Shock Wave Permeabilization with Ribosome Inactivating Proteins: A New Approach to Tumor Therapy

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ABSTRACT

Extracorporeal shock waves are high-pressure pulses of microsecond duration clinically used for lithotripsy. Recently, shock waves have been shown to cause a transient increase of the permeability of the cell membrane. We therefore hypothesized that shock waves might be able to transfer tumoricidal agents into tumor cells and examined this in vitro and in vivo. In vitro, the ribosome inactivating proteins gelonin and saporin were transferred into L1210, SSK2, and HeLa cells, and dose-response curves were established. The drug concentration that reduced the cell proliferation by 50% (IC₅₀) was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and the enhancement factors from shock wave application were calculated. It was found that shock waves enhanced the action of gelonin from 900-fold in L1210 cells to 40,000-fold in HeLa cells and the action of saporin from 300-fold in L1210 cells to 15,000-fold in HeLa cells. In vivo, the effect of gelonin and saporin was assessed in a murine tumor model. SSK2 fibrosarcoma tumors locally grown in C3H mice were treated with shock waves after i.p. administration of gelonin or saporin. Shock wave application delayed the tumor growth, and long-term remissions lasting >180 days were induced in 40% of the animals. In conclusion, shock waves enhanced the action of ribosome inactivating proteins and led to complete tumor remissions. The local transfer of toxic substances by shock waves into tumor cells constitutes a new approach to a local tumor therapy.

INTRODUCTION

Extracorporeal shock waves are microsecond acoustic pressure pulses with peak pressures of 400-1200 atmospheres (1). Shock wave treatment was introduced by Chaussy et al. (2) and is the treatment of choice worldwide for urinary tract stones and a treatment alternative for most other types of stones in the human body, such as gallstones and pancreatic and salivary duct stones (3). In addition, shock waves have been applied recently to treat orthopedic diseases, such as nonhealing fractures or heel spurs (4, 5).

There have been attempts to use shock waves to treat solid tumors in animal experiments. These resulted in delayed tumor growth, yet complete remissions were rarely achieved (6, 7). Recently, shock waves have been shown to cause a transient increase in the permeability of the cell membrane when administered to cells in vitro (8). It does not lead to cell death and enables the transfer of large molecules and also of nucleic acids into cells (9). We hypothesized that shock waves might also act on tumor cells by transferring cytotoxic molecules into their cytoplasm. Ribosome inactivating proteins (10) were considered suitable for this purpose. These M₇₀, 30,000 plant proteins are not very toxic to cells, because they have no receptor at the cell surface. Once in the cytosol, they inhibit protein synthesis in minute concentrations and lead to cell death. Linking a ribosome inactivating protein with an antibody generates an immunotoxin against a surface constituent of a tumor cell. Immunotoxins are presently applied in clinical trials as a new approach to tumor therapy (11).

In the following report, we examined whether the ribosome inactivating proteins gelonin and saporin showed an enhanced action on tumor cells when shock waves were used to transfer them directly into the cytoplasm. The result of the in vitro experiments encouraged us to test in a further step whether a similar effect could be observed in vivo in a tumor model when shock waves were applied after systemic administration of gelonin or saporin.

MATERIALS AND METHODS

Cell Lines. L1210 mouse leukemia cells were cultured in RPMI 1640, HeLa cells in DMEM, and SSK2 mouse fibrosarcoma cells in MEM, all with 10% FCS added. The SSK2 cell line had been induced in a C3H mouse by methylcholanthrene for radiotherapy experiments (12) and has been used previously for in vivo experiments with shock waves (7). Its immunogenicity had been tested according to a standard protocol (13) by J. Kummermehr (GSF-Institute of Radiobiology, Neuherberg, Germany) and had been found to be very low. L1210 cells were kept as suspension culture, and HeLa and SSK2 cells were grown in subconfluence and harvested by trypsination.

