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THE EFFECTS OF ULTRASOUND ON THE GROWTH AND FUNCTION OF BONE AND PERIODONTAL CELLS

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Running title: Ultrasound effects on cells

THE EFFECTS OF ULTRASOUND ON THE GROWTH AND FUNCTION OF BONE AND PERIODONTAL CELLS

Abstract - In order to understand the effects of ultrasound on the growth and function of connective tissues *in vivo*, we have examined the effects of a single 5 min CW exposure of 3.00 MHz on a human osteoblast-like cell line (MG63 cells) and on human periodontal ligament (PDL) cells *in vitro*. Although cell proliferation was found to be largely unaffected by ultrasound intensities between 140 - 990 mW / cm² ISA, flow cytometry (FCM) analysis showed that there were pronounced and differential effects on cell function. Thus, in the MG63 cells, bone-associated proteins were down-regulated whereas collagen type I (COL I) was unaffected and fibronectin (FN) was up-regulated at low intensities. In contrast, in the PDL cells, bone protein expression was found to be dose-dependant, while FN and COL I were down-regulated. These findings demonstrate that therapeutic ultrasound can have a marked influence on tissue repair and regeneration processes, and suggest that the parameters of ultrasound could be modulated to elicit effective and predictable wound healing responses.

Key Words: Ultrasound, Flow cytometry, Bone cells, Periodontal ligament cells, Extracellular matrix proteins, *In vitro*.

INTRODUCTION

Ultrasound has had extensive application for diagnostic purposes, becoming the modality of choice for many soft tissue radiological examinations. While the clinical value of ultrasound as a diagnostic tool has been overwhelming, there has also been increasing interest regarding ultrasound-induced biological effects, which have thus far demonstrated the potential to both damage and stimulate tissues.

Biological responses of a deleterious nature have been widely demonstrated *in vivo*, and include thermal lesioning (Clarke and Ter Haar 1997) and lung haemorrhage and hind limb paralysis (Frizzel et al. 1994). Investigations at the cellular level have also reported damage to the cell membrane (Fahnestock et al. 1989) and cell survival and lysis (Dooley et al. 1983). In addition, sister chromatid exchange has been widely studied regarding the possibility of damage at the genetic level, although the results of such investigations have been inconclusive (Stella et al. 1984; Ciaravino et al. 1985).

In marked contrast, many studies have suggested that ultrasound can generate beneficial effects under certain conditions, leading to the development and widespread use of therapeutic ultrasound by physiotherapists for a number of types of injury to soft connective tissues, such as tendon and ligament (Ter Haar et al. 1987). *In vivo* studies on soft tissue at physiotherapy doses have demonstrated improved rates of healing (Byl et al. 1992) and tissue strength (Enwemeka et al. 1990). Such therapeutic levels have also indicated stimulatory effects *in vitro*, including increased protein synthesis (Edmonds and Ross 1988) and enhanced calcium uptake (Mortimer and Dyson 1988). Furthermore, several common wound sites have shown evidence of beneficial response to ultrasound,

such as venous ulcers (Johannsen et al. 1998), lateral epicondylitis ('tennis elbow') (Binder et al. 1985) and particularly bone fractures (Kristiansen et al. 1997). Thus, double-blind, placebo-controlled studies on ultrasound-treated human fractures have reported improvement in healing rates by approximately 30-40% (Heckman et al. 1994; Kristiansen et al. 1997). Other studies *in vivo* have supported these findings, with significant improvement in fracture healing at low intensity values of 30 - 100 mW / cm² (Pilla et al. 1990; Yang et al. 1996; Zorlu et al. 1998). Additional stimulation has been demonstrated at higher levels, such as 500 mW / cm² (Dyson and Brookes 1983), although Tsai et al. (1992) demonstrated a deleterious response at the higher value of 1000 mW / cm². A recent *in vitro* study reported increased prostaglandin E₂ production with low intensity exposure in mouse osteoblasts (Kokubu et al. 1999), and suggested this as a possible mechanism in ultrasound-assisted fracture healing. However, few other studies have examined the precise effects of ultrasound on fundamental cellular and molecular processes involved in the repair and regeneration of either hard or soft connective tissues, profoundly limiting the development of new strategies that may significantly improve wound healing of damaged tissues.

In the present study we have therefore used, for the first time, the technique of flow cytometry (FCM) to determine the effects of ultrasound on specific extracellular matrix (ECM) proteins at an ultrasonic frequency and intensity range representative of current clinical therapeutic doses (Ter Haar et al. 1987). The FCM technique permits accurate measurement of the relative levels of selected components of the ECM, which plays a fundamental role in connective tissue integrity and function, and may therefore be of great value in understanding ultrasound-induced healing mechanisms and the optimum

conditions for the clinical application of ultrasound therapy.

