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THE INFLUENCE OF HIGH-ENERGY SHOCK WAVES ON THE DEVELOPMENT OF METASTASES

Department of Urology, University Hospital, Nijmegen, The Netherlands

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Abstract—The hypothesis that exposure of a solid tumor to high-energy shock waves (HESW) could lead to an increase of metastases was investigated in an animal model. The highly metastatic AT-6 Dunning R3327 rat prostate cancer subline was implanted in the hind limb of a Fisher Copenhagen rat and was exposed to 6000 shock waves delivered by an experimental lithotripter, or sham-treated, as soon as the tumor had reached a volume of 175–225 mm³. The tumor-bearing leg was amputated 24 h later and the number of metastases was examined 12 weeks thereafter at autopsy. Metastases were seen in 82% of the animals exposed to HESW and in 25% of the sham-treated animals. There was no significant difference in weight of the lungs that contained metastases, between sham and treated animals. These results were confirmed in a second experiment. We conclude that the metastatic spread of tumors with a high metastatic potential may be enhanced by shock-wave exposure.

Key Words: High-energy shock waves, Metastases, Dunning rat prostate cancer.

INTRODUCTION

Extracorporeally generated focused HESW are clinically used in the noninvasive treatment of most renal and ureteral calculi (Chaussy et al. 1980). Several other applications of HESW have been reported, such as disintegration of salivary gland stones and gall bladder stones, treatment of fractures and tumor treatment (Cornel et al. 1994a; Iro et al. 1989; Sackman 1988; Sauerbruch et al. 1986; Valchanou and Michailov 1991). In particular, since HESW can be focused into a small area of interest within the body, the efficacy of HESW as a new experimental noninvasive antitumor therapy has been extensively investigated.

Temporary and complete suppression of local tumor growth has been demonstrated in animal models, after local administration of HESW (Debus et al. 1991; Delius et al. 1989; Hoshi et al. 1991; Oosterhof et al. 1990b; Russo et al. 1985; Weiss et al. 1990). Furthermore, the combination of HESW and chemotherapy or cytokines has been found to produce additive or synergistic antitumor effects (Holmes et al. 1990; Lee et al. 1990; Oosterhof et al. 1990a, 1991). Until now, no severe local or systemic side effects, in particular no enhanced risk for metastases, have been seen in animal studies (Gamarra et al. 1993; Geldhof et al. 1989; Holmes et al. 1990; Hoshi et al. 1991; Oosterhof et al. 1990a, 1990b, 1991). However, none of these studies specifically aimed to examine the potential risk of increased metastases after HESW exposure. Before HESW will ever become clinically applicable in (urological) oncology, not only the effective dosage and methods of administration have to be established, but also the question whether HESW influence the development of metastases has to be answered. Since HESW cause structural and functional disturbances of the vascular system with necrosis, it is conceivable that exposure of a tumor to HESW may lead to an increase of metastatic spread (Debus et al. 1991; Gamarra et al. 1993; Hoshi et al. 1991; Oosterhof et al. 1990b; Russo et al. 1987; Smits et al. 1991, 1994).

In this study, we therefore wanted to investigate this hypothesis more in detail. We used the highly metastatic AT-6 Dunning R3327 prostate cancer subline and exposed the tumors at such a volume that changes in metastatic rate, after HESW or sham treatment, could easily be detected.
METHODS

Animals

Adult male F1 hybrid rats (F344C0pFl/OLA/ HSD, Copenhagen male and Fisher female) were purchased from Harlan OLAC (Bicester, England). The Fisher—Copenhagen rats were housed three animals per cage and subjected to a 12 h cycle of light and darkness. They were provided free access to a standard pelleted diet (Hope Farms, Woerden, The Netherlands) and acidified water (pH 3). The experiments were in accordance with institutional and legal requirements.

Tumors

The AT-6 Dunning R-3327 rat prostate cancer subline was established in our own laboratory and has been described previously (Bussemakers et al. 1992). In brief, AT-6 tumors are anaplastic, hormone nonresponsive and highly metastatic to the lungs. AT-6 tumors were excised from tumor-bearing rats and, after removal of normal and necrotic tissue, 20—25 mg pieces of tumor were transplanted subcutaneously in the hind limb of anaesthesitized Fisher—Copenhagen rats.