Preparation of Ribosome Inactivating Proteins. Gelonin was prepared from Gelonium multiflorum seeds by the method of Stripe et al. (14). Briefly, seeds (Celo, Zweibrücken, Germany) were ground in 0.14 M sodium chloride with 5 mM Na₂HPO₄ and stirred overnight at 4°C. After removal of solids, the extract was spun for 20 min at 30,000 × g. The supernatant was dialyzed twice overnight against 5 mM Na₂HPO₄ (pH 6.5) and then centrifuged at 30,000 × g. After passing through a 0.45 µm filter, it was applied to a 40 × 26-mm column with Fractogel EMD COO⁻ 650 (Merck, Darmstadt, Germany) equilibrated with Na₂HPO₄ buffer. Gelonin was eluted with a Merck HPLC system in a stepwise 0–0.5 M NaCl gradient at a flow of 5 ml/min. The resulting four peaks were dia lyzed again overnight, lyophilized, and stored at −70°C. From 100 g of seeds, the average yield was 200 mg of protein (Lowry method).

Saporin was prepared from Saponaria officinalis seeds as described (15). The seeds (Jelitto, Schwarmstedt, Germany) were again ground in 5 mM Na₂HPO₄ (pH 7.2), stirred overnight at 4°C, and then spun for 20 min at 30,000 × g. After dialyzing twice overnight against 5 mM Na₂HPO₄ (pH 6.5) and centrifuging again at 30,000 × g, the extract was filtered through a 0.45 µm filter, applied to a 140 × 26-mm CM cellulosic column (Pharmacia, Freiburg, Germany) equilibrated with the Na₂HPO₄ buffer and eluted with a linear 0–0.5 M NaCl gradient at a flow of 3 ml/min. There were four peaks that were dialyzed, lyophilized, and stored at −70°C. Seeds (100 g) yielded an average of 800 mg of protein (Lowry method).

SDS-PAGE. All peaks were examined by electrophoresis and revealed single bands with M₇₀, 29,000 and M₇₀, 31,000. All were then further examined by MTT test (see below) with HeLa cells and shock waves to determine their specific activity. Briefly, 10⁶ cells were incubated with a 10⁻⁶ M dilution of lyophilizate of each peak and subjected to 250 shock waves at 25 kV. The subsequent proliferation of the cells was assessed as described in “Results.” All peaks were found to have near-identical activity against each other; this enabled pooling of them. Screening by reticulocyte lysate assay by F. Stripe (Institute of Experimental Pathology, University of Bologna, Italy) revealed a suppression in the expected range (10).

Shock Wave Generation. Shock waves were generated by an XL1 experimental lithotripter (Dornier Med Tech, Gernering, Germany) by underwater spark discharge in a set-up shown in Fig. 1. This set-up had been created originally for patient lithotripsy.

In Vitro Treatment of Tumor Cells. Dose-response curves were established for gelonin and saporin over the whole concentration range, both with

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and without shock wave exposure. Cell suspension and medium with gelonin or saporin were mixed together to a final concentration containing $1 \times 10^6$ cells/ml and $10^{-3}$ to $10^{-12}$ M toxins/ml. Ricin ($10^{-6}$ to $10^{-12}$ M) was used in an additional experiment. The solution was filled into 2-ml polypropylene vials (Nunc, Roskilde, Denmark). They were closed without residual air with a silicone stopper perforated with a butterfly to prevent the generation of excess pressure within the vial (16). Shock wave exposure was in the lithotripter with 250 waves at a voltage of 25 kV. The protocol was similar for the specimens without shock wave exposure. To normalize for the effect of shock wave exposure, which lowers cell proliferation slightly, vials with cells without toxin were exposed to shock waves in the same way, and additional vials with cells were not exposed to them. The exposure period of the cells to the ribosome inactivating protein was 40 min both with and without shock wave application. Preliminary experiments had shown that shock wave application at the beginning or the end of the exposure period had an identical effect.

After exposure to shock waves as well as after exposure without them, the cells were washed three times to remove any residual toxin and counted in a hemocytometer. In general, 70–95% of the cells were recovered after shock wave exposure, and 5–30% were lost. The cell viability was determined by trypan blue exclusion; after shock wave exposure, it was 80–95%, and without shock waves, 90–100%. There was no difference between vials exposed with toxin or without it.

### Table 1

**In vivo determination of the toxicity of gelonin and saporin as described in “Materials and Methods”**

The agent was administered in 100 μl of PBS as a single i.p. injection. Death occurred 5–12 days later, and survival lasted >50 days.

