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The in vitro effect of electromagnetically generated shock waves (Lithostar) on the Dunning R3327 PAT-2 rat prostatic cancer cell-line

A potentiating effect on the in vitro cytotoxicity of Vinblastin

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Summary. High energy shock wave lithotripsy has proven to be an effective tool in the management of renal calculi. The effects of electrohydraulically generated high energy shock waves (HESW) on tumor cells were described only recently. Here we present data on the experimental design for treatment of tumor cells, using electromagnetically generated shock waves. The dertermination of the focal area, in which pressures are at least 50% of the maximum pressure, appeared to be essential. In vitro HESW treatment resulted in a dose dependant anti-proliferative effect on Dunning R-3327 PAT-2 rat prostate cancer subline, determined by temporal growth curve analysis after plating of treated cells in soft agar. Furthermore, it was shown that HESW treatment had a potentiating effect on Vinblastin treatment. The combination of HESW with Vinblastin appeared to have an additive in vitro anti-proliferative effect on PAT-2 prostatic cancer cells.

Key words: High energy shock waves – In vitro cytotoxicity – Prostatic cancer – FCM analysis – Soft agar

Introduction

High energy shock waves (HESW) generated electrohydraulically (Dornier Lithotriptor) can alter growth characteristics of tumor cells in vitro and in vivo [2, 4, 14, 17, 19]. Thus, different tumor lines derived from prostatic carcinoma [16, 17, 18, 19], bladder tumors [4, 13], and ovarian tumors [2, 5] were studied. A direct tumor cytotoxic effect of electrohydraulically generated HESW was established by a decrease in cell viability as determined by trypan-blue dye exclusion and impaired clonogenic capability in soft agar.

Flowcytometric analysis of DNA content in Dunning R-3327 AT-3 cells 24 h after HESW exposure showed a decrease in the population of cells in the G2 and M phases of the cell cycle [6, 15, 17, 18, 20]. More recent studies, however, did not confirm these results [3, 14]. A direct effect on the cell membrane, mitochondriae and nuclear chromatin was found [14].

Additionally, cells pretreated with HESW can become more sensitive to cytotoxic chemotherapy as was shown by Chaussy et al. [4] and Lee et al. [7] in the treatment of bladder tumors and by Berens et al. [2] for ovarian tumors.

All these studies were done using the Dornier Lithotriptor (electrohydraulically generated shock waves) and no experiments with electromagnetically generated HESW's previously have been described.

We investigated the anti-proliferative effect of shock waves produced by the Lithostar (Siemens) extracorporal shockwave lithotriptor. To achieve an optimal experimental set-up for the HESW treatment, pressure profiles at the focus of the lithotriptor were determined. As a model system the Dunning R-3327 rat prostatic cancer subline PAT-2 was used for in vitro studies using HESW. Furthermore, to study whether HESW treatment made cells more sensitive to cytotoxic treatment the combination of HESW with Vinblastin was tested.

Materials and methods

Cell-line

The Dunning R-3327 PAT-2 rat prostatic carcinoma, was generously provided by Dr. John T. Isaacs (Baltimore, Md, USA).

The cells were cultured at 37°C and 6% CO_2 in RPMI-1640 medium (Gibco) enriched with 10% fetal calf serum, 1% L-glutamine, 1% penicillin/-streptomycin and 2.2% HEPES. Cells were fed every other day. When the mono-layer growth became confluent, the cells were trypsinized, using 0.25 mg/ml trypsine and 0.1% EDTA. Just before the HESW exposure a single cell suspension

containing 5×10^6 cells/ml was made by trypsinizing. This cell suspension was kept in a polyethylene test-tube (Greiner), submerged in water warmed to 37°C and positioned in the radiological focus of the lithotriptor.

Administration of HESW

For this study the Siemens Lithostar was used. For a precise and reproducable positioning of the test-tube a water-filed perspex container was developed in our labarotory. The shock wave tube was in contact with the water bath over a silicon membrane in the lateral side of the container. To ensure an optimal contact between the shock wave tube and the membrane of the container a lubricating gel was applied to the membrane. A specially constructed apparatus attached to this container was used to hold the test-tube in the axis of the focal area. For the in vitro experiments with the PAT-2 cell line 80 shock waves/min, at 19.0 kV (equivalent to 375 bar) were given. The exposed cells received different number of shock waves (1,000–3,000) and were compared with a control group which was placed outside the focal area.

