchased from CLONTECH Laboratories, Inc. Palo Alto, CA. Just prior to the ultrasound treatment, GFP plasmids were added to group B suspensions to a final working concentration of [GFP]=1.0μg/ml (or 44.5nM of 4.73 kilobase DNA). Each group was further subdivided into ten T-25 flasks (5ml suspension/flask) which represented nine different ultrasound treatment conditions in the absence or presence of plasmids. The nine ultrasound treatment conditions comprised of eight different tone burst repetition frequencies in the range 10Hz$\leqslant$ RF $\leqslant$ 10kHz and one CW exposure. The remaining (unexposed) flask served as the control sample condition in the absence (or presence) of plasmids. Experiments were preformed as three independent replicates.

Consistent with present clinically employed ultrasound diagnostic and therapeutic parameters (7,8), a 20% duty cycle (i.e., t_{on}/ (t_{on} + t_{off})) was selected for all burst signals in this study. Comparison between several different ultrasonic burst signals in mediating gene transfer were made under the following ultrasound energy exposure constraint conditions. The net ultrasound energy of exposure, i.e., (spatial average temporal average) intensity $\times$ surface area of exposure $\times$ time of exposure was selected as the parameter to be conserved for each treatment condition. A moderately low, though clinically relevant therapeutic intensity level of I(SATA) = 0.33 W/cm^2 or I(Spatial Avg.Temporal Peak) = 1.67 W/cm^2, with 20% duty cycle was selected in this study to mediate gene transfer and minimize cell killing via thermal mechanisms. Temperature measurements within the sample chamber's 5 ml cell-free growth medium with 10% FBS revealed no significant temperature changes (< 0.3°C) immediately after the 24 seconds of CW exposure (I_{SATP} = 1.67 W/cm^2), or after the 120 second tone burst exposures (I_{SATP} = 1.67 W/cm^2) and (I_{SATA} = 0.33 W/cm^2). To maintain sterility, the underside of the T-25 flasks were liberally washed with 70% ethanol and dried, after the completion of all exposure conditions. All treatment conditions were thereafter incubated in the original T-25 flasks, without further modifications, for two complete days.

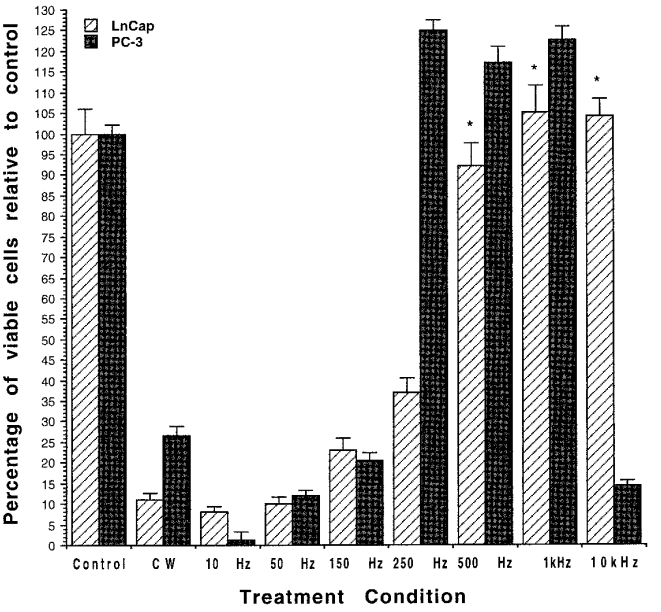

FIG. 1. Effect of ultrasound treatment on viability of LnCap and PC-3 cells. Survival was assayed five days after the ultrasound treatment (24 seconds CW or a 2 minute tone-burst) with a net energy of deposition of 328.5 J for each condition. [LnCap]=30k/ml, [PC-3]=30k/ml, ultrasound carrier Freq. = 932.7kHz, Ultrasound intensity = 0.33W/cm^2. Results shown for n=3, percentage of mean survival relative to sham ultrasound ± S.D. Statistical significance relative to the control condition was determined through unpaired student T-test of equal sample variance with a confidence level greater than 95% ($P< 0.05$). All treatment conditions were found to be statistically significant for PC-3 relative to its control. LnCap treatment conditions which yielded statistical insignificance relative to its control are denoted by an asterisk (*).

