Effects of therapeutic ultrasound on osteoblast gene expression

J. HARLE
University Department of Radiology, The Radcliffe Infirmary, Woodstock Road, Oxford, OX2 6HE, UK

V. SALIH, J. C. KNOWLES
Department of Biomaterials, Eastman Dental Institute, University College London, 256 Gray's Inn Road, London, WC1X 8LD, UK

F. MAYIA
Department of Medical Physics, The Churchill Hospital, Headington, Oxford, OX3 7LJ, UK

I. OLSEN
Department of Periodontology, Eastman Dental Institute, University College London, 256 Gray's Inn Road, London, WC1X 8LD, UK

Ultrasound (US) is commonly used as a physiotherapy aid for a number of types of injury to soft connective tissues and for fracture healing. However, the precise effects of therapeutic US on tissue healing processes are not clearly understood, although they are likely to involve changes in key cellular functions. The present study has therefore examined the effects of several US intensity levels on the activity of two bone-associated proteins, alkaline phosphatase (ALP) and osteopontin (OP) in a human cell line, MG63, using RT-PCR. ALP showed progressively higher expression with increasing US intensities, whereas OP responded differently, showing down-regulation at 120 mW/cm^2, the lowest US exposure. OP expression was considerably less affected overall compared with the relative response of ALP to the same US doses. The results show that there is a differential response to therapeutic levels of US, since ALP and OP clearly exhibited gene-specific response profiles. These findings suggest that modifying the parameters of US exposure could be used to improve repair and regeneration processes and enhance the clinical efficacy of implanted biomaterials for tissue engineering.

1. Introduction

Therapeutic ultrasound (US) is a common form of treatment for the repair of damaged tissues. This non-invasive application of acoustic energy at low megahertz frequencies (up to 3000 mW/cm^{-2}) has been utilized in more than one million patient visits each year in the UK, constituting 20% of all treatments within hospital physiotherapy departments [1].

Beneficial responses to the application of US have been demonstrated in several soft tissue animal models, with improved healing rates [2] and tissue strength [3]. Clinical trials have also reported beneficial results at soft tissue wound sites such as venous ulcers [4] and the elbow joint [5]. US treatment of hard tissue injuries, such as bone fractures, have also proved highly successful, with markedly improved healing rates of human fractures (approximately 30–40%) [6, 7], while animal studies have provided evidence of enhanced fracture healing, particularly at the lower range of clinical intensity values [8, 9]. Moreover, the potential benefit of applying therapeutic US to improve the clinical efficacy of implant materials has been demonstrated by the improved rates of bone regeneration and bioabsorption of US-treated DP-Bioglass implants [10]. Improved rates and extent of bone ingrowth have also been reported using US-treated porous-coated implants [11].

Many additional studies and widespread clinical experience strongly suggest that the use of US to enhance tissue repair and regeneration processes is clearly of clinical value. However, application of this non-invasive procedure is limited because the mechanisms involved in initiating favorable cellular responses by ultrasonic stimulation are poorly understood and little is known about the optimum acoustical conditions which elicit beneficial responses. The aim of this study was therefore to determine the relationship between intensity levels of applied US and the activity of two bone-associated genes, alkaline phosphatase (ALP) and osteopontin (OP), which play fundamental roles in the growth and function of bone.

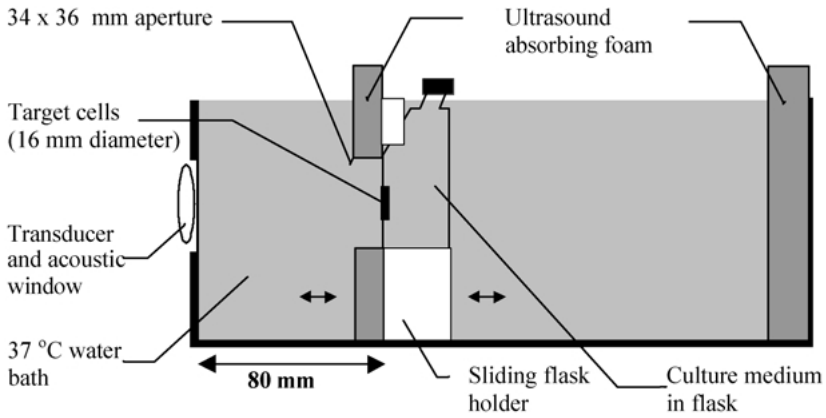

Figure 1 A schematic diagram describing the US exposure apparatus.