Pressure measurements

Pressure measurements were determined using a piezoelectric crystal transducer (Imotec) connceted with a 100 mHz oscilloscope (Gould DSO, 4072). Pressures at different kilovoltages (kV) were registred and a field of relative pressures around the focus was determined by positioning the transducer at different sites.

Trypan-blue dye exclusion

Cell viability was determinded by adding 15 µl trypan-blue solution (25 mg in 5 ml 3% acetic acid) to 15 µl cell suspension and simultaneously counting coloured and not coloured cells using a Bürker Türk haemocytometer.

Cloning in soft agar

The anchorage-independent clonogenic potential was evaluated by the modified double layer soft agar culture system [22] originally described by Hamburger and Salmon [10]. In brief, 24 35 mm petridishes were seeded (2,000 cells/dish) with the cells in doubly enriched CMRL-1066 (Gibco) with 0.3% agar in the top layer, on a bottom layer of doubly enriched McCoy's-5A (Gibco) medium with 0.5% agar. Finally 0.2 ml doubly enriched CMRL with or without Vinblastinesulfate was administered to the dishes by an overlayer technique. The cultures were incubated at 37°C and 6% CO_2 in a humidified atmosphere. As a cytotoxic control $HgCl_2$ was used.

All colony counting was performed using the Omnicon FAS-2 automated colony counter [11]. Counting of the dishes at intervals of 3–4 days resulted in temporal growth patterns over a period of 21 days [7].

Flowcytometric analysis

A sample of approximately 3×10^6 cells was fixed for flowcytometric analysis immediately after treatment. Also, treated cells were incubated under standard conditions for 24 h. and 48 h. respectively, and then fixed for DNA analysis using flowcytometry (FCM). After centrifugation at room temperature, the cells were resuspended in 70% ethanol at −20°C by vortexing. As internal standard chicken red blood cells (CRBC) fixed in 70% ethanol were added to the tumor cell suspension up to a concentration of 10% [10, 21]. As an external standard, human lymphocytes were used for daily calibration of the flow cytometer. After removal of the fixative, cells were stained with propidiumiodide (A grade, Calbiochem-Behring, La Jolla, CA) in a 0.15 M sodium phosphate buffer (pH = 7.40).

Cells were incubated for 10 min with RNase (Sigma, St. Louis, MO) at 37°C and stored in the dark. Flow cytometric analysis was performed using a cytofluorograph 50H (Ortho Instruments, Westwood, MA) equiped with an Argon ion laser (Spectra Physics, Mountain View, CA) [8, 10]. Data were stored on a PDP 11/34 computer (Digital Equipment, Maynard, MA) and subsequently the cell cycle distribution was analysed by the method of Baisch et al. [1].

Results

Pressure measurements

Determination of the focal area. In order to determine an optimal and reproducable set-up for our experimental in vitro and in vivo studies we measured the pressures at different sites of the focus of the Lithostar. These measurements revealed that the site of maximum pressure was not identical with the radiological focus, but 10 mm away from the shock wave tube in the axis of the focus (Fig. 1 a). Moreover, still considerable pressures could be found several centimeters away from the radiological focus (Fig. 1 a), while in the lateral plane the pressures rapidly decreased 2–4 mm away from the radiological focus (Fig. 1 b).

As expected, the pressure depended largely on the voltage (kV) discharge applied (Fig. 1 a and b).

These pressure measurements indicate that it is not correct to speak about a focus, suggesting a high and effective pressure in a very limited area. We, therefore, defined the focal area at a certain kV discharge as the area limited by pressures which are half of the maximum pressure ($P_{max}\,50$) in that area (Fig. 1 c).

It appeared that at 18.1 kV the maximum pressure was 372 bar (measured in the axis of the shock wave tube, 10 mm distal from the radiological focus). Its focal area ($P_{max}\,50$), with pressures above 186 bar appeared to be about 100 mm long in the axial plane (Fig. 1 a) and 9 mm wide in the lateral plane (Fig. 1 b).