Cell viability assay. The cytotoxic and proliferative response of cells were assessed with a non-radioactive colorimetric cell proliferation tetrazolium compound (MTS) assay (Promega, Madison, WI) (9) in three independent replicates. Five days after the ultrasound treatment, 5 ml of culture medium in each flask was aspirated from each cell culture. Two milliliters of fresh RPMI 1640 with 10% FBS and dissolved MTS reagent at a working concentration of 333 μg/ml was replaced per flask and incubated for 90 minutes in a standard 5% CO_2 incubator at 37°C. The percentage of cell survival was measured relative to the sham ultrasound corrected average absorbance value. Statistical significance relative to the control condition was determined through unpaired student T-test of equal sample variance with a confidence level greater than 95% (viz., $P<0.05$).

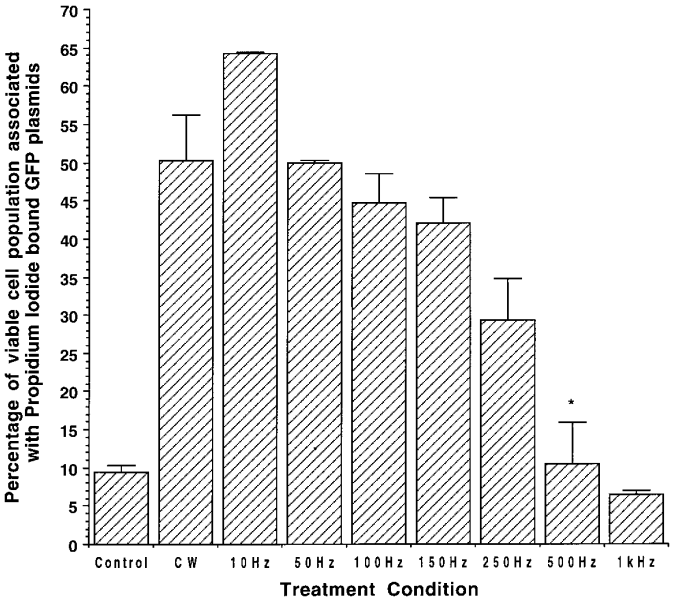

FIG. 2. Effect of ultrasound on transgene association / uptake in LnCap cells. The percentage of viable LnCaps cells associated with Propidium Iodide labelled GFP plasmids was determined by flow cytometery. [LnCap]=30k/ml, [GFP+PI]= 1μg/ml = 44.5nM, [PI]/[GFP] = 500. Results shown for n=3, mean ± S.D. Statistical significance relative to the control condition was determined through unpaired student T-test of equal sample variance with a confidence level greater than 95% ($P < 0.05$). Statistical insignificance relative to the control condition is denoted by an asterisk (*).

RESULTS AND DISCUSSION

The findings from this investigation reveal that exposure of prostate cancer cells to low intensity ultrasound of identical net energy per treatment condition, operating within the currently employed clinical therapeutic parameters, resulted in differential (i) cell viability, (ii) gene transfer across the cell membrane and (iii) expression of the GFP plasmid reporter. As shown in Fig. 1 cell survival is strongly dependent on tone-burst frequency. The greatest cytotoxicity was found at the low tone-burst frequency end of the spectrum, viz., 10Hz, and was independent of the cell type. LnCap cells were approximately 2.5 fold more sensitive to the CW mode ultrasound application than PC-3 cells; whereas, at the 10Hz burst frequency PC-3 cells were more sensitive than LnCap cells. No significant cytotoxic effects were found within the 500 to 1kHz range. At 1kHz, PC-3 cells proliferated approximately 1.25 fold greater than that of untreated cells, while no enhancement in proliferation was observed for the LnCap cells. Interestingly, the PC-3 line was found to be highly prone to cell death at the 10kHz burst frequency of application than its androgen sensitive counterpart.