METHODS

Exposure apparatus

An unfocussed, circular PZT piezoelectric crystal of 22 mm diameter, driven by an amplified signal from a TG1010 function generator (Thurlby Thandar; Huntingdon, UK), was used to insonate the cell cultures. Regulation of crystal output was achieved by monitoring voltage across the two electrode sides of the crystal, using a TDS220 digital oscilloscope (Tektronix; Marlow, UK) and adjusting the voltage to levels applied during dosimetry measurements. Output voltage was adjusted by a 150LA RF amplifier (Amplifier Research; Souderton, PA, USA). A frequency of 3.00 MHz was used for all experiments, and continuous wave sinusoidal signals applied in all cases.

Dosimetry

A 1.0 mm diameter submersible PVdF needle hydrophone (Precision Acoustics; Dorchester, UK) calibrated at the time of purchase, was used to measure the acoustic field produced by the crystal. Hydrophone measurements were made within a carpeted glass water tank (450 x 300 x 300 mm), fitted with a Plaiton acoustic window and containing approximately 40 l of tap water which was de-gassed overnight. Ultrasound-scattering bubbles were carefully removed from within the tank and from the acoustic gel by compression of the gel-occupying space between the crystal and acoustic window. To measure acoustic pressure values within the beam, the needle hydrophone was aligned

using the method of Duck et al. (1985). A beam axis profile of acoustic pressure was recorded, at 5 mm intervals, to locate near and far field regions within the ultrasound beam. Radial beam profiles were subsequently recorded, at 2 mm intervals, with separations from the transducer ranging from 30 mm to 120 mm in order to select an optimum distance for placing the cell culture monolayer and was ultimately selected at 80 mm. This crystal-monolayer separation had the least detrimental beam profile properties of near field peaks and troughs and far field decreased beamwidth. Thus, all cells within a 16 mm diameter target region of the monolayer experienced an acoustic signal without significant fall-off of signal intensity towards the edges of the region.

Radial profiles at 2 mm intervals were then measured for various crystal excitation voltages at the selected monolayer-crystal separation, and values of continuous wave spatial average intensity (I_{SA}) calculated for a 16 mm diameter circular target region within the monolayer. Values were calculated by averaging intensity recordings at equal distances from the centre of the beam profile, and weighting this value by the area of the target monolayer to which this measurement was the most proximal reading, namely areas corresponding to 2 mm thick concentric rings. Doses with values of approximately 125, 250, 500 and 1000 mW / cm² I_{SA} were applied to the target region.

Exposure assembly

The exposure assembly is shown in Fig. 1. A 12.5 cm² culture flask (Falcon, Becton Dickinson; Cowley, UK) was filled beyond the flask neck with 40 ml of culture medium, as outlined below, which was pre-heated to 37°C. This flask was held in place, with top above water level, in a foam-fronted plastic sliding assembly containing an

aperture of matching dimensions to the monolayer. The 22 mm diameter crystal was aligned to the centre of the 16 mm diameter cell target region, for which I_{SA} values were calculated. The monolayer was positioned 80 mm from the crystal surface, its plastic substrate the first object to encounter the ultrasonic signal. Foam was placed at the end of the 350 mm length tank to help remove standing waves and unwanted reflection from the flask holder. The tank water was maintained at $37.0 \pm 0.5^\circ\text{C}$ during the exposure and the crystal left for approximately 5 min to reach operating temperature prior to initial exposure. Finally, medium in the flask was decanted, discarded and fresh medium added.

Cell culture

MG63 cells (a gift from Dr L. Di Silvio, Institute of Orthopaedics, University of London, UK), which were originally derived from an osteogenic sarcoma of a 14-year old male (Heremans et al. 1978), have previously been shown to exhibit certain characteristics of bone cells (Clover and Gowen 1994). They were incubated as adherent monolayers in Dulbecco's Minimum Essential Medium (DMEM) (Gibco Life Technologies Ltd; Paisley, UK), 10% heat-inactivated fetal calf serum (FCS) (PAA Laboratories; Linz, Austria), 2 mM L-glutamine (Gibco), 100 U/ml of penicillin (Gibco) and 100 $\mu\text{g/ml}$ of streptomycin (Gibco) at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was replaced twice weekly until the cells reached high density. They were detached from the monolayer by incubating with trypsin-EDTA (0.25% trypsin, 1 mM EDTA) (Gibco) for 5 min at 37°C , centrifuged and recultured in DMEM until required.

Human periodontal ligament (PDL) cells were grown from periodontal ligament obtained from the middle third of clinically healthy molar teeth of patients undergoing routine extractions at the Eastman Dental Hospital, as previously described (Kuru et al.