Absorption by the test tube. In vitro treatment of tissue culture cells requires the use of a container in which the cells are exposed to the HESW. Ideally, such a container should not absorb energy applied by the shock waves. It appeared that a polyethylene test-tube with a thickness of approximately 1 mm and rounded bottom

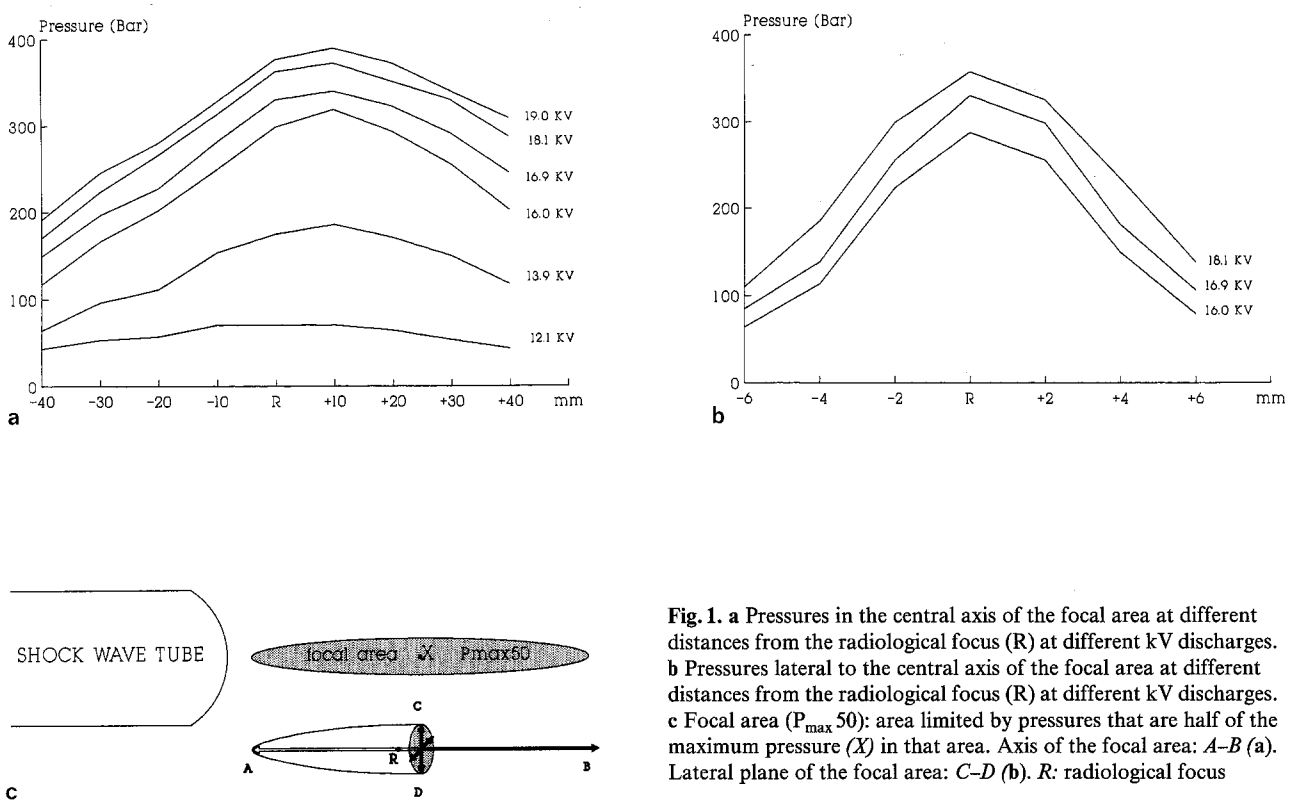

Fig. 1. a Pressures in the central axis of the focal area at different distances from the radiological focus (R) at different kV discharges. **b** Pressures lateral to the central axis of the focal area at different distances from the radiological focus (R) at different kV discharges. **c** Focal area ($P_{max}50$): area limited by pressures that are half of the maximum pressure *(X)* in that area. Axis of the focal area: *A–B* (**a**). Lateral plane of the focal area: *C–D* (**b**). *R:* radiological focus

met these demands. The pressure profile was measured inside the test-tube and at the same point without a test-tube. No significant differences in pressure were found; hence this polyethylene container with rounded bottom was used for our further studies.

In vitro HESW treatment of PAT-2 rat prostatic cancer cells

We have chosen the Dunning R-3327 derived PAT-2 prostatic cancer cell line for our first in vitro HESW experiments. This cell line grows with a doubling time of approximately 16 hours [12]. Upon injection of 1×10^7 tumorcells in Fisher-Copenhagen rats, an androgen independent growing tumor is palpable after 10 days. Therefore, this tumor is also useful for in vivo studies using HESW. Histologically the tumor is anaplastic, and it has a high metastatic capacity to lymph nodes and lungs [12].

