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The Cytocidal Effect of High Energy Shock Waves on Human Prostatic Tumour Cell Lines

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Abstract—*This report describes the effect of high energy shock waves (HESW) generated by a Siemens Lithostar on four human prostatic carcinoma cell lines* in vitro. *The effects of temperature, shock wave energy, cell density and the number of HESW were investigated. Pressure measurements were carried out in the focus of the lithotriptor and inside test tubes that were placed in the focus.*

Direct cell kill was inversely related to temperature, whereas a linear relationship was found with shock wave energy. Cell kill appeared to be independent of cell density. All four cell lines were sensitive to the treatment with HESW, but displayed a different dose–response pattern. In vitro *treatment of PC-3 cells retarded their growth upon injection into nude mice. It is concluded that human prostatic tumour cells are killed by HESW. Therefore, HESW could be of potential value in tumour treatment.*

INTRODUCTION

THE USE of high energy shock waves (HESW) has gained widespread application for the disintegration of renal and ureteral stones. Also, studies are in progress for the application of HESW to the treatment of patients suffering from stone disease of other organs, such as gall bladder and biliary duct [1, 2]. Treatment with HESW is generally considered to be a relatively safe procedure. The first reports on animal and clinical studies noted only mild damage to the surrounding tissues [3, 4]. Later reports, however, did mention more severe tissue damage, including focal fibrosis, haemorrhages, etc. [5–7]. Russo *et al.* [8–11] were the first to report on an inhibitory effect of HESW on tumour tissue. Their experiments included the assessment of the clonogenicity of the R3327-AT3 rat prostatic carcinoma and the SK-Mel human melanoma following HESW treatment. Both tumour lines were found to be highly sensitive to the administration of HESW. Meanwhile, several other authors have confirmed the cytocidal and cytostatic effect of HESW on various tumour cell lines [12–16]. Moreover, Randazzo *et al.* [14] reported a greater *in vitro* sensitivity to HESW of a renal cell carcinoma compared to a normal embryonic kidney cell line.

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In the present study the cytocidal effects of electromagnetically generated HESW on four human prostatic carcinoma lines *in vitro* are described. Data are presented concerning the shock wave energy, the effect of the temperature, the material of the test tubes containing the cells, the number of cells present during HESW treatment as well as the influence of the number of HESW administered.

MATERIALS AND METHODS

Cell lines

Four human prostatic carcinoma cell lines (DU-145, PC-3, PC-93, LNCaP-LNO) were used to study the effects of HESW. The cell lines were grown and maintained under standard culture conditions in RPMI-1640 medium (Gibco Europe, Breda, The Netherlands) supplemented with 10% foetal calf serum (Gibco) in a humidified incubator at 37°C and 5% CO_2 in air. Single cell suspensions were harvested from subconfluent cultures with 0.05% Trypsin–0.02% EDTA solution (Gibco). Viable cell counts were performed in a haemocytometer using trypan-blue exclusion as a parameter for viability.

Shock wave administration

HESW were generated electromagnetically with a Siemens Lithostar, which was made available to our laboratory by the Siemens company (Medical Division, Erlangen, F.R.G.). HESW were administered to cell suspensions in RPMI without further

additions in a Nunc 3-63401 cryotube completely submerged in a specially constructed and thermostatically controlled water bath. Control experiments indicated a large influence of air present in the test tube on the cell kill. This cell kill ranged from 60% in an air-free, completely filled tube (approx. volume 3 ml) to 100% in a tube only filled with 1 ml of suspension. Therefore great caution was taken against any air being trapped in the tube when closing the cap of the tube. Control tubes were placed in the corner of the water bath, outside the focus. In all experiments treated cells were returned to an incubator for 1 h before cell counts were performed.