GFP plasmid uptake immediately following ultrasound treatment was determined by flow cytometery analysis of fluorescent Propidium iodide (PI) tagged DNA. Ten micrograms of propidium iodide was intercalated into 150μg GFP plasmids in a non-specific manner (10). All of the propidium iodide was observed to be bound to the DNA as evidenced by its change in color, from red to deep purple red, and by electrostatically moving the precipitated DNA+PI along the side wall of the microcentrifuge tube. Just prior to the ultrasound treatment, the PI bound GFP plasmid was added to cell suspensions at a final working concentration of [GFP+PI]=1.0μg/ml or 44.5nM. Immediately following the ultrasound exposure, cells were analyzed in a flow cytometry (Becton-Dickinson FACS Star). The PI bound GFP fluorescence signal was restricted to live cells by gating on forward and side angle light scattering. The percentage of viable LnCap cells associated with the GFP plasmids can be seen in Fig. 2. This experiment revealed a strong ultrasound tone-burst frequency dependence on the association of GFP plasmids in the viable LnCap cell population. GFP transfer accross the cell membrane immediately after ultrasound treatment is found to be most prevalent at the low tone-burst frequency end, viz., 10Hz. Progressive higher repetition frequencies yielded fewer viable cells associated with the GFP plasmids. A small percentage of viable sham exposed cells were were observed to associate with the GFP plasmids. Interestingly, at the

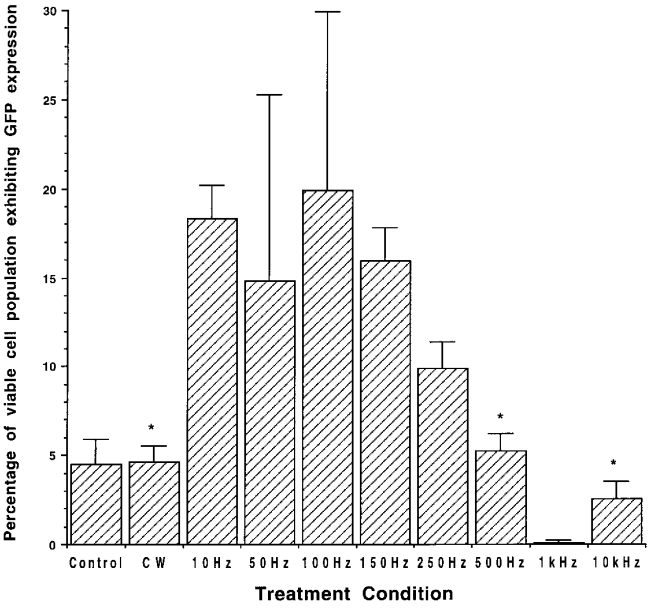

FIG. 3. Expression of GFP following ultrasound mediated transfection. Burst frequency dependence was assessed in viable LnCap cells by FACS analyses two days after ultrasound treatment. [LnCap] = 30k/ml, [GFP]=1μg/ml = 44.5nM, ultrasound carrier Freq.=932.7kHz, Ultrasound intensity = 0.33W/cm^2. n=3, mean ± S.D. Statistical significance relative to the control condition was determined through unpaired student T-test of equal sample variance with a confidence level greater than 95% ($P < 0.05$). Treatment conditions which yielded statistical insignificance relative to the control are denoted by an asterisk (*).