Cell suspensions of the R-3327 derived PAT-2 rat prostatic carcinoma subline were exposed in vitro to different numbers of shock waves with 19.0 kV discharge. The effect of HESW on viability was established by trypan-blue dye exclusion and evaluation of clonogenic potential in double layer soft agar using a automatic colony counter. Trypan-blue dye exclusion tests were done immediately after the HESW exposure. Trypan-blue dye exclusion after 1,000, 1,500, 2,000 and 2,500 HESW showed a viability of 89%, 80%, 74%, and 67% respectively, compared to a 97% viability in the non-exposed control group.

Temporal growth curve analysis after plating HESW treated cells in soft agar showed a decrease in clonogenic potential, dependant on the number of shock waves exposed to the cells (Fig. 2).

After treatment of cell suspensions with 3,000 shocks, the clonogenic capacity after lating the cells, was completely impaired. This indicates that, while according to the trypan-blue dye exclusion assay 65% of the cells was viable, these cells had no clonogenic potential in soft agar, a characteristic feature of tumorcells. Therefore, the decrease in viability measured by trypan-blue dye exclusion was less informative than the decrease in clonogenicity established in soft agar.

Flowcytometric analysis

Several studies [6, 15, 17, 18, 20] indicated that HESW treatment resulted in a specific reduction of the frac-

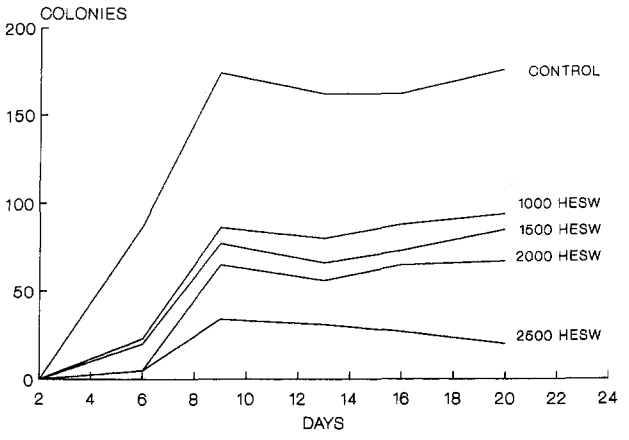

Fig. 2. Clonogenic assay (double layer soft agar) of the PAT-2 prostate cancer cell line after different number of shock waves

tion of cells in the G2M and S phase. The high proliferative capacity of the PAT-2 line makes this line very useful for a study on the influence of shock waves on cells in different stages of the cell cycle. Flowcytometric analysis was performed immediately after HESW exposure and 24 h and 48 h later. The results are shown in Fig. 3a and b. Only marginal effects of HESW on the fraction of cells in respectively G2M- (Fig. 3a), and G2M-plus S phase (Fig. 3b) are found. Unlike earlier findings [6, 15, 17, 18, 20] no decrease of cells that are proliferating could be established. Alternatively a slight increase of the fraction of cells in G2M plus S phase was observed, most noticeable after 24 h. This phenomenon however was also seen in the control group. Furthermore, HESW dosedependent increase of the fraction of cells in G2M-plus S phase was evident after 48 h. This effect was only found after administration of 2,000 and 2,500 shock waves.