The tumor volume was determined by measuring the three dimensions with a precision sliding caliper: maximum diameter ($L$), width ($W$) and height ($H$). No corrections were made for skin thickness. The tumor volume ($V$) was calculated from the formula $V = \pi/6 \times L \times W \times H$. In this study, tumors were allowed to grow to a tumor volume of $175—225 \text{ mm}^3$. When the tumors had reached this desired volume, the rats were randomly divided in two groups: one control group ($N = 16$ rats) and one HESW-exposed group ($N = 17$ rats). The experiments were later repeated with 10 animals in each group, thus a total of 27 rats were treated with HESW and 26 animals were sham-treated.

HESW

HESW were generated by an experimental set-up of the commercially available electromagnetic shock wave source Lithostar Plus (Siemens AG, Erlangen, Germany). The physical and technical characteristics of this experimental shock-wave source have been described earlier (Cornel et al. 1995; Steinbach et al. 1992). In short, the positive pressures range from 24 to 63 MPa, the corresponding energy densities, defined as the time integral over the duration of a pulse, range from 0.08 to 0.6 mJ/mm$^2$. The main frequency of the pulse was 200 kHz (resonance frequency of the system), but frequencies up to 10 MHz are included. Negative pressures range from 5 to 10 MPa. The pressure rise time, defined as the time for the pressure to rise from 10% to 90% of the value of $P_{\text{max}}$, ranged from 30 to 120 ns, respectively. The half-width time, defined as the half-amplitude width of the initial positive pressure half cycle was about 5 $\mu$s. The pulse-to-pulse variation was about 2—3%. The HESW were focused centrally on the tumors. In this way, we ensured that, according to pressure measurements, all parts of the exposed tumors experienced more than one third of the maximal pressure applied centrally. The shock waves were applied with a frequency of 2 Hz and with an energy density of 0.47 mJ/mm$^2$. Prior to the application of HESW, rats were anaesthetized with 30 mg/kg phenobarbital (Apharma, Arnhem, the Netherlands). Hair in the tumor area was removed using a depilatory cream (Reckitt and Collman, Naarden, The Netherlands). Rats were kept in a fixed position in a plastic tube which was placed in the water bath containing degassed water at 37°C. The tumor-bearing leg was projected through a hole in the base of the plastic tube and the centre of the tumor was positioned in the focal area through a three-dimensional positioning system. Each HESW administration consisted of, in total, 6000 pulses. The rats in the sham-treated group were anaesthetized, shaved and positioned in the same fixed position, in a water bath containing degassed water at 37°C in the same way as the HESW-exposed animals but did not receive the HESW exposure.

Surgical procedures

Surgical removal of the primary tumor was performed 24 h after HESW exposure or sham treatment according to the technique described by Kadmon et al. (1982). In brief, the tumor was removed by disarticulation at the hip joint of the tumor-bearing left hind limb. Haemostasis was obtained by using a 3/0 silk ligature on the femoral artery and vein. Additional haemostasis was performed, if needed, by electrocautery. The skin incisions were then closed with the use of skin clips (7.5 × 1.75 mm, Aesculap, Germany).

Evaluation of metastasis rate

All animals were evaluated at 12 weeks postamputation or earlier in case of death. At the time of killing or death, a complete autopsy was performed. Rats were carefully examined macroscopically for metastases in lymph nodes and visceral organs (in particular the lungs). The lungs of all rats were excised, weighed and the total number of metastases was macroscopically counted. The lungs were then fixed in buffered 4% formalin and 4 µm sections were cut and stained with haematoxylin and eosin. All lungs were microscopically examined to confirm the macroscopically visible metastases and to rule out micrometastases in the lungs that macroscopically were not visible.
Statistics
The data were analyzed for statistical significance between the two groups using the t test and logistic regression analysis was performed to compare the number of metastasis for each group (Agresti 1990). p-Values less than 0.05 were considered significant.

RESULTS
In a pilot study we observed a 100% metastatic rate to the lungs if the AT-6 tumor-bearing legs were amputated with tumor volumes greater than 400 mm³. However, when the tumors were removed at a volume of 200 mm³, only 40% of the rats had lung metastases at 12 weeks postamputation. Moreover, the size of the metastases in this group of rats was much smaller when compared with the lung metastases in the other three groups. We therefore used 175–225 mm³ as the tumor volume to determine whether HESW enhance metastases.