1998). Briefly, the periodontal ligament was cut into 1-2 mm³ pieces, washed twice with phosphate-buffered saline (PBS) (Gibco), supplemented with 100 U/ml of penicillin (Gibco), 100 µg/ml of streptomycin (Gibco) and 2.5 µg/ml of amphotericin B (Gibco) and placed into 6-well tissue culture plates (Falcon). Sterile glass coverslips were placed over the explants to anchor them to the wells. The explants were incubated with α-MEM (Gibco) containing 10% FCS and supplements as described, until the outgrowth of the adherent cells reached confluence. They were recultured as described above for bone-derived cell lines and used between passages 6 and 8.

Proliferation

A light microscope fitted with a graticule eyepiece was used to measure proliferation within the 16 mm target region of cells. Growth rates were calculated by counting cell numbers each day within four identical locations on the target region of each flask. Each region corresponded to an area of approximately 1.5 mm², which could be located exactly by lining up the graticule with markings on the flask.

1 x 10⁴ MG63 and PDL cells were seeded into 12.5 cm² flasks (Falcon) in 2 ml of medium, a sufficiently low density which enabled individual cells to be counted throughout the culture period. After 24 h, 3 replicate flasks were exposed to a single 5 min CW ultrasound exposure at 3.00 MHz for each intensity level. Flasks were then incubated for a further period of four days, with cell counting only performed within the target region on each day for each flask in order to assess post-insonation growth rate.

Flow cytometry (FCM)

FCM is a technique that measures certain physical and chemical properties of

individual cells by analysis of scattered light and emitted fluorescence from cells in suspension flowing past a laser and optical detection assembly. The FACScan flow cytometer (Becton Dickinson) utilizes an argon-ion laser of wavelength 488 nm and 15 mW output, from which values of forward scatter (FSC) and side scatter (SSC) are recorded for each individual cell. FSC and SSC have been shown to be related to cell size and granularity (intracellular complexity), respectively (Shapiro 1988). In addition, the application of fluorochrome-conjugated antibodies to the cells enables specific antigens to be measured within each cell via emitted fluorescence recordings.

For the FCM procedure, 5×10^4 cells were seeded into 12.5cm² flasks (Falcon) and incubated for 24 h, at which time the target region was marked on the outside of the flask and cells outside this central circular zone removed using a sterile cell scraper (Falcon). These cells were decanted and discarded to ensure that the cells undergoing subsequent FCM analysis arose only from within the exposed target region. The flasks were then washed 3 times with PBS, fresh medium added and recultured for 24 h. They were then filled with culture media at 37°C and recultured for 5 days following exposure. On day 5, the cells were washed twice with PBS (Gibco) and detached using 20 mM EDTA in PBS (pH 7.2) (Sigma, Poole, UK) for 10 min at 37°C. Trypsin was not applied because its protease activity would be likely to remove cell surface-associated antigens. The cells were then fixed for 30 min with 1% w / v paraformaldehyde (BDH; Poole, UK) in PBS, containing 0.1% w / v saponin (Sigma), a non-ionic detergent which permeabilises the cell membrane and allows the subsequent entry of the detecting antibodies (Sumner et al. 1991; Bou-Gharios et al. 1994). They were centrifuged, resuspended and washed in a buffer containing 2% FCS in PBS and 0.1% saponin and

centrifuged at 400 x g for 10 min. Approximately 1×10^5 cells were placed into separate tubes and each tube used to measure the relative level of each antigen. These were selected as representative antigens characteristic of hard and soft connective tissues, as follows: rabbit polyclonal antibodies (pAbs) against osteonectin (ON), osteopontin (OP), bone sialoprotein (BSP) (kindly provided by Dr L. Fisher, NIDCR, USA) and fibronectin (FN) (Dako; Glostrup, Denmark); a mouse monoclonal antibody (mAb) against collagen type I (COL I) (Chemicon; CA, USA). These were diluted 1:100 in washing buffer and incubated with the fixed cell suspensions for 60 min at room temperature. Normal rabbit serum (Dako) and mouse IgG1 (Dako) served as negative controls for the pAb and mAb, respectively. After washing the cells, fluorescein isothiocyanate (FITC)-labeled swine anti-rabbit IgG, diluted 1:20 in PBS, was then added for 30 min at the pAb-treated cells. FITC-labeled rabbit anti-mouse IgG, diluted 1:50 in PBS, was added for the same time to the mAb-treated cells. The cells were resuspended in 500 μ l of wash buffer and the FSC, SSC and fluorescence intensities of 10 000 individual cells measured.

FCM analysis

The results were analysed using CELLQuest Software (Becton Dickinson Immunocytometry Systems) and are presented as arbitrary units of FSC, SSC and fluorescence intensity. The actual scattered light and fluorescence values obtained were dependant on the electronic input and detection settings of the FACScan, which are constant in the experiments described, and on the specific antibody used. Since each of the antibodies has unique reactivity with each corresponding antigen, the fluorescence levels obtained using different antibodies cannot be compared with each other.