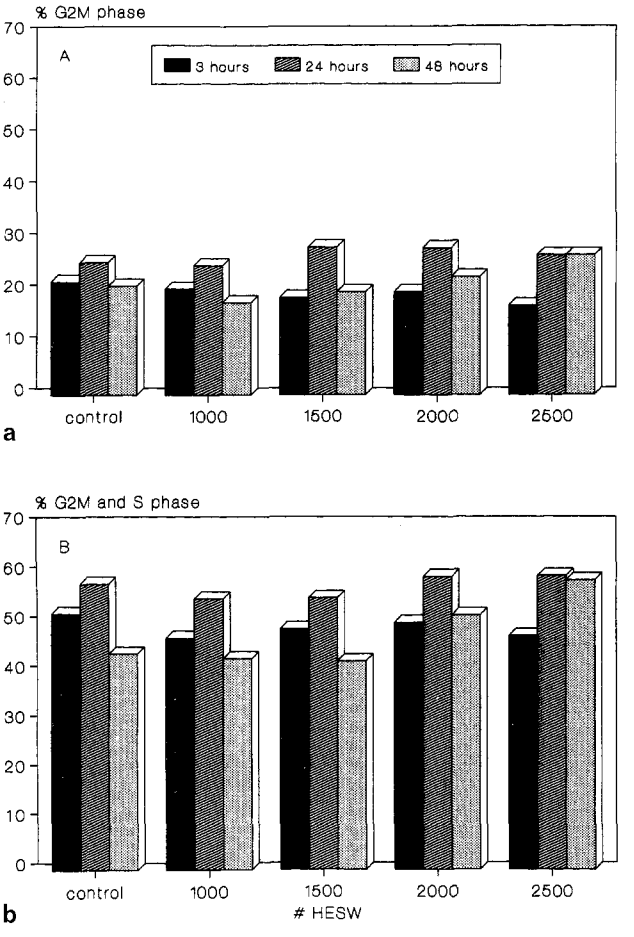

Fig. 3a and b. Flowcytometric analysis of the PAT-2 prostate cancer cell line at different hours (3, 24 and 48) after different number of shock waves. Percentage of cells in the G2M phase (**a**) and in the G2M plus S phase of the cell cycle (**b**)

Combination of in vitro HESW and Vinblastin treatment

The rapidly proliferating PAT-2 line, with a high fraction of cells in G2M plus S phase is likely to be sensitive to chemotherapy. In vitro studies done in our own labarotory on the Dunning R-3327 PAT-2 prostatic cancer cell line showed a cytotoxic effect of Vinblastin (unpublished data).

In order to be able to establish a possible potentiating effect of HESW on Vinblastin treatment, we exposed the PAT-2 tumor cell to the following treatment protocols, cell suspensions received 1,500 or 2,000 HESW, were plated in soft agar and after 24 h 0.005 or 0.01 μg Vinblastin/dish was administered by an overlayer technique. Other cell suspensions were

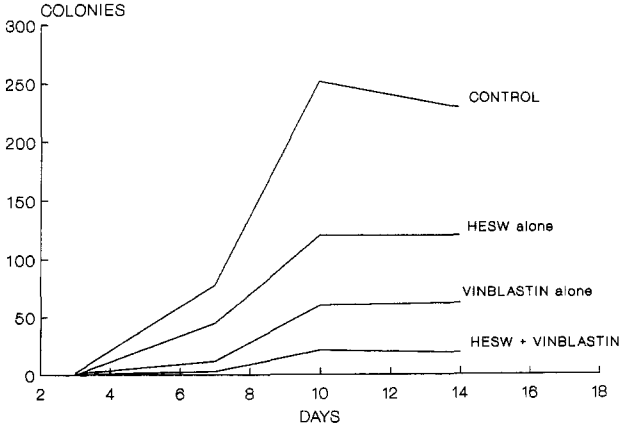

Fig. 4. Clonogenic assay (double layer soft agar) of the PAT-2 prostate cancer cell line exposed on day 0 to 1,500 shock waves, 0.01 μg Vinblastin dish or combination of both, compared to a control group, that received no treatment

Table 1. Growth potential (clonogenicity on day 14) as a percentage of an untreated control group. PAT-2 prostate cancer cell line exposed in vitro to different number of shock waves (1,500 or 2,000), different dosages of Vinblastin (0.005 µg/dish or 0.01 µg/dish) or combination of both

	1,500 HESW + Vinblastin 0.005 µg/dish	1,500 HESW + Vinblastin 0.01 µg/dish	2,000 HESW + Vinblastin 0.005 µg/dish	2,000 HESW + Vinblastin 0.01 µg/dish
Control	100	100	100	100
HESW alone	55	55	40	40
Vinblastin alone	42	27	42	26
HESW + Vinblastin	34	8	20	14

not treated with HESW and served as a control or were plated with the same dosages of Vinblastin. In this way, we could determine the clonogenic potential in soft agar after Vinblastin alone, HESW alone, the combination of both and compare it with the untreated tumor cells. The colony counting on day 14 was considered as the end point of this study, since the culture medium became exhausted by that time. As we knew from the in vitro no additional decrease in viablity could be expected.

These experiments indicate that HESW treatment can potentiate the cytotoxic effect of Vinblastin. Especially, 0.01 µg Vinblastin/dish showed a 3 fold enhanced colony inhibition in cells pretreated with 1,500 HESW (Fig. 4). Vinblastin (0.005 µg/dish or 0.01 µg/dish) could achieve a 50% decrease in the growth potential of cells exposed to 2,000 HESW (Table 1). The combination of 1,500 HESW and 0.005 µg Vinblastin/dish proved to be less effective (Table 1).

Discussion

Shock wave lithotripsy has proven to be a safe and effective treatment for nephrolithiasis, with few complications for the patient.