Pressure measurements

In order to determine the relative acoustic impedance of the test tube and the attained pressure inside it, measurements were carried out in a water bath equipped with a tube holder with a three-dimensional micro-adjustment. The pressure was converted to an electrical signal by a piezoelectric micro-sensor (Imotec, Würselen, F.R.G.) which was further processed by a Gould 1072 oscilloscope. With the aid of this oscilloscope the waveform could be visualized and studied. The focus was determined by measuring the area with the highest pressure. The attained pressure in the focus was measured at different kV settings of the shock-wave generator between 13.3 and 19.0 kV. At 18.1 kV pressures were measured 10 times inside five different test tubes; a Nunc 3-63401 cryotube, a Greiner 162161 culture tube, a Costar 25310 culture tube, a Nunc RIA tube and a Falcon 2052 test tube.

Effect of the shock wave energy

This experiment was performed with the LNCaP line at a cell concentration of 2×10^6 cells/tube. Shock waves were given to the suspension as described above. The energy of the shock wave was controlled by altering the kV setting of the shock-wave generator from 13.6 to 18.1 kV with intervals of 0.6 kV.

Influence of temperature

Cells were submitted to 400 HESW at 10, 20, 30 or 37°C at a concentration of 2×10^6 per tube. The temperature of the water bath was regulated thermostatically. Control tubes for each temperature were placed outside the focus in a corner of the water bath and processed in a similar way as the treated tubes.

Influence of the cell concentration

To investigate the possible influence on cell damage of the cell density present during the administration of HESW, different quantities of cells were brought into the Nunc tubes. The cell concentrations tested included 1.0, 2.0, 3.0 and 4.0×10^6 cells/tube. Either 400 or 1000 HESW were given to the cell suspensions in the above described manner.

Dose–response curves

For all four cell lines, dose–response to HESW was determined. Nunc cryotubes containing 2×10^6 cells per tube were placed in the water bath in the focus of the Lithostar and 100, 200, 400, 600, 800 or 1000 HESW were delivered to the cell suspension at 18.1 kV and 37°C.

In vivo *experiment*

Subconfluent PC-3 cultures were harvested and 400 or 1000 HESW were delivered to the cells at a viable cell density of 2×10^6. The viable cell concentration after treatment was adjusted to 5×10^6 cells/ml for both control and treated cells, then 0.1 ml of this suspension was injected subcutaneously into nude mice. Tumour growth was monitored biweekly by caliper measurements.

RESULTS

A typical pressure wave profile generated at 18.1 kV is displayed in Fig. 1, as measured in water in the focus of the Lithostar. Characteristically, a large positive pressure peak is followed by a much smaller negative pressure peak.

Figure 2 demonstrates the relationship of the generated pressure in the focus with an increasing voltage setting of the shock wave generator. An increase from 18.1 to 19.0 kV did not yield a great rise in pressure, whereas the generator membranes seemed to wear out faster at this voltage. Therefore 18.1 kV was chosen as a standard kV setting for the generator.

Figure 3 shows the mean positive and negative pressure values of 12 consecutive measurements

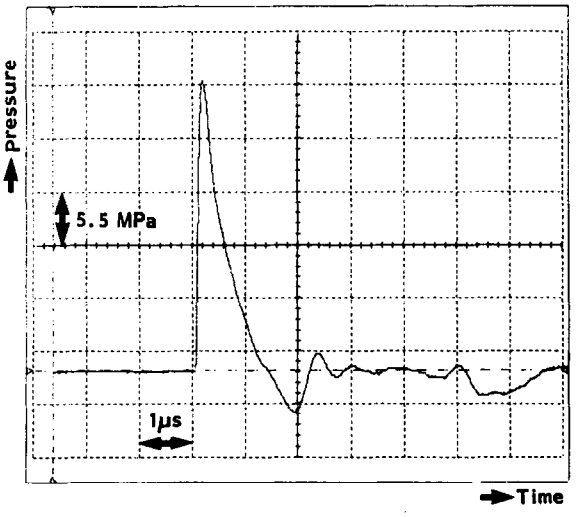

Fig. 1. Characteristic pressure wave profile as generated by the Siemens Lithostar at 18.1 kV.