<table>
<thead>
<tr>
<th>Gelonin (mg/mouse)</th>
<th>Survival after 2 weeks</th>
<th>Saporin (mg/mouse)</th>
<th>Survival after 2 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 mg (75 mg/kg)</td>
<td>0/5</td>
<td>1 mg (25 mg/kg)</td>
<td>0/4</td>
</tr>
<tr>
<td>2 mg (50 mg/kg)</td>
<td>4/5</td>
<td>0.3 mg (8 mg/kg)</td>
<td>1/4</td>
</tr>
<tr>
<td>1 mg (25 mg/kg)</td>
<td>5/5</td>
<td>0.1 mg (2.5 mg/kg)</td>
<td>4/4</td>
</tr>
<tr>
<td>0.3 mg (8 mg/kg)</td>
<td>5/5</td>
<td>0.03 (0.8 mg/kg)</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.01 (0.25 mg/kg)</td>
<td>4/4</td>
</tr>
</tbody>
</table>

**MTT Test.** Viable, trypan blue-negative cells were adjusted with medium to a concentration of $10^6$ cells/ml, and $10^3$ cells were plated in 10 μl with 50 μl medium into microtiter plates. L1210 cells were incubated for 72 h, and SSK2 and HeLa cells for 96 h, to allow more than six cell divisions. Cyto-toxicity was examined by measuring cell viability by the tetrazolium salt MTT test as described (17). Fifty μl MTT (2.5 g/l in 0.9% sodium chloride; Sigma, Taufkirchen, Germany) was added and incubated for 4 h at 37°C. After dissolving the precipitated dye in 100 μl of DMSO (Merck, Darmstadt, Germany), the absorption was read at 540 nm in an ELISA reader (SLT Lab Instruments, Crailsheim, Germany).

**In Vivo Tumor Model and Shock Wave Treatment.** The animal experiments were approved by the Government of Upper Bavaria in Munich. The animals were cared for at the Institute according its approved guidelines. The SSK2 fibrosarcoma is an invasively growing tumor adapted to the *in vivo* situation that does not metastasize (7). In a brief ether anesthesia, $2 \times 10^3$ SSK2 tumor cells were injected s.c. into the lower back of C3H mice (Charles River, Kissing, Germany). Seven or eight days later, the tumor under the dorsal skin had reached a diameter of 5–7 mm. Anesthesia was performed with 1.65 mg of pentobarbital (Nembutal; Sanofi, Hanover, Germany) and in 110 μl of saline i.p. Gelonin or saporin was injected i.p.; the animal was then placed into a chamber shielded with a styrofoam pad, and the tumor and the dorsal skin were pulled out of a slit and fixed as in Fig. 1B. The tumor was then put exactly into the focus of the lithotripter water bath, and 500 shock waves were administered at a rate of 100 waves/min. Previous experiments had established that lung hemorrhage, a common consequence of shock wave administration, did not occur with this protocol.

Three experiments were conducted, two with gelonin and one with saporin. Each experiment consisted of four treatment groups. Two groups were treated with shock waves, one with ribosome inactivating protein and the other with saline. The two remaining groups were treated without shock waves, one with ribosome inactivating protein and the other with saline. In the first experiment, treatment was performed with 500 shock waves at 20 kV; in the second and third experiments, three treatments were performed with 500 shock waves at 20 kV and a 2–3-h interval between treatments.

The toxicity of gelonin and saporin was determined in a preliminary experiment in C3H mice by i.p. administration of the doses shown in Table 1. Twenty-five mg/kg of gelonin and 2.5 mg/kg of saporin did not lead to death of an animal. This was in agreement with values reported previously (18). No animal death was observed after these doses in all three experiments.

**Evaluation of Tumor Growth.** Tumor diameters in the long (l) and short perpendicular (b) axes and tumor height (h) were determined twice weekly with calipers, and the tumor volume was calculated by the formula $V = \frac{1}{2} \times b \times h \times 0.873$ as reported (7). A growth curve was recorded for each animal. When the tumor reached a volume of $\geq 2.5 \text{ cm}^3$, the animal was killed. In cases of complete tumor regression, there was a 200-day observation period for possible tumor recurrence.