The relative level of expression of each antigen is shown as the average fluorescence intensity (AFI). This value is calculated from the overall distribution of cell fluorescence values in the culture, after eliminating the signals due to cellular clumping and debris. A contour plot of cell population distribution within a graph of FSC against SSC was used as a guide to establishing a measurement region that contained $\geq 90\%$ of the total population of intact, single cells. This same region was used for all exposure groups within the same cell type. The net AFI values for each antigen were then calculated after subtraction of the AFI of the relevant negative control samples (non-specific serum and IgG) from the AFI of the test samples (cells treated with specific antibody).

Statistical analysis

Three separate culture experiments were performed for all the proliferation and FCM studies, in which the selected target areas of cells in at least two separate flasks were used for cell counting and for FCM analysis at each exposure level. The results are reported as the arithmetic mean \pm standard error of the mean (SE). Statistical analysis of the results was performed using the Students' *t*-test, with $p < 0.05$ considered to be significant.

RESULTS

Effects of ultrasound on cell proliferation

Cell growth in response to ultrasound was measured by direct cell counting, as described in the Materials and Methods. The results in Fig. 2. show the proliferation rates of MG63 and PDL after exposure to increasing doses of ultrasound, relative to the growth of non-exposed cells used as controls. While growth of the MG63 cells appeared

to be somewhat reduced at day 2 but recovered, and even somewhat elevated at day 3 post-insonation (Fig. 2a), these differences were not statistically significant ($p > 0.05$). Moreover, the apparent progressive inhibition of MG63 proliferation by increasing doses of ultrasound (at days 3 and 4) was also found to be non-significant. The growth of the PDL cells (Fig. 2b) was also only slightly affected at all intensities of ultrasound, even the maximum increase of 16% observed at day 3 (at 990 mW / cm²) not being statistically different from the non-treated control cells.

Effects of ultrasound on antigen expression

ON, OP and BSP are connective tissue antigens which are closely associated with bone function and integrity and FN and COL I are both very widely distributed in all connective tissues, with many studies having reported the expression of these antigens in both bone and PDL. In the present study we have therefore measured the response of each antigen to ultrasound, as described in Materials and Methods. Fig. 3. is a representative experiment showing FN expression by the MG63 and PDL cells. These results indicate that, in the former, FN is markedly up-regulated (compared with the control cells) by the lowest dose of ultrasound (140 mW / cm²) and to a lesser extent at 540 mW / cm², but not at the other two doses (Fig. 3a). In contrast, in this experiment all intensities of ultrasound appeared to substantially reduce the expression of FN by the PDL cells (Fig. 3b).

The results of all the FCM experiments in Fig. 4a show that, in the MG63 cells, there was a very notable difference between the response of the bone-associated proteins ON, OP and BSP and the soft connective tissue-associated antigens FN and COL I. Thus, among the former, ON expression was down-regulated at all intensities, reaching significantly lower levels at the highest and lowest doses ($p < 0.05$), while the expression of OP demonstrated a dose-dependant reduction, reaching a significantly lower value of 40% of the control cells at the maximum dose of 990 mW / cm². BSP was also

significantly down-regulated, to approximately 50% of control values at all intensity levels (Fig. 4a). In contrast, FN expression by the MG63 cells demonstrated clear up-regulation at the two lowest intensities (100% and 50% of control at 140 and 230 mW / cm², respectively), whereas COL I expression appeared unresponsive to all intensities of ultrasound, as shown in Fig. 4a.

The response of the PDL cells to ultrasound was found to differ from that of the MG63 cells, although there were notable differences between the effects of ultrasound on each of the bone-associated proteins and also between these and FN and COL I (Fig. 4b). Thus, while ON was progressively down-regulated by increasing doses of ultrasound (22% less than the control at 990 mW / cm²), OP expression was strongly and significantly elevated at the highest dose, reaching a level which was 38% higher than the control cells. However, there was no clear pattern of modulation of BSP expression in response to increasing doses of ultrasound, which appeared to be somewhat reduced at the two intermediate levels but unchanged at both the highest and lowest doses of ultrasound, as shown in Fig. 4b. In contrast, reduced expression of FN was found at all four intensities, reaching a maximum and statistically significant decrease of 35% at 230 mW / cm². Similarly, COL I was also significantly down-regulated in the PDL cells, by approximately 20% of control level at 140, 230 and 540 mW / cm² ($p < 0.05$), although it was reduced by only 6% at 990 mW / cm².