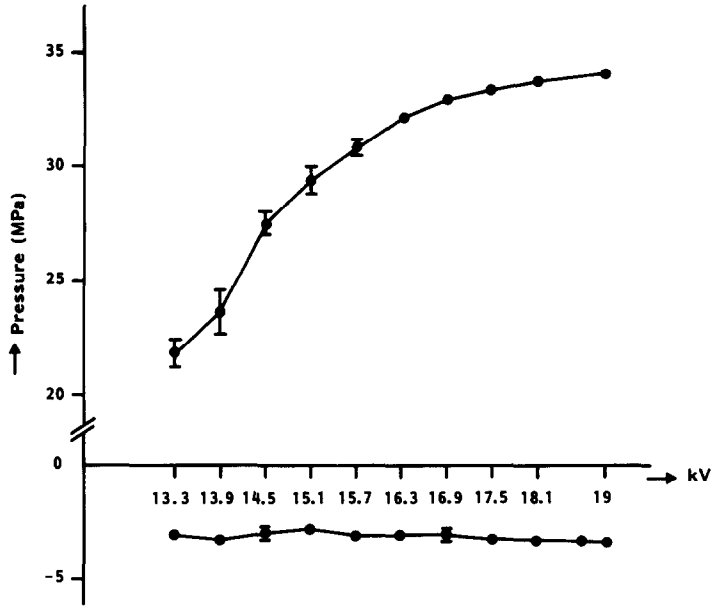

Fig. 2. The generated positive and negative pressure (MPa) *is shown as a function of the voltage* (kV) *of the generator. Each point represents the mean ± S.D. of* 10 *measurements.*

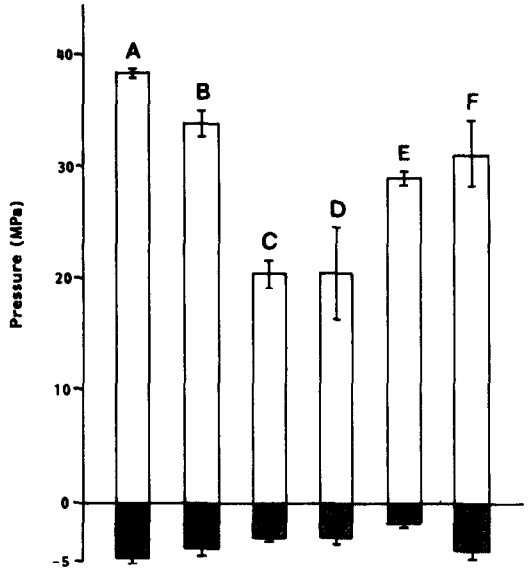

Fig. 3. Mean positive and negative pressures measured in the centre of a Nunc 3-63401 *cryotube (B), a Greiner* 162161 *culture tube (C), a Corning* 25310 *culture tube (D), a Falcon* 2052 *test tube (E) and a Nunc RIA tube (F). Bar A shows the pressure in the focus without tube. Each bar represents the mean ± S.D. of* 12 *consecutive measurements.*

inside five different types of test tubes in the focus. The Nunc 3-63401 cryotube appeared to have the best acoustic properties. It is also convenient in volume and fits completely within the focus of the Lithostar. Hence, this tube was chosen for all further experiments.

The inverse relationship between the cytocidal effect of 400 HESW and the temperature during treatment is evident from Fig. 4. Obviously, temperature strongly affects HESW-induced cell kill, since the effect at 10°C (54%) was almost three times as large as the effect at 37°C (19%).

The number of surviving cells decreased drastically with increasing shock wave energy. Figure 5 clearly illustrates a decrease for the LNCaP cell line from 63% survival at 13.1 kV to 11% survival at 18.1 kV.

The four assayed cell lines demonstrated a different sensitivity to HESW as illustrated in Fig. 6, where the percentage of surviving cells is given as a function of the number of administered HESW at 18.1 kV and 37°C. For all cell lines, an opposite relationship was found between the number of HESW and the number of surviving cells. The number of non-viable, trypan-blue stained cells observed after treatment was more or less constant, regardless of the number of HESW administered. Comparison of total cell counts (i.e. viable cells plus stained cells) after treatment with the initial cell number indicated that a number of cells had disintegrated.