In the sham-exposed group, 16 rats were included with an average tumor burden of 204 ± 19 mm³. The HESW-exposed group consisted of 17 rats with tumor volumes of 197 ± 17 mm³ (p = 0.45, t test).

All rats survived the 12 week follow-up postamputation except that, in the HESW-exposed group, one rat died 9.5 weeks postamputation which resulted in a survival rate of 97%. In the sham-exposed group 4 of the 16 (25%) rats had macroscopically and microscopically visible lung metastases at the time of killing (p = 0.001 sham vs. HESW, logistic regression analysis). There was a widespread variation in the number and size of the lung metastases per animal (Fig. 1). The number of metastases per animal ranged from 1 to 79 with a median of 3. The average weight of the lungs that contained metastases was 3.90 ± 4.80 g. There was no relation between tumor volume at the time of HESW exposure and the metastatic tumor burden at time of death/killing. Apart from the lungs, no metastases could be macroscopically detected in other visceral organs or in lymph nodes.

These results were confirmed in a second experiment where 10 rats were included in each treatment group (Table 2). Complete data from both experiments are summarized in Table 3.

DISCUSSION
Animal studies using different tumor model systems have shown that temporal growth-suppressive effects can be achieved after treatment of tumors with HESW. Moreover, HESW combined with chemotherapy or cytokines have been found to produce additive or synergistic antitumor effects (Debus et al. 1991; Delius et al. 1989; Holmes et al. 1990; Hoshi et al. 1988).
ically evident lung metastasis at time of killing (Table 1). The total number of metastases per animal was macroscopically difficult to score. One rat had 17 nodules, whereas the lungs in the other 3 animals contained 5-10 very tiny nodules. The tumor burden in those positive lungs was minimal to moderate. This is expressed by the average weight of 2.38 ± 1.61 g in those positive lungs versus 1.69 ± 0.17 g of lungs of normal age-matched Fisher-Copenhagen rats.

Several studies have been performed to elucidate the mechanisms underlying the antitumor effects of HESW (Debus et al. 1991; Gamarra et al. 1993; Hoshi et al. 1991; Oosterhof et al. 1990a, 1990b; Russo et al. 1987; Smits et al. 1991, 1994).

Histological examinations in various tumor models indicate that the vascular damage caused by HESW leads

Fig. 1. From left to right: rat lung containing no metastasis; rat lung containing a moderate number of metastases; and a rat lung containing many metastases of different sizes.
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14 (82%)

Not done (60%)

6 17

HESW 197 (17)

(29)

10
done

3.9 (4.8)

4.3

2.4

191 (5.1)

4 (25%)

p

Significance

Table 1. Results of first experiment. Tumor volume (mm$^3$) and weight of lungs (g) are given in mean values with standard deviations in parentheses.

<table>
<thead>
<tr>
<th>Number of treated rats</th>
<th>Sham</th>
<th>HESW</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor volume (SD) at time of exposure</td>
<td>16</td>
<td>17</td>
<td>$p = 0.45$</td>
</tr>
<tr>
<td>Number of rats with metastases at killing (%)</td>
<td>4 (25%)</td>
<td>14 (82%)</td>
<td>$p = 0.001$</td>
</tr>
<tr>
<td>Weight of lungs (SD) of rats with metastases at killing</td>
<td>2.4 (1.6)</td>
<td>3.9 (4.8)</td>
<td>$p = 0.55$</td>
</tr>
</tbody>
</table>

to necrosis of the tumor (Debus et al. 1991; Hoshi et al. 1991; Oosterhof et al. 1990b; Russo et al. 1987). Evaluation of HESW-exposed tumors with nuclear magnetic resonance spectroscopy revealed a significant temporary dose-dependent acidification and depletion of energy-rich metabolites of the tumor (Smits et al. 1991, 1994). Furthermore, several investigators have shown that HESW exposure of tumors leads to a temporary impairment of tumor blood flow (Gamarra et al. 1993; Smits et al. 1991, 1994). Recently, a threefold local increase in the concentration of a systemically given drug has been found after the local administration of HESW, related to a temporary impairment of the tumor blood flow (Cornel et al. 1994b).

