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 determination 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 dependent 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 24h 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) extracorporeal 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% CO₂ 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 trypsin and 0.1% EDTA. Just before the HESW exposure a single cell suspension
containing $5 \times 10^6$ 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 reproducible positioning of the test-tube a water-filled perspex container was developed in our laboratory. 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 petridishes were seeded (2,000 cells/dish) with the cells in doubly enriched CMRL medium, 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 HgC1$_2$ was used.

**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 petri dishes 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 HgC1$_2$ 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 $3 \times 10^6$ 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 ($\phi H = 7.40$).

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) equipped 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 Baiisch et al. [1].

**Results**

**Pressure measurements**

Determination of the focal area. In order to determine an optimal and reproducible 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. 1a). Moreover, still considerable pressures could be found several centimeters away from the radiological focus (Fig. 1a), while in the lateral plane the pressures rapidly decreased 2-4 mm away from the radiological focus (Fig. 1b).

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

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. 1a) and 9 mm wide in the lateral plane (Fig. 1b).

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...