For all four cell lines it was investigated whether the density of cells present in the original suspension is of importance for the number of cells killed after exposure to a fixed number of HESW at 18.1 kV. As shown in Fig. 7 for DU-145 cells there are no significant differences between the number of cells killed at cell concentrations in the range of $1–4 \times 10^6$ cells per tube.

The *in vitro* treatment of PC-3 cells with 400 or 1000 HESW resulted in a considerable growth retardation upon subcutaneous injection into nude mice (Fig. 8). The time required to develop palpable tumours (100 mm^3) was delayed from 44.4 ± 5.8 days for the controls to 62.5 ± 15.7 days and

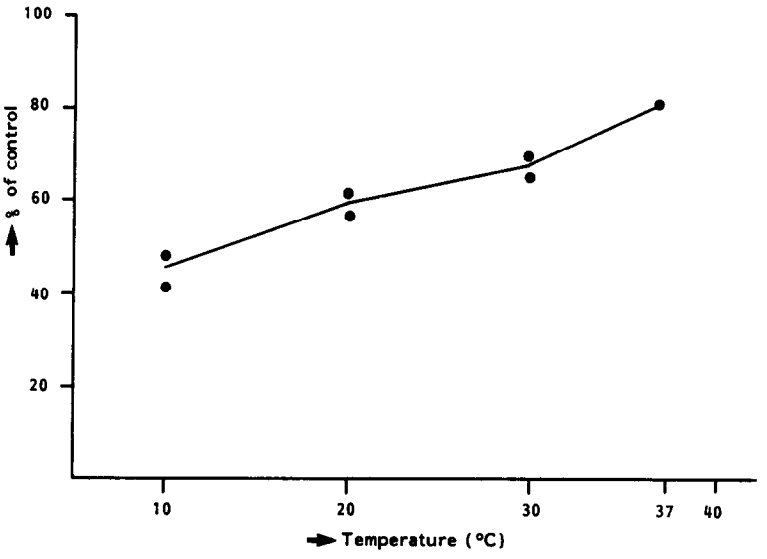

Fig. 4. Temperature dependence of DU-145 cell survival after 400 HESW expressed as a percentage of untreated controls.

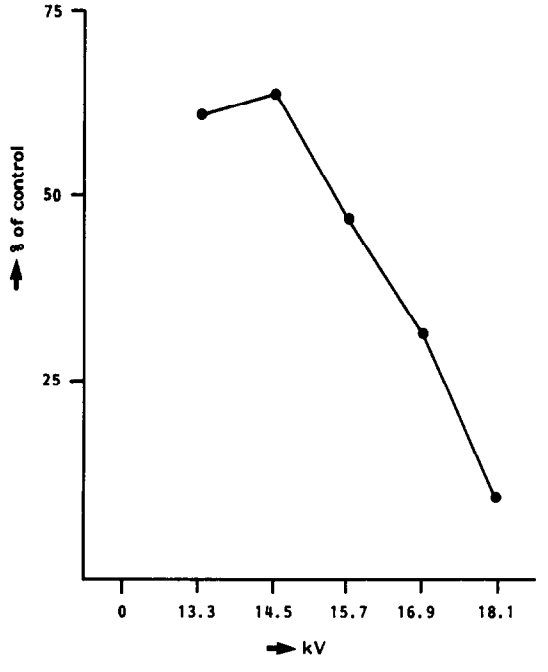

Fig. 5. Relation between shock wave energy (kV) *and LNCaP cell survival (percentage of initial cell number) at a fixed number of HESW of* 600.

59.0 ± 16.1 days for the groups that were inoculated with cells treated with 400 HESW and 1000 HESW respectively. The doubling time was not significantly delayed (9.8 ± 2.4 days for control vs. 13.8 ± 6.1 respectively 9.1 ± 3.9 days for treated cells).