DISCUSSION

The clinical treatment of wound sites by ultrasound is now widespread for soft connective tissue injuries (Ter Haar et al. 1987), while the repair of damage to hard connective tissue by ultrasound has also shown promise in clinical trials (Kristiansen et al. 1997) and *in vivo* studies (Pilla et al. 1990). However, the cellular and molecular mechanisms responsible for the apparently beneficial effects of ultrasound on both soft

and hard connective tissue, and the precise effects of ultrasound on the fundamental processes that promote repair and regeneration in such tissues, has hitherto received very little attention. Study of the biological responses to ultrasound is therefore essential not only for establishing guidelines for the safe use of diagnostic ultrasound (WFUMB 1998), but also for improving, developing and applying effective therapeutic strategies (Dyson 1987).

The present study has examined the effects of different intensities of ultrasound on the level of certain selected connective tissue components. These have previously been shown to be of major importance in the structure, integrity and function of the ECM and thus in wound healing and tissue remodelling. Osteoblasts and fibroblasts were selected as 'target' cells since they comprise a substantial proportion of hard and soft connective tissue and have demonstrated beneficial responses to therapeutic ultrasound in histological studies (Singh et al. 1997). The acoustic parameters used, encompassing values of 250, 500 and 1000 mW / cm², were comparable to those previously applied in clinical physiotherapy (Ter Haar et al. 1987), while the lowest intensity value, 140 mW / cm², is close to values for worst case B-mode diagnostic exposure (Henderson et al. 1995).

Our findings indicate that although none of the intensities used here appeared to have any deleterious effect on the proliferation of the human cells in culture, all doses of ultrasound nevertheless clearly and selectively influenced the expression of the ECM antigens by the MG63 and PDL cells. This demonstrates the sensitivity, and value, of the FCM technique in detecting ultrasound-induced changes at intensity levels which do not affect the growth of the cells. Moreover, the present study also identified cell-specific responses to ultrasound, since the expression of the bone-associated proteins ON, OP and

BSP by the MG63 cells was adversely affected at all doses, whereas the response of these antigens in the PDL cells was variable and in some cases, such as with ON and OP, was even up-regulated at some doses. In addition, FN expression was markedly up-regulated in the MG63 cells but clearly blocked in the PDL cells and, while COL I was also substantially down-regulated in these latter cells, this antigen was unaffected in the MG63 cells. These apparent differential cell responses to ultrasound *in vitro* suggest that the application of specific and different ultrasound parameters may be of fundamental importance for achieving optimal therapeutic efficacy at hard and soft connective tissue sites *in vivo*.

Antigen-specific responses to ultrasound were also clearly noted, in particular with respect to differences between the bone-associated proteins and FN and COL I. For example, while ON, OP and BSP were all down-regulated in the MG63 cells by ultrasound, FN was markedly up-regulated at low intensities and COL I remained unchanged. In addition, while ultrasound-induced changes were variable in the PDL cells, all doses down-regulated both FN and COL I. Further studies are required, however, to delineate whether these antigen-specific responses to ultrasound are effected directly at the level of activity of the corresponding genes. Alternatively, since ultrasound has been reported to have a potent influence on the integrity and transport properties of the plasma membrane (Dinno et al. 1989), it is possible that the effects we have observed are at least partly due to modulation of specific post-transcriptional processes which affect secretion from the cell.

It is notable that we also observed that FN in the MG63 cells and ON in the PDL cells were significantly up-regulated at the lowest intensity (140 mW / cm²). This

observation is consistent with reports of low level stimulation at 100 mW / cm² *in vivo* (Yang et al. 1996; Zorlu et al. 1998), and at lower intensities (30 mW / cm²) with increased exposure times and multiple applications (Pilla et al. 1990; Heckman et al. 1994; Krisitansen et al. 1997). Fracture healing effects at intensities up to 500 mW / cm² have also been noted, with Dyson and Brookes (1983) reporting such stimulation at 5 min exposures, although attenuation-correction for overlying tissue will have reduced the actual intensity applied.

The use of isolated cells and cell lines *in vitro* has allowed the use, for the first time, of the FCM technique to investigate precise cellular responses to ultrasound and has additionally enabled accurate delivery of different and well-characterised ultrasonic doses, which is difficult to achieve *in vivo*. This is particularly pertinent when comparing soft and hard connective tissues, which have different acoustic properties and consequently transmit different intensity levels to component cells *in vivo*. Moreover, cavitation and shear processes on cells in tissue culture are generally enhanced beyond those found in bodily tissues (WFUMB 1998), and studies are now in progress to determine the cavitation, heating and radiation forces within the culture flasks in order to assess their effects on biological responses.

In conclusion, this paper demonstrates that FCM can be used to measure the precise effects of ultrasound on the production of key antigens by connective tissue cells *in vitro*, and thus on the functional activities of these cells. Thus the use of this technique may be of real value for assessing potentially beneficial and deleterious effects of ultrasound exposure at the clinical level, in order to improve the efficacy of therapeutic ultrasound for wound healing and tissue repair processes.