2. Materials and methods

The osteoblast-like cell line MG63 was seeded onto 10 cm^2 slide chamber flasks (Nunc, Naperville, IL, USA) and grown to confluence in medium consisting of 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 mg/ml of streptomycin (Gibco) at 37 °C in a humidified atmosphere of 5% CO_2 in air. The flasks were filled with medium and then exposed to 3.0 MHz ultrasound for 10 min, at intensity levels of 120, 390 and 1490 mW/cm^2. Control cultures received no exposure to US. Exposure was performed in a water bath exposure assembly, as shown in Fig. 1, and the cells were recultured. After 6 h, cells from within a 16 mm diameter central circular target region of the flask were detached by treatment with 0.25% trypsin/0.02% EDTA (Gibco) and analyzed as described below. A 1.0 mm-diameter-PVdF needle hydrophone, housed within a purpose built plotting assembly, was used to calibrate the ultrasonic field from a 25 mm diameter ultrasonic probe within the target area.

The reverse-transcription polymerase chain reaction (RT-PCR) was used to examine the expression of osteoblast genes by measuring the relative levels of specific mRNA gene transcripts in the cells. In this rapid and sensitive technique, a complimentary DNA (cDNA) strand is generated from the mRNA and amplified many times, producing sufficient material for semiquantitative analysis following gel electrophoresis of the RT-PCR product.

Cells within the slide chamber target region were harvested by trypsinization, centrifuged and RNA isolated by the single step method of Chomcynski and Sacchi [12]. The isolated RNA was then reverse-transcribed into first strand cDNA and the cDNA then amplified using oligonucleotide primers specific for the bone-associated proteins ALP and OP, as previously described [13]. Glyceraldehyde-3-phosphate de hydrogenase (GAPDH), a ''housekeeping'' gene whose activity remains constant, was also amplified as an internal control. The components of the reaction were then taken through a series of different temperatures for varying amounts of time, at the end of one cycle of amplification the amount of specific cDNA product having been doubled (10 cycles theoretically multiplying the product by a factor of approximately one thousand, 20 cycles by more than a million). The amplified products were added to individual lanes of an agarose gel and subjected to electrophoresis in order to separate, by molecular size, the amplified ''target'' gene cDNA from other components. Ethidium bromide was added to the gel as it binds to the DNA, which can then be visualized under UV illumination. RT-PCR of the GAPDH gene was carried out at the same time and under the same conditions as those for ALP and OP and all the gene products analyzed together, as described below.

After electrophoresis, image analysis of the gels was carried out using Scion Image software to record band intensities of the target genes relative to the background intensity level of the gel. From the area of the histograms, the ratio of ALP and OP band intensity (i.e. gene expression) in each sample was compared with that of GAPDH, at each dose of ultrasound, and is presented as the expression index (EI):

$$EI = (\text{Bone marker intensity/GAPDH intensity}) \times 100\%$$

For each EI, the relative expression of ALP and OP at each US exposure was calculated relative to the non-exposed control, defined as 100%.

3. Results

The results in Fig. 2 show agarose gels of the RT-PCR products of the ALP and OP genes and the corresponding GAPDH gels, obtained from the mRNA transcripts in cells incubated for 6 h after insonation with 0 (control), 120, 390 and 1490 mW/cm^2 at 3.0 MHz. The GAPDH band intensities remained at a very similar intensity at all doses of US and the same as control cultures which received no US exposure. However, the intensities of both the ALP and OP genes were found to vary markedly at different US intensities, as shown in Fig. 2. Thus, at US intensities 390 and 1490 mW/cm^2, the band intensities of both bone-related genes appeared to be elevated compared with the lowest (120 mW/cm^2) and no US dose. This effect of US on ALP is shown by the image analysis of the gels (Fig. 3A), and demonstrates that 120 mW/cm^2 had only little effect on the expression of this gene (10% increase). In contrast, the higher two doses progressively increased ALP gene activity,