DISCUSSION

The present results indicate that electromagnetically generated high energy shock waves can induce cell death *in vitro*. This is in accordance with earlier findings of Russo *et al.* [8–10], who noted an inhibition of clonogenic potential of rodent tumours by HESW. Other investigators also noticed a cytocidal effect of HESW produced by the electrohydraulic principle on tumour cells [12–16].

In the present study the cytocidal efficacy of electromagnetically generated HESW on four human prostatic carcinoma cell lines was investigated. All four cell lines showed sensitivity to some degree (i.e. in all cases cells were killed) to the administration of HESW.

With regard to their dose–response pattern, however, a marked difference existed between the PC-3 and the LNCaP cell lines. After receiving 200 HESW the surviving LNCaP cells seem to be resistant to HESW even up to 1000 HESW, whereas the disruption of PC-3 cells continues until, at 1000 HESW, only a few cells survive. The other two cell lines, DU-145 and PC-93, display an intermediate dose–response pattern compared with PC-3 and LNCaP. The outcome of these experiments is in accordance with the sensitivities for PC-3 and DU-145 reported by Russo *et al.* [9] following treatment with electrohydraulically generated HESW. The fact that with all cell lines, for all numbers of HESW, the amount of non-viable cells after treatment remains approximately equal, indicates that the destruction of the cells may proceed step by step. The absence of an influence of the number of cells present during HESW treatment does not support the hypothesis that the *in vitro* effects of HESW are mainly due to cell–cell and cell–tube collisions as postulated by Brümmer *et al.* [17]. The presence of more cells would presumably give more collisions and thus a higher cell kill.

From this study no conclusion can yet be drawn with respect to the mechanism of action of HESW-

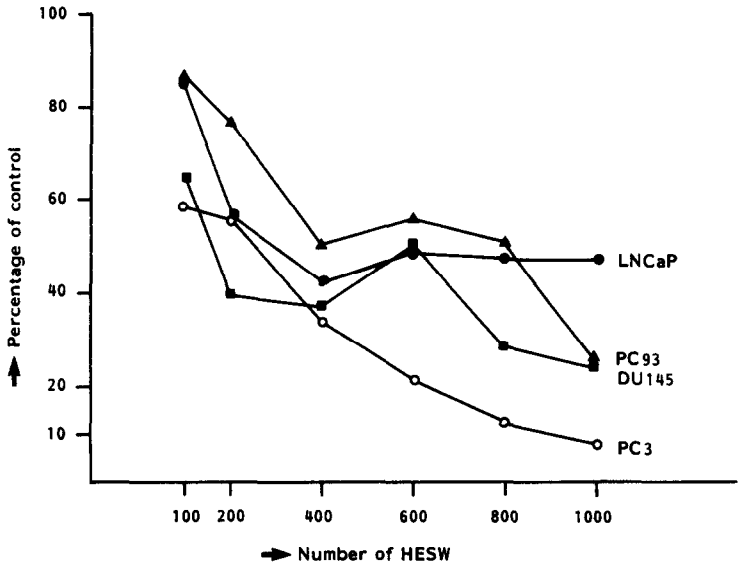

Fig. 6. Dose-dependence of cell survival on the number of HESW. Cell survival of the DU-145, LNCaP, PC-93 and PC-3 cell lines are expressed as percentage of untreated controls, at 37°C *and* 18.1 kV.