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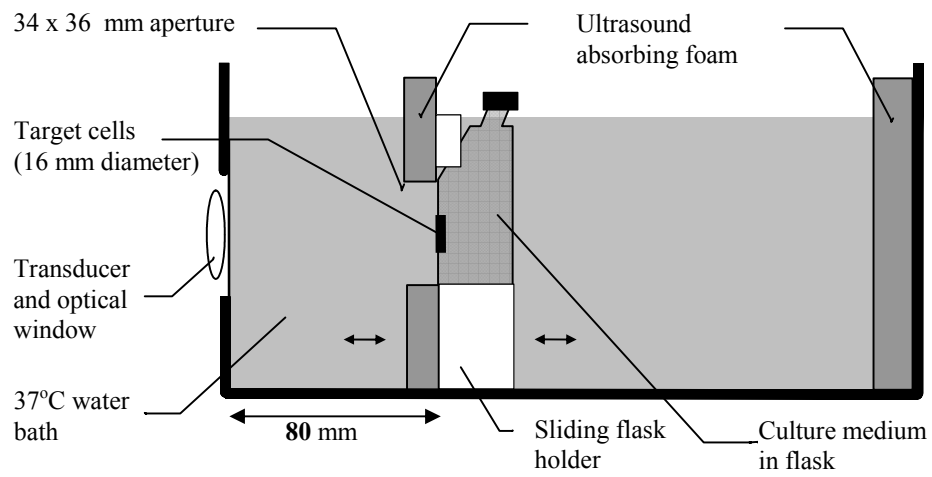
FIGURE LEGENDS

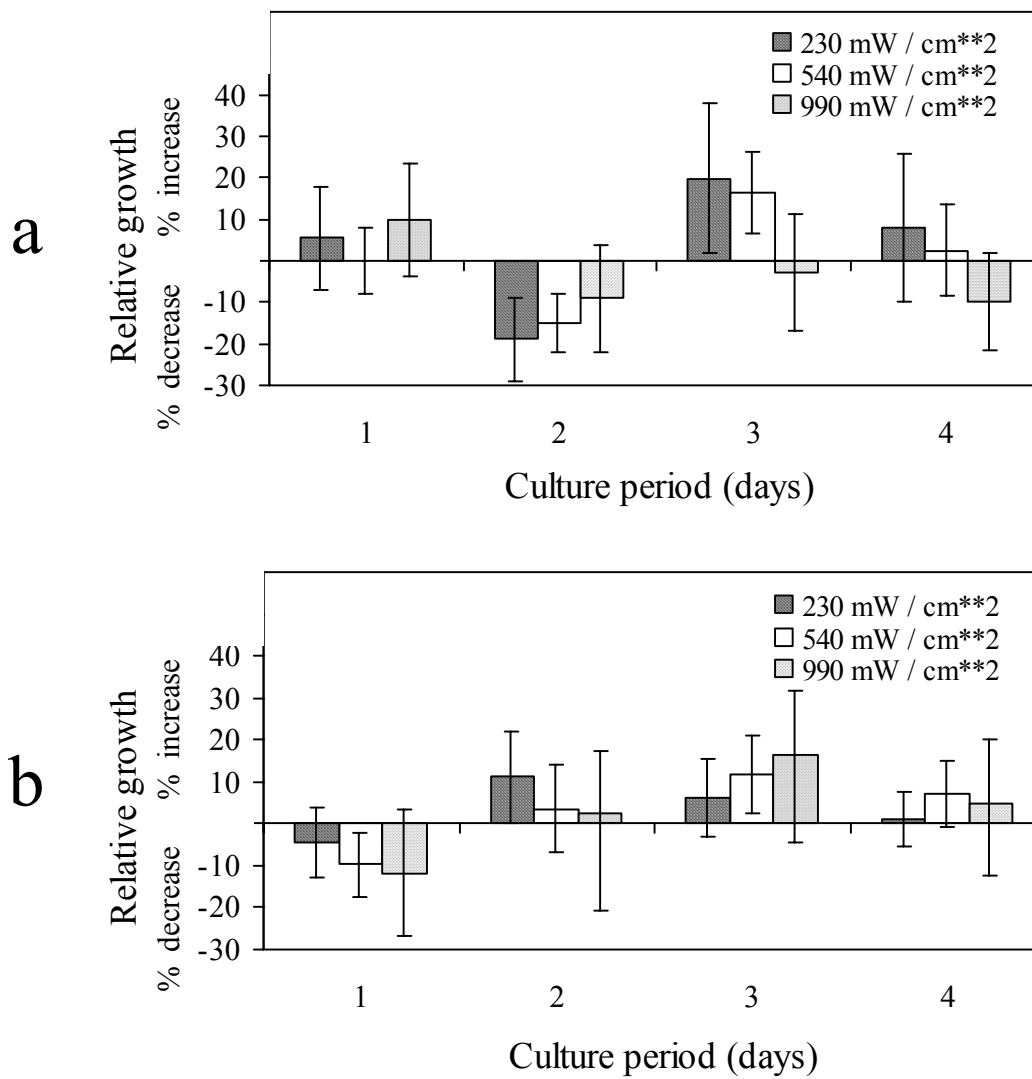
Fig. 1. Ultrasound exposure assembly. The culture flask, filled with medium, was positioned within the ultrasound at a distance (80mm) that optimised beam uniformity across the target cell region.