**Statistical Analysis.** The number of experiments for the dose-response curves was less than or equal to five for each substance, each cell line, and each drug concentration, both with and without shock wave treatment. Nonlinear regression analysis for a sigmoidal dose-response with variable slope was performed with the software PRISM (Graphpad Software, San Diego, CA) to generate curves and to calculate IC$_{50}$. In the *in vivo* experiments, the software PRISM was used to generate Kaplan-Meier curves, with the event being a...
RESULTS

In Vitro Enhancement of Gelonin and Saporin by Shock Waves.

Shock wave exposure of cells without toxin reduced the cell proliferation to 80–95% of the nonexposed cells. For the evaluation of the interaction of shock waves and toxin, the proliferation of the exposed cells was normalized for the shock wave effect. Fig. 2 shows the dose-response curves of gelonin and saporin with shock waves. Each row in the figure depicts one of the three cell lines; the two columns delineate which protein was used. Each curve depicts, on the right, the cell proliferation in the MTT test without shock wave application in comparison with the curve after shock wave application on the left. Without shock waves, all three tumor cell lines had IC50s in the $10^{-4}$ M range with no obvious difference between gelonin and saporin. Shock wave permeabilization shifted the proliferation curves to considerably lower IC50s. The enhancement factors obtained by shock waves with gelonin and saporin and their confidence intervals are shown in Table 2. In L1210 cells, they were 890 and 290. In the other cell lines, they were higher and reached four orders of magnitude. The factors obtained with shock waves and gelonin were about three times lower than those with saporin. The largest factor was found in HeLa cells, where the action of gelonin was found to be 40,000-fold enhanced. The cell proliferation was inhibited with pm concentrations of the proteins. A similar result was obtained with K562 cells, where the enhancement factor exceeded 50,000 (data not shown).

Gelonin and saporin are type 1 ribosome inactivating proteins and have no cell surface lectin receptor. High concentrations in the millimolar range were needed to detect cytotoxicity in the controls, and they are achieved by the excellent solubility of these proteins. Their low toxicity requires the uptake of a very high amount of

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Gelonin</th>
<th>Saporin</th>
</tr>
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<tbody>
<tr>
<td>L1210</td>
<td>890</td>
<td>290</td>
</tr>
<tr>
<td>SSK2</td>
<td>18750</td>
<td>6776</td>
</tr>
<tr>
<td>HeLa</td>
<td>39580</td>
<td>12022</td>
</tr>
</tbody>
</table>

Table 2. Enhancement factors of gelonin and saporin by shock waves in the three cell lines examined

The data were obtained by nonlinear regression analysis as described in “Materials and Methods.”

- Mean (IC50) from shock wave application.
- CI, confidence interval.
molecules by endocytosis to give a few of them the opportunity to reach the cytoplasm and lead to cell death (19). To examine this further, the effect of ricin was evaluated. This is a type 2 ribosome inactivating protein and one of the most potent toxins known. It is composed of two protein chains, an A and a B chain. The former binds to a surface receptor that is internalized and reaches the cytoplasm via the trans-Golgi network (20). To test whether the direct transfer by shock waves was more effective than receptor-mediated transport, we examined ricin with and without shock waves in L1210 cells. According to Fig. 3, there was only a marginal effect because the enhancement factor by shock waves was only three.

In Vivo Action of Gelonin and Saporin with Shock Waves on a Small Animal Tumor. In vivo experiments were conducted after it became obvious that membrane permeabilization with shock waves was effective in vitro. A fibrosarcoma invasively growing under the dorsal skin of mice was treated by shock waves. The ribosome inactivating protein was given i.p. before shock wave treatment.

In the first experiment, gelonin was injected, and a single shock wave treatment was performed. The resulting tumor growth curves in the four treatment groups are shown in Fig. 4. Shock wave treatment only or gelonin injection only had no influence on the tumor growth when compared with the untreated controls. The combination of the two therapies had also no influence on it. The end point of the experiment, a tumor volume >2.5 cm³, was reached in all groups after a similar period of time.