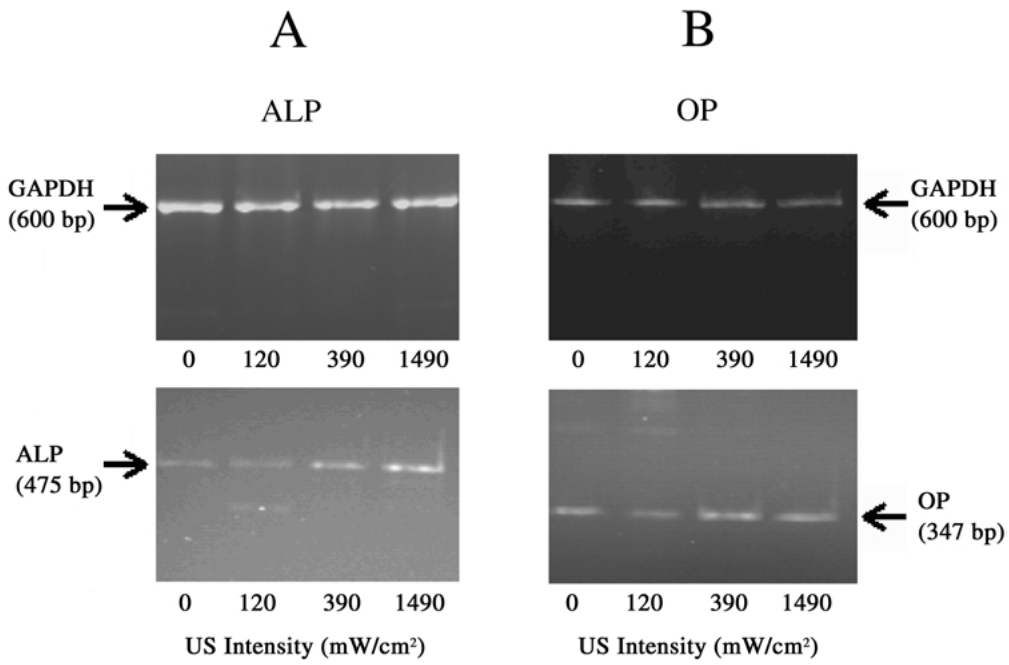

Figure 2 Agarose electrophoresis gels of the RT-PCR products of the ALP (A) and OP (B) genes in MG63 cells exposed to different intensities of US (0, 120, 390, 1490 mW/cm^2). The corresponding gels of the housekeeping gene, GAPDH, amplified from the same cDNA samples and used as an internal standard, are also shown. The size, in base pairs (bp), of each product is indicated.

reaching a level which was, after 1490 mW/cm^2 exposure, 420% greater than the non-insonated cells.

It is notable, however, that OP expression (Fig. 3B) responded differently to increasing US intensities. Thus, the activity of this gene was down-regulated at 120 mW/cm^2, to approximately 60% of the control cells which did not receive any US exposure. Moreover, at the higher doses of 390 and 1490 mW/cm^2, OP gene expression increased by 1.3- and 1.6-fold, respectively, considerably less than the relative response of the ALP gene to the same doses of US.

4. Discussion and conclusion

The clinical treatment of injured tissue sites by US is widespread, but little is known about the precise effects of US modalities on the fundamental processes that promote tissue repair and regeneration. We have previously shown, using flow cytometry, that US influences the production of extracellular matrix components *in vitro*, suggesting that it is likely to affect connective tissue integrity and function *in vivo* [14]. In the present study, RT-PCR was used to determine whether US also affected gene expression, by measuring the activities of 2 selected bone-related genes at different intensities of US. The acoustic parameters applied, encompassing values of 120, 390 and 1490 mW/cm^2, are comparable to those previously applied in clinical physiotherapy [1]. Exposure to these therapeutic doses of US was found to alter the expression of both the ALP and OP genes at 6 h post-insonation in osteoblast-like cells. The two highest doses showed that ALP and OP expression were clearly up-regulated, particularly ALP, whereas at the lowest dose of 120 mW/cm^2, the OP gene was down-regulated.