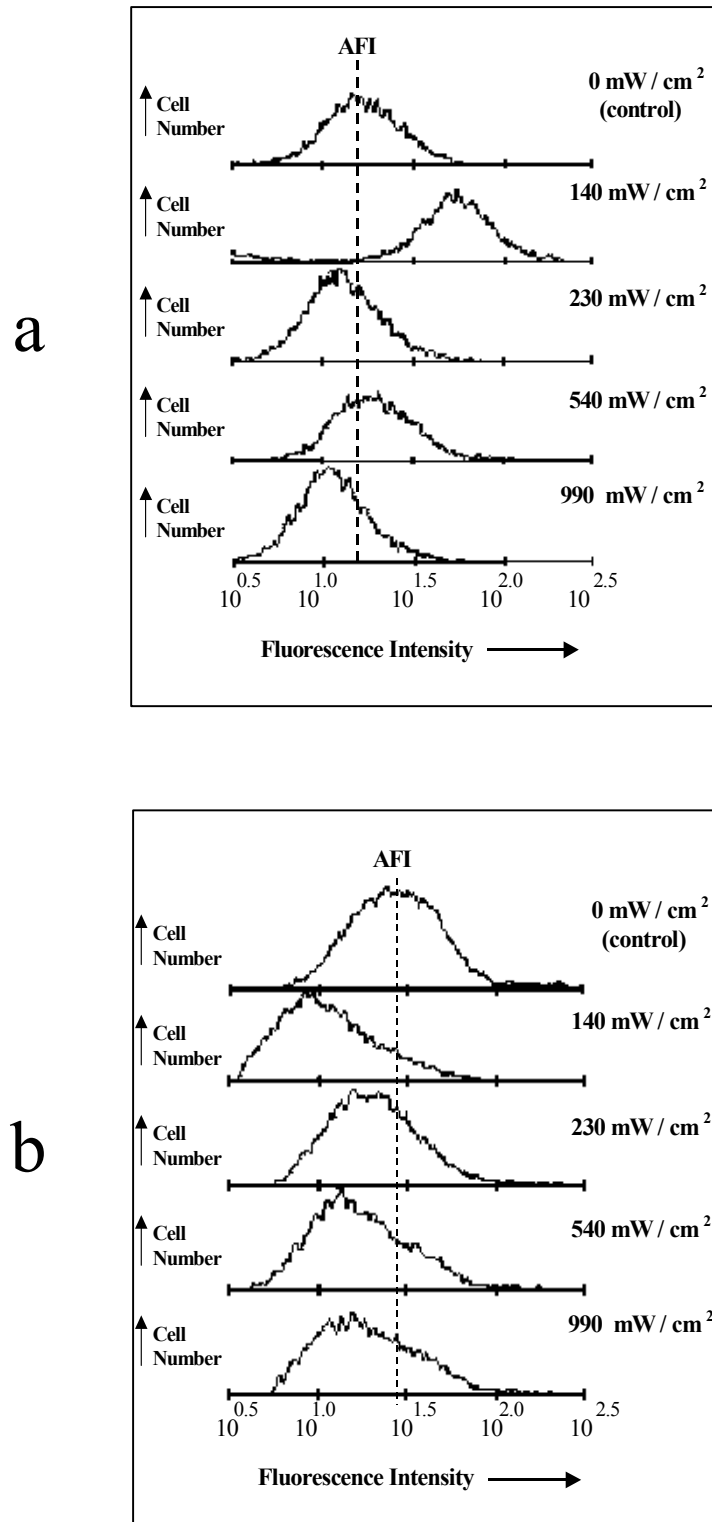
Fig. 2. Growth of MG63 cells (a) and PDL cells (b) following exposure to ultrasound. The rates of proliferation are shown relative to the control cells on each day following exposure to 230, 540 and 990 mW / cm² intensities of ultrasound. There was no significant difference in growth at any intensity level, compared with the respective non-exposed control cells ($p > 0.05$). Vertical lines are \pm SE.