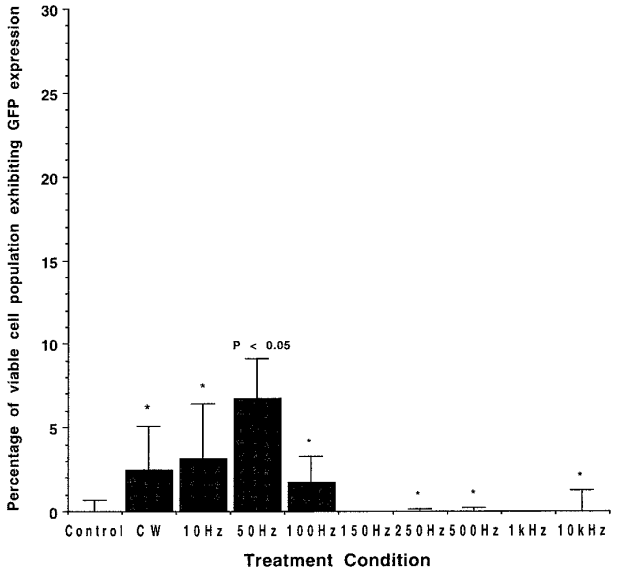

FIG. 4. Clinical ultrasound mediated transient GFP transfection burst frequency dependence in viable PC-3 cells determined by FACS analyses two days after ultrasound treatment. [PC-3] = 30k/ml, [GFP]=1μg/ml = 44.5nM, ultrasound carrier Freq.=932.7kHz, Ultrasound intensity = 0.33W/cm^2. n=3, mean $\pm$ S.D. Statistical significance relative to the control condition was determined through unpaired student T-test of equal sample variance with a confidence level greater than 95% ($P < 0.05$). Treatment conditions which yielded statistical insignificance relative to the control are denoted with an asterisk (*).

1kHz repetition frequency, ultrasound is incapable of mediating gene transfer through the cell membrane.

The transient expression of GFP, due to ultrasound facilitated gene transfer, was measured through a "two-color" fluorescence flow cytometry technique. The GFP signal collection was restricted to live cells by gating on cells which did not exhibit the fluorescence uptake of free PI (1.0μg/ml). The findings revealed a strong and distinctive ultrasound tone-burst frequency dependence on the GFP expression for the two cell lines (Figs. 3 and 4). Approximately 20% to 30% in GFP transfection efficiencies were found within an ultrasound burst frequencies range 10Hz to 150Hz (Fig. 3). The optimal transfection efficiency for LnCap was observed at the 100Hz treatment. Transfection efficiencies were found to diminish dramatically at a burst repetition frequency treatment greater than 150Hz. In the presence of plasmid, with the 1 kHz treatment, no viable cells were found to be transfected. Interestingly, treatment of LnCap cells with the plasmid alone and without ultrasound yielded 5% of the cell population expressing GFP. The CW+plasmid did not yield any statistically significant enhancement to that of the plasmid alone.

An important energy constraint of this investigation served as a foundation for making comparisons among several different types of clinically relevant ultrasound signals. Each exposure condition comprised a net identical ultrasound energy of 328.5 J. In this study the CW energy delivery parameters, viz., I_{SPTA}=5.27W/cm^2, I_{SATA}=1.67W/cm^2 with radiation rate ($I_{SATA} \times$ irradiated surface area) = 13.3W, are quite similar to the clinical therapeutic ultrasound parameters (7,8), and interestingly do not yield statistically significant increases in the transfection efficiency level relative to the sham ultrasound condition, even though 50% of the viable cell population was observed to take up the GFP plasmids immediately following ultrasound exposure, as determined via FACS analysis and fluorescence microscopy, (Figs. 2 and 3). However, applications of several tone-burst RF parameters not commonly utilized in ultrasound clinical therapy (10Hz to 150Hz) do exhibit a strong differential gene transfer and expression dependence. It is noteworthy that the most commonly employed clinical ultrasound RF parameter of 1kHz is not capable of mediating gene transfection.

The fact that low intensity clinical therapeutic ultrasound is non-invasive, with an excellent penetration depth through soft tissues, makes it a potential target-site specific gene transfer modality. These *in-vitro* findings suggest that there may exist an *in-vivo* tone-burst frequency "window" for clinical ultrasound to mediate site-specific gene transfer and expression of a therapeutic gene of choice.

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