In 1985, Russo first described the in vitro and in vivo cytotoxic effect of HESW on tumor cells [15, 16, 17]. Since then many communications dealing with this topic have been presented at international urological congresses and have been published in abstract form than. Therefore, it was necessary to establish the pressures that are generated by the electronmagnetic lithotriptor and, to try to reproduce the cytotoxic effects of HESW on tumor cells as was described for electrohydraulically generated shock waves.

Pressure measurements on the Lithostar during shock wave exposure showed that, depending on the voltage discharge used, high pressures were generated in an area several centimeters long and several mm wide. With a 18.1 kV discharge (mostly used in stone treatment) approximately 5 cm before and behind the radiologically focussed point, pressures up till 185 bar could be measured. We therefore prefer to speak about *focal area* (Fig. 1c) rather than focus, which suggests a limited region of high pressure. If one defines the focal area as the field limited by pressures which are half of the maximum pressure (P_{max} 50) in that area, at 18.1 kV the focal area measures 100 mm × 9 mm (Fig. 1a and b).

At 14 kV the maximum pressure reaches 185 bar and the focal area measures 80 mm × 8 mm.

In order to be able to reproduce and compare results it is important to define the force exerted on tumor cells not only by the number of HESW but also by the pressure (bar) that reaches the cells. We could prove that in our experiments there was no absorption of energy by the polyethylene test-tube and, thus, the pressure applied to the cells was 375 bar (19.0 kV in the radiological focus).

Also for in vivo experiments it is of great interest to know the exact pressures in the focal area because even low pressures may cause damage to the lungs. In our first experimental animal studies it proved necessary to focus the tumor in the axial top of the focal area to avoid this complication, especially in fragile animals like the nude mice.

The dose-related reduction in viability from shock waves has extensively been demonstrated in different publications [17, 18, 19]. Our results also show that the electromagnetically generated shock waves (Lithostar) cause a reduction in viability, and an impaired clonogenic capacity as was described for the electrohydraulically generated shock waves.

In some publications viability was judged only by trypan-blue dye exclusion or colony counting after plating in soft agar at one specific moment. Trypan-blue dye exclusion proved to be less informative in determining the effect of HESW treatment on tumor cells.

The mechanism of action of HESW is unclear, and moreover it is unknown whether proliferating cells are

more sensitive than resting cells. The high proliferative capacity of the PAT-2 line, with about 50% of the cells in G2M plus S phase, makes this line very useful to study the influence of HESW on the fraction of cells in these phases of the cell cycle. Russo and Fair observed a decrease of the percentage of the cells in G2 and M phase of the cell cycle [6, 15, 17, 18, 20]. However Berens [13] and Loening [14] could not reproduce these findings. We also found that the percentage of cells in G2M- or G2M-plus S phase did not change significantly after shock wave exposure. There are indications that the cytotoxic effect of shock waves on the tumor cells is caused by damage to the cell membrane, mitochondriae and nuclear chromatin [14]. These findings need to be confirmed.

Vinca alkaloids like Vinblastin are known to be of little use in the treatment of disseminated prostatic cancer in man. The low fraction of proliferating cells in prostate cancer is most likely the explanation for the fact that, in general, human prostatic cancer is refractive to most chemotherapeutic treatments. Vinblastin had a cytotoxic effect in vitro on the PAT-2 cell line. Cells exposed to 1,500 or 2,000 HESW showed a 2 to 3 fould enhanced sensitivity to Vinblastin (0.005 or 0.01 μg/dish) treatment (Fig. 4 and Table 1) as was shown by temporal growth curve analysis after plating in soft agar. The effect of HESW plus Vinblastin appeared to be additive. The mechanism of action of this phenomenon is not clear. If there was direct damage of the cell membrane due to shock waves it is reasonable that any cytotoxic drug may cause an additional damage to the tumor cell. Further experiments are needed to confirm this hypothesis.

Since these results were obtained in vitro it is obvious that the damage caused by the shock waves is on a cellular level. In vivo, an additional effect of HESW on tumor growth may be found where shock waves cause damage to blood vessels. In pilot studies on rats we could not establish a significant effect of HESW on tumor growth, but histological examination showed extensive bleeding in the tumor.

Many technical problems remain to be solved before these results can be reproduced in the experimental animal model. It is, however, an exciting and challenging idea that, at least in vitro, electromagnetically generated shock waves may alter tumor cells in such a way that they become more sensitive to cytotoxic drugs.

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