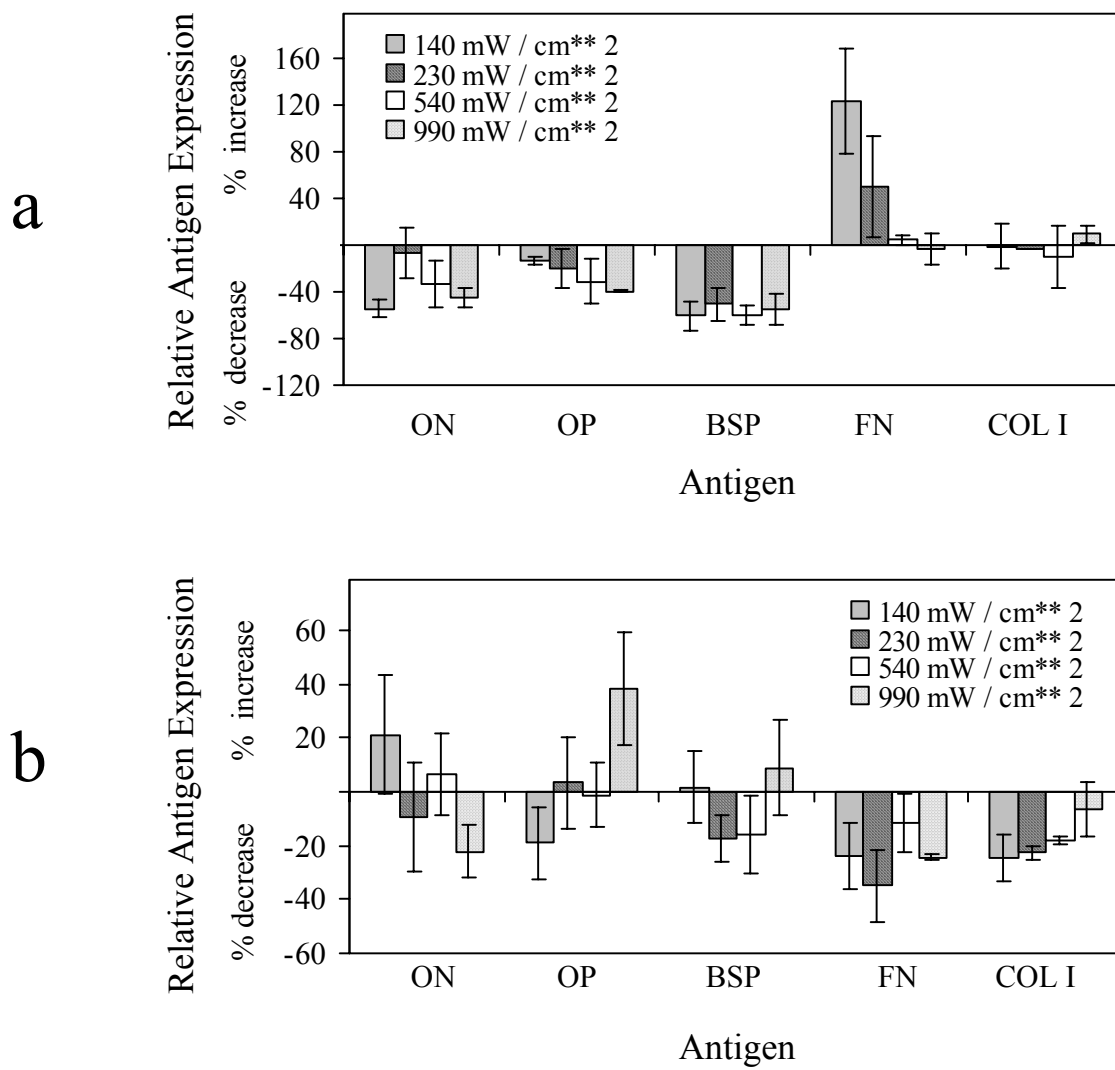
Fig. 3. Representative FCM analysis of FN expression by the MG63 cells (a) and PDL cells (b) five days after ultrasound exposure at 140, 230, 540 and 990 mW / cm². In (a), note the up-regulation of FN at low intensities and, in (b), the reduced expression of this antigen by the PDL cells at all ultrasound intensities, compared with the non-exposed control cells.

Fig. 4. Effects of ultrasound on expression of connective tissue antigens by the MG63 cells (a) and PDL cells (b), five days after exposure. The data shows the relative expression of each antigen in response to a range of ultrasound intensities, as described in Fig. 3, compared with non-exposed control. Note the antigen- as well as cell- specific responses to ultrasound. Vertical lines are \pm SE.

**Fig. 1.**

**Fig. 2.**

**Fig. 3.**

**Fig. 4.**