The Potential of Lithotripter Shockwaves for Gene Therapy of Tumors

Douglas L. Miller,* Richard A. Gies,* Brian D. Thrall,* and Shiping Bao†

*Battelle Pacific Northwest National Laboratory, PO Box 999, Richland WA 99352 and
†US Transuranium and Uranium Registries, Washington State University, Richland, WA 99352

Abstract: The shockwave-induced effects of cell lysis and sonoporation of surviving cells were investigated for possible application to anti-tumor therapy. Shockwaves were generated by a system similar to a Dornier HM-3 lithotripter. *In vitro* exposures of B16 melanoma cell suspensions containing a DNA reporter plasmid indicated significant transfection. Results were enhanced by leaving an air space in the exposure chambers to promote cavitation activity. *In vivo*, plasmids and air were injected into melanoma tumors before exposure. Significant luciferase production occurred for 200, 400, 800 and 1200 shockwaves with air injection. Results are encouraging for future development of simultaneous shockwave treatment and gene therapy of tumors.

INTRODUCTION

Lithotripter shockwaves cause cell lysis *in vitro*(1). In recent studies of whole blood, shockwave-induced hemolysis *in vitro* approached 10 % after 500 shockwaves, but could be enhanced to 59 % by addition of an air bubble to the exposure chamber(2). The latter observation indicates that the effect is indirectly induced by the shockwaves *via* cavitation activity, which is augmented by the added bubble. Cell lysis results from irreversible cell membrane damage which, in the case of erythrocytes, releases the hemoglobin into the surrounding medium. External molecules can also leak into injured cells, and remain trapped inside if the cells survive. This cell-permeabilization and re-sealing effect has been termed sonoporation. Shockwave-induced loading of cells with large fluorescent dextran molecules has been demonstrated in whole blood *in vitro* when a bubble was added to the exposure chamber to enhance cavitation activity (3). The sonoporation of erythrocytes was associated with hemolysis and also with microsphere formation. The number of surviving cells decreased exponentially, but the fraction of the surviving cells which were loaded with the macromolecule tended to increase with increasing numbers of shockwaves. This process can be described by a simple theoretical model, which explains the otherwise puzzling observation that the percentage of the initial cell number becoming fluorescent remained roughly constant for the range 250 to 1000 shockwaves (3).

Other large molecules including DNA, which are normally excluded by cells, can be loaded into cultured cells by shockwave exposure in the presence of the molecule(4). The transfer of external DNA into cells opens the possibility of gene transfer into the cells, and utilization of this phenomenon for gene therapy. However, the destructive cell lysis effect of shockwave exposure tends to limit the range of acceptable targets. One target for which significant tissue destruction can be acceptable is cancer. Research into applications of shockwave lithotripsy has suggested some promise in treatment of malignant tumors, which suffer mechanical damage *via* shockwave-induced acoustic cavitation(1). Gene transfer has received wide attention as a potential method of tumor therapy, but new ways to accomplish gene transfer to targeted regions *in vivo* are needed (5). Since shockwave treatment can produce both tissue destruction and gene transfer, the simultaneous application of gene therapy during shockwave treatment may be a plausible prospect for tumor therapy. In this paper, progress in understanding sonoporation and its application for tumor gene therapy are discussed.

METHODS

The B16 mouse melanoma, an established tumor model which has received attention in gene therapy research, was used to evaluate the potential for gene transfection into cancer cells(6). Cells were cultured by standard methods in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum. The cells could be exposed *in vitro* in suspensions or *in vivo* in tumors implanted in female C57BL/6 mice. The cells were implanted toward the left side of the abdomen, at 0.1 ml with 2 x 10^6 cells per mouse. A luciferase reporter vector (Control plasmid pGL3, Promega, Madison, WI) was utilized as the plasmid for insertion and subsequent transient expression within the cells, as described in earlier work (7).