Metastasis formation is a process of linked sequential steps (Fidler 1991). One of the major steps is embolisation of tumor cells in the bloodstream. Weiss (1990) demonstrated that development of necrotic and haemorrhagic areas facilitates entry of cells in the circulation. HESW exposure leads to structural and functional disturbances of the vascular system with necrosis (Debus et al. 1991; Gamarra et al. 1993; Hoshi et al. 1991; Oosterhof et al. 1990b; Russo et al. 1987; Smits et al. 1991, 1994). Embolisation of (non)viable tumor cells may thus be facilitated by HESW. Fidler (1970) demonstrated that, although tumor cells are quickly destroyed within the bloodstream, the greater the number of cells released by the primary tumor, the greater the chance that tumor cells will survive and form metastases. Moreover, Young and Hill (1990) found that when tumor cells are reoxygenized after a period of acute hypoxia they have a 1.5–3 times increased metastatic ability compared to aerobic cells. Our group has shown that HESW tumor exposure induces a period of acute hypoxia by a temporarily decreased tumor blood flow (Cornel et al. 1995; Smits et al. 1994). Therefore, it is conceivable that exposure of a tumor to HESW leads to an increase of metastatic spread due to the liberation of tumor cells and transient acute hypoxia. Animal studies carried out so far have never shown severe local or systemic complications (e.g., induction or enhancement of metastases), after the local HESW administration on tumors. Geldhof et al. (1989) used the highly metastatic MatLyLu rat prostate cancer Dunning subline to address the issue of whether HESW affect the metastatic spread and no relation could be seen. Also Hoshi et al. (1991) found no effects on metastasis formation in a HESW-sensitive rabbit bladder cancer model. In earlier studies, we described HESW administration in several tumor model systems with different biological behaviour and induction or enhancement of metastases was never seen (Oosterhof et al. 1990a, 1990b, 1991). Although all these aforementioned studies did not demonstrate enhancement or induction of metastases after HESW administration, the results should be interpreted critically for several reasons. First, some of the tumor models used have never shown any metastatic potential and it is therefore questionable whether such a tumor is appropriate for evaluating the influence of HESW on metastasis formation. Second, potentially metastasising tumors should be exposed to HESW in the early phase of the metastasising process to be able to quantify the size and number of (lung) metastases. Third, only tumors that metastasise to only one organ should be used to assure that all metastases are taken into account.

Table 2. Results of second experiment. Tumor volume (mm$^3$) and weight of lungs (g) are given in mean values with standard deviations in parentheses.

<table>
<thead>
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<th>Number of treated rats</th>
<th>Sham</th>
<th>HESW</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor volume (SD) at time of exposure</td>
<td>199 (29)</td>
<td>191 (17)</td>
<td>$p = 0.47$</td>
</tr>
<tr>
<td>Number of rats with metastases at killing (%)</td>
<td>0 (0%)</td>
<td>6 (60%)</td>
<td>$p = 0.003$</td>
</tr>
<tr>
<td>Weight of lungs (SD) of rats with metastases at killing</td>
<td>Not done</td>
<td>4.3 (5.1)</td>
<td></td>
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</table>
This "window" was also determined for the highly metastatic AT-6 used in our study. A pilot study revealed that tumors with a volume of 200 mm$^3$ had led to metastases in 40%, whereas in tumors greater in size than 400 mm$^3$ metastases were present in 100%.

AT-6 tumors were transplanted in such a way on the hind limb of Fisher-Copenhagen rats that the tumor and the implantation "area" could be removed completely by disarticulation of the tumor-bearing leg. By doing so we did not encounter any local recurrence after amputation, neither in the sham nor in the HESW-exposed group.

Tumors were sham treated or exposed to 6000 HESW at a tumor volume of 200 mm$^3$. The tumor-bearing legs were removed 24 h after sham or HESW treatment. The only variable between the two groups was HESW administration. Differences in metastatic rate, evaluated 12 weeks postamputation, can therefore only be related to the HESW exposure. Our results clearly demonstrate a statistically significant enhancement of metastases after HESW exposure. A total of 82% of the rats in the HESW-exposed group had metastases, compared with 25% of the rats in the sham-exposed group. This 60% higher metastasis rate in the HESW-exposed group was confirmed in a second experiment. As indicated before, embolisation of tumor cells by HESW and hypoxia, leading to an increased metastatic capacity, may explain this adverse effect of HESW.

We conclude that, although tumor treatment with HESW results in growth-suppressive effects, the increased risk for metastases obstructs the clinical application of HESW as a new antitumor modality.

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