Previous experiments had demonstrated that multiple shock wave treatments can influence the tumor and inhibit its growth (7). Therefore, the experimental protocol was changed in the next experiment, and three consecutive shock wave treatments were performed. The resulting growth curves of the tumors after administration of shock waves and gelonin are depicted in Fig. 5. Administration of gelonin alone or shock wave application alone had no influence on the growth of the tumor when compared with the untreated controls. The combination of gelonin and shock waves, on the other hand, delayed the tumor growth. There was no tumor regrowth over the following 180 days in 40% of the animals. This difference was significant in the log-rank test for trend \((P = 0.004)\). The experiment was repeated with saporin, and its result is shown in Fig. 6. There was again no tumor regrowth in 40% of the animals. The number of animals per group had, however, been slightly smaller in this experiment, and the difference in survival was not significant in the log-rank test for trend \((P = 0.14)\).

DISCUSSION

In Vitro Results. Extracorporeal shock waves could be used in vitro to transfer highly toxic molecules into tumor cells, where they...
enhanced the cytotoxicity by four orders of magnitude. The enhancement factors exceeded by far those found previously with other cytostatics such as anthracyclines and cisplatin, when shock waves were applied to different tumor cell lines (21, 22). A similar enhancement of a drug action by acoustic means has not been described to our knowledge. In principle, shock wave permeabilization with direct transfer of a drug or metabolite into the cytoplasm enables a broad screening of almost any other water-soluble chemical compound for a possible toxic activity in the cytoplasm of tumor cells. This might aid drug design and help to find out mechanisms of action.

The mechanism of shock wave action is cavitation, which is the generation and movement of bubbles in a fluid. Gas bubbles move with high velocities when they oscillate (23) and generate shear forces that violate transiently the integrity of the cell membrane. Their movement can also be recorded in vivo. Permeabilization by shock

Fig. 5. Growth of SSK2 tumors after administering gelonin and 3 × 500 shock waves. Protocol, abbreviations and Kaplan-Meier curve as in Fig. 4. Straight lines, no tumor regrowth.

Fig. 6. Growth of SSK2 tumors after administering saporin and 3 × 500 shock waves. Protocol, abbreviations and Kaplan-Meier curve as in Fig. 4. Straight lines, no tumor regrowth.
waves is completely suppressed by minimal static excess pressure in the container in which cells are exposed (16). The suppression was confirmed in experiments with gelonin and saporin.4

Further improvement of the transfer of toxins into cells should be achieved by downsizing the shock wave generator and by modifications of the technology of shock wave generation. It should be possible to construct small generators that fit easily on a laboratory table.

**In Vivo Results.** The *in vivo* experiments demonstrated that the local application of shock waves was only effective after systemic administration of the ribosome inactivating protein. No effect on tumor growth was demonstrated in groups treated with shock waves without toxin and in groups with toxin administered without shock waves. No other part of the animal’s body than the tumor was exposed without toxin and in groups with toxin administered without shock waves. No other part of the animal’s body than the tumor was exposed to the high-pressure field of the shock waves. The toxins were probably transferred into tumor cells, where they acted.

As a consequence, a higher level of ribosome inactivating protein should be demonstrable in the tumor. New approaches to measure picomolar amounts of a ribosome inactivating protein by its N-should be demonstrable in the tumor. New approaches to measure picomolar amounts of a ribosome inactivating protein by its N-glycosidase activity have been described recently (24, 25); their quantitative determination in tumor cells remains to be established. The amount of toxin might be below the detection limit, as a previous experiment with ribosome inactivating proteins and own data suggested, that a few molecules of toxins within a cell might suffice to kill the cell (26).

Unfortunately, not all tumors went into remission, and this indicates that a small fraction of tumor cells survived. Improvements of the shock wave technology and of the administration protocol should yield better results.

Direct access to the cytoplasm is also gained by high voltage electric pulses. Electroporation has been shown to enhance the action of bleomycin and has recently been applied clinically to s.c. tumor nodules (27, 28). A major problem of electrochemotherapy is that the electrodes have to be in close local contact over the whole tumor surface. This prohibits its use in internal organs. Shock waves can, in contrast, be directly administered to internal organs such as the liver or the gut, and no surgical intervention is necessary.

In summary, shock waves enable a defined local delivery of drugs into defined body regions and organs and represent a new approach to tumor therapy.

**ACKNOWLEDGMENTS**

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4 Unpublished observations.
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