These results suggest that the clinical effects of US

A (ALP)

Intensity (mW/cm^2)	Band Intensity GAPDH mRNA expression	Band Intensity ALP mRNA expression	E.I.	% Change E.I.
0			13.2	100
120			14.7	110
390			32.6	240
1490			55.8	420

B (OP)

Intensity (mW/cm^2)	Band Intensity GAPDH mRNA expression	Band Intensity OP mRNA expression	E.I.	% Change E.I.
0			18.9	100
120			11.7	60
390			24.4	130
1490			30.7	160

Figure 3 Image analysis of the expression of the ALP, OP and GAPDH mRNA transcripts at different US intensities. The areas under the histograms have been measured and the EI calculated relative to GAPDH for each US dose. The % change is calculated relative to the control, defined as 100%.

could be mediated, at least partly, by altered expression of such genes, for example by up-regulating ALP and OP at higher US doses. However, the down-regulation of OP, which was observed at the lowest intensity exposure, suggests that the response of cells to US is highly dose-specific, as was shown by studies of fracture healing *in vivo* at the lower end of the therapeutic US intensity range [7].

The present findings also indicate that there is a differential gene response to US, since ALP and OP clearly exhibited gene-specific response profiles. The precise mechanisms underlying these differential changes in the expression of two fundamental bone components are still unclear. Nevertheless, it is possible that US exposure at clinical intensities may modulate gene transcription processes, perhaps by a mechano-transduction pathway, and suggests that modifying US exposure parameters could be utilized to optimize the clinical efficacy of US for engineering new bone and improving implant biocompatibility.

References

1. G. TER HAAR, M. DYSON and E. M. OAKLEY, *Ultrasound. Med. Biol.* **13** (1987) 659.
2. N. N. BYL, A. L. MCKENZIE, J. M. WEST, J. D. WHITNEY, T. K. HUNT and H. A. SCHEUENSTUHL, *Arch. Phys. Med. Rehabil.* **73** (1992) 656.
3. C. S. ENWEMEKA, O. RODRIGUEZ and S. MENDOSA, *Ultrasound. Med. Biol.* **16** (1990) 801.
4. F. JOHANNSEN, A. N. GAM and T. KARLSMARK, *Wound. Repair. Regen.* **6** (1998) 121.
5. A. BINDER, G. HODGE, A. M. GREENWOOD, B. L. HAZLEMAN and D. P. P. THOMAS, *Brit. Med. J.* **290** (1985) 512.
6. J. D. HECKMAN, J. P. RYABY, J. MCCABE, J. J. FREY and R. F. KILCOYNE, *J. Bone Joint Surg.* **76A** (1994) 26.
7. T. K. KRISTIANSEN, J. P. RYABY, J. MCCABE, J. J. FRAY and R. L ROE, *J. Bone Joint Surg.* **79A** (1997) 961.
8. A. A. PILLA, M. A. MONT, P. R. NASSER, S. A. KHAN, M. FIGUEIREDO, J. J. KAUFMAN and R. S. SIFFERT, *J. Orthop. Trauma.* **4** (1990) 246.
9. M. DYSON and M. BROOKES, *Ultrasound. Med. Biol.* **9** (1983) 61.
10. F. H. LIN, C. C. LIN, C. M. LU, H. C. LIU and C. Y. WANG, *Med. Eng. Phys.* **17** (1995) 20.
11. M. TANZER, E. HARVEY, A. KAY, P. MORTON and J. D. BOBYN, *J. Orthop. Res.* **14** (1996) 901.
12. P. CHOMCZYNSKI and N. SACCHI. *Anal. Res.* **162** (1987) 156.
13. D. J. RICKARD, M. KASSEM, T. E. HEFFERAN, G. SARKAR T. C. SPELSBERG and B. L. RIGGS, *J. Bone. Min. Res.* **11** (1996) 312.
14. J. HARLE, V. SALIH, F. MAYIA, J. C. KNOWLES and I. OLSEN, *Ultrasound in Med. Biol.* **27** (2001) 579.

Received 14 May
and accepted 30 May 2001