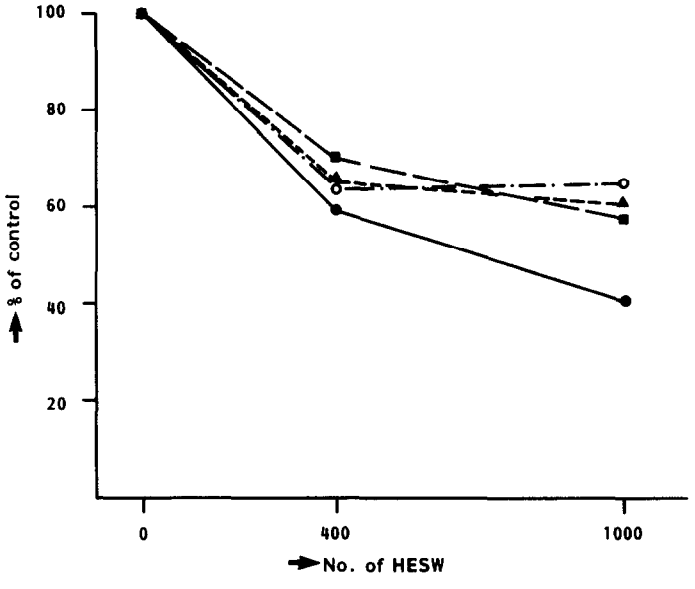

Fig. 7. Sensitivity of DU-145 to 400 *and* 1000 *HESW at cell concentrations of* 1 (○), 2 (●), 3 (▲) *and* 4 × 10^6 (■) *cells per tube at* 37°C *and* 18.1 kV.

induced cell kill. Morgan *et al.* [18] suggested that the formation of free radicals, caused by microcavitation, might be involved. These investigators were, in fact, able to show the formation of free radicals during HESW treatment. At the same time, however, they found no effect on the tumour cells treated with HESW, whereas those tumour cells were shown to be sensitive to ionizing radiation. This might indicate that the formation of such radicals is of minor importance to the direct cytocidal effect of HESW. The microcavitation by itself might on the other hand cause damage to membranes, as documented by Ellwart *et al.* [19] in studies on the effect of ultrasound, which is a form of energy related to HESW.

The inverse relationship between cell death after HESW treatment and the temperature during treatment is clearly demonstrated (Fig. 4), and is in accordance with the findings of Berens *et al.* [20] who reported a 10-fold enhancement of sensitivity to HESW of the SkMel cell line at 18°C vs 37°C. These observations suggest that the fluidity of the membranes may play a role, rather than hyperthermic or metabolic effects.

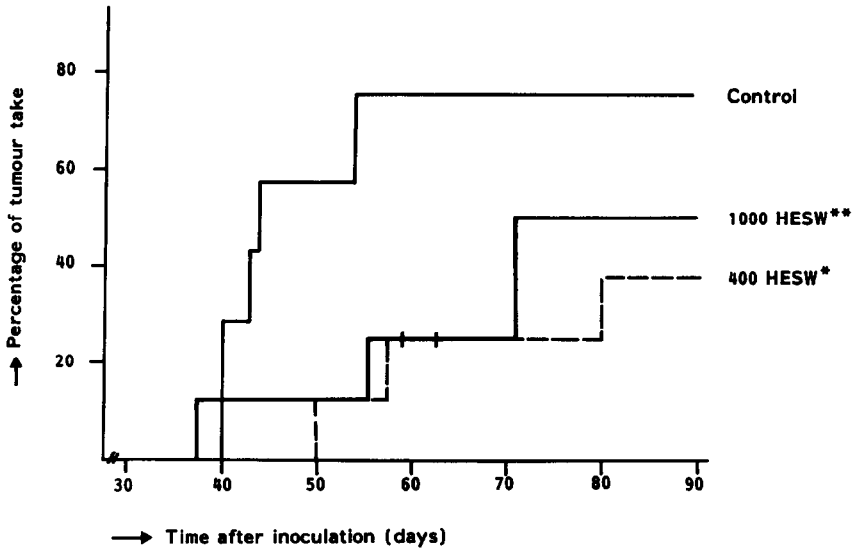

*Fig. 8. Cumulative tumour take rate of PC-3 cells injected subcutaneously into nude mice after treatment with 400 and 1000 HESW. Take was considered positive when a volume of 100 mm³ was reached (n = 8 for each group). *P < 0.05 for control vs. 400 HESW. **n.s.*

Further studies on the mechanism of HESW-induced cell kill are in progress. The results of the present investigation suggest the potential value of high energy shock waves in tumour treatment and encourage further investigations in this field, including studies of the possible synergistic action of cytostatic drugs and HESW.

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