The lithotripter system employed in this study was similar to a Dornier HM-3 lithotripter, and produced shockwaves

at a 2 Hz rate with a spatial peak pressure amplitude of 24.4 MPa (5.2 MPa standard deviation) peak positive and 5.2 MPa (1.3 MPa s.d.) peak negative. The water in the exposure bath was degassed and continuously filtered to minimize the occurrence of cavitation in the water, and maintained at 37 °C. Cultured cells were harvested and suspended at 2.5×10^6 ml^{-1} together with a final DNA concentration of 20 μg/ml. For *in vitro* exposure, 1 ml of the suspension was loaded into sterile polyethylene transfer pipettes, which constituted the exposure chambers. The pipette bulbs held about 1.2 ml, but only 1 ml of the suspension was loaded into the bulb, leaving a 0.2 ml air bubble which rose to the top of the chamber and served to enhance cavitation activity. After exposure, the cell suspensions were cultured for one day before assay of luciferase production.

Tumors growing *in vivo* were subjected to shockwave treatments 10-14 days after cell implantation. All *in vivo* procedures throughout the study were in accord with the guidance and approval of the institutional Animal Care Committee. After the tumor area was shaved and depilated, the volume of each tumor was estimated and a volume of 2 mg ml^{-1} DNA solution equal to 10% of the tumor volume was injected into the tumor. For some experiments, a volume of air equal to 10 % of tumor volume was also injected into the tumor to enhance cavitation activity. Finally, the mouse was mounted on a plastic board in the water bath for shockwave exposure. Either immediately or one day after exposure *in vivo*, mice were sacrificed by CO_2 asphyxiation for cell isolation and gene expression assay.

RESULTS AND DISCUSSION

In vitro exposure produced a dramatic reduction in one-day survival of B16 cells, similar to cell lysis produced in whole blood. Luciferase activity found after one day of culture increased with increasing numbers of shockwaves. These *in vitro* results confirmed the shockwave-induced transfection of the plasmid into B16 melanoma cells and the subsequent expression of the reporter gene product.

Results for exposure of tumors *in vivo*, followed by immediate isolation and culture of tumor cells for one day were less than for *in vitro* exposure but clearly demonstrated a statistically significant increase in luciferase production after shockwave exposure relative to sham exposures. Transfection was detected with DNA injection only, but air injection gave approximately a seven-fold enhancement in the results. Exposure to 200, 400, 800 or 1200 shockwaves with air injection yielded significantly increased luciferase production for all treatments relative to shams, but the effect was approximately constant over this range. The roughly constant transfection was consistent with the roughly constant cell loading found in erythrocytes and with the simple model of the lysis and sonoporation effects (4).

Exposure with the isolation of tumor cells delayed for a day to allow expression of the reporter gene within the growing tumors allowed the antitumor effect of the shockwaves, which can greatly reduce cell viability, to play a role in the results. Luciferase production was increased relative to shams with or without air for 100 and most 400 shockwave treatments.

These results demonstrated that transient transfection of reporter genes into melanoma cells can be induced by lithotripter shockwaves both *in vitro* and *in vivo*. The expression was maintained at higher exposures, which implies that higher treatment levels do not eliminate the transfected cells. Finally, the reporter expression persisted in most treated tumors for at least a day, which indicates a potential for carry-over of a gene-therapeutic effect through the tissue-ablation phase of the shockwave treatment. These results are encouraging indications for future development of simultaneous gene therapy and shockwave treatment of cancer.

ACKNOWLEDGMENTS

This investigation was supported by PHS Grant CA42947 awarded by the National Institutes of Health, DHHS.

REFERENCES

1. Delius, M. *Shock Waves* **4**, 55-72; 1994.
2. Miller, D. L. and Thomas R. M. *Ultrasound Med. Biol.* **22**, 1089-1095 (1996).
3. Miller, D. L., Williams, A. R., Morris, J. E. and Chrisler W. B. *Ultrasonics.* (in press).
4. Lauer, U., Burgelt, E., Squire, Z., Messmer, K., Hofschneider, P. H., Gregor, M. and Delius M. *Gene Ther.* **4**,710-715 (1997).
5. Roth, J. A. and Cristiano, R. J. *J. Natl. Cancer Inst.* **89**, 21-39 (1997).
6. Bao, S., Thrall, B. D., Gies, R. A. and Miller, D. L. *Cancer Res.* **58**, 219-221 (1998).
7. Bao, S., Thrall, B. D. and Miller, D. L. *Ultrasound Med. & Biol.* **23**, 953-959 (1997).