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Effects of high-energy shock waves combined with biological response modifiers or Adriamycin on a human kidney cancer xenograft

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Summary. We have studied the effect of high-energy shock waves (HESW) alone or in combination with biological response modifiers (BRMs) or Adriamycin on the growth of the NU-1 human kidney cancer xenograft. When HESW are administered repeatedly (four sessions of 800 shock waves on days 0, 2, 4 and 6) a prolonged delay in tumor growth was found compared with that following a single administration. This effect was temporary, and several days after stopping the HESW administration the tumor regained its original growth potential (same doubling time). Tumor growth was suppressed for a longer period by the combination of 4 sessions of HESW and a single administration of Adriamycin, 5 mg/kg. Combination of HESW treatment with interferon alpha (5.0 ng/g body weight, three times/week) and tumor necrosis factor alpha (500 ng/g body weight, 5 days/week) s.c. around the tumor resulted in a complete cessation of tumor growth. While Adriamycin had an additive effect on HESW treatment, the combination with BRMs was highly synergistic.

Key words: High-energy shock waves – In vivo cytotoxicity – Biological response modifiers – Adriamycin

Electromagnetically generated high-energy shock waves (HESW) can alter the growth characteristics of tumor cells in vitro [8]. Furthermore, HESW can provoke suppression of tumor growth in vivo [10–13]. The tumor growth suppression observed in vivo is temporary and results rather in an elongated lag-phase than in a permanent effect. This clearly indicates that HESW is not likely to be useful as monotherapy. The number of shock wave sessions, the number of shock waves per session and the role of tumor volume at start of treatment appeared to be relevant in determining the antiproliferative effect of HESW in vivo [9]. Also, the effects depend on the tumor line used, which may be related to doubling time and vascularization of the tumor [9].

Alternatively, HESW exposure can potentiate the effect of cytotoxic drugs [3, 6, 9, 10]. Our studies clearly revealed the enhancement of cytotoxicity by vinblastine or Adriamycin after shock wave exposure [8, 9].

Biological response modifiers (BRMs) are known to have an antiproliferative effect on human renal cancer xenografts [2]. We now studied the combination of HESW and Adriamycin or BRMs (Interferon alpha + tumor necrosis factor alpha) on the NU-1 human kidney cancer xenograft to establish possible additive or synergistic effects on tumor growth of this combination therapy.

For the in vivo studies the (commercially available) Siemens lithotriptor (Lithostar) was used. The pressure profile of the electromagnetically generated shock waves differs from that of electrohydraulically generated HESW and has been described elsewhere [8].

Materials and methods

Animals

Xenografts were transplanted in 6-week-old male Balb C nu/nu (Bornholt Gårt, Rye, Denmark). The mice were kept in groups of five in PAG type 2 cages (IFFA Credo) covered with an iso-cap for sterile conditions. The mice were fed ad libitum with irradiated SRM food (Hope Farms, Woerden, The Netherlands), and drinking water was acidified with 0.7 ml concentrated HCl/ml.

Tumors

The human renal cell carcinoma NU-1 xenograft was used. This tumor was established in our laboratory by serial s.c. transplantation of tumor pieces after original s.c. transplantation of small primary tumor pieces directly after nephrectomy. The tumors were transplanted s.c. as trocar pieces in the hind limb of each animal under ether anesthesia. This human renal adenocarcinoma is well vascularized and grows with a doubling time of 3–4 days. Histologically the tumor is characterized as sarcomatous and shows spontaneous hemorrhagic necrosis (Fig. 1A).

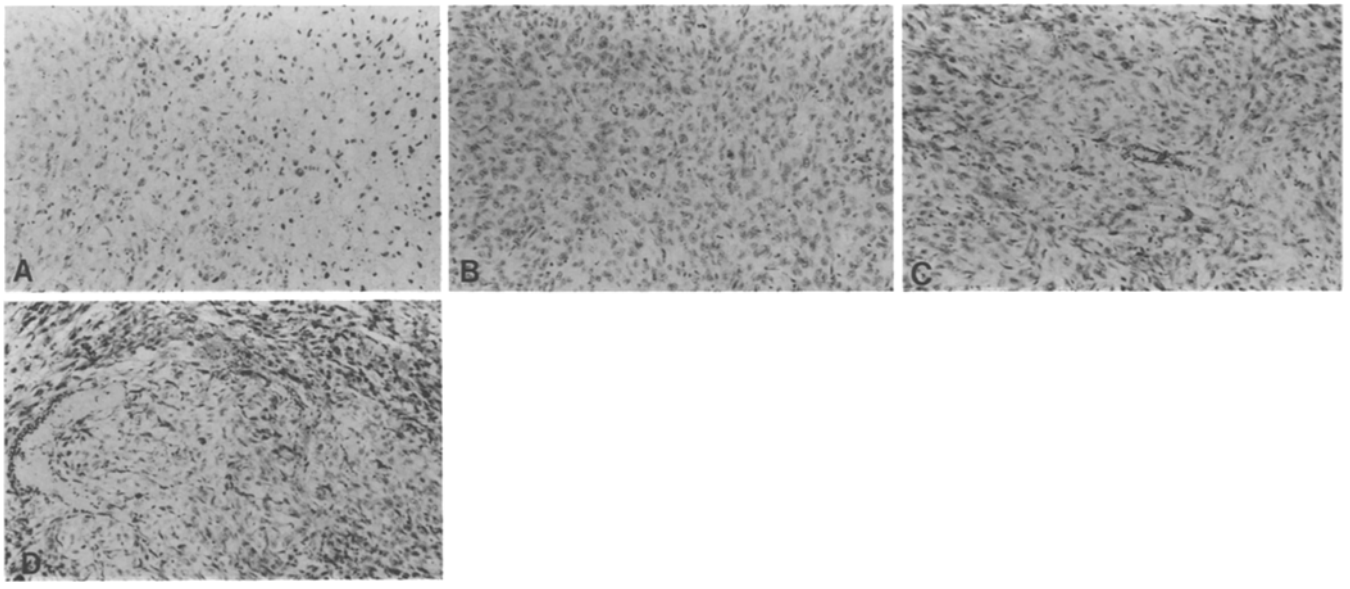

Fig. 1. **A** NU-1 human kidney adenocarcinoma. H&E, 40×. **B** NU-1 kidney tumor after exposure to four sessions of 800 HESW: vasodilation, hemorrhage, some neutrophilic migration and pyknotic tumor cells. H&E, 40×. **C, D** NU-1 kidney tumor after exposure to four sessions of 800 HESW combined with BRM treatment. Pronounced necrosis, neutrophilic migration and infiltration, fibrin clots within the dilated capillaries. H&E, 40×

HESW treatment

The shock waves were generated electromagnetically by the Lithostar (Siemens). The experimental set-up and way of administration of the shock waves have been described in detail elsewhere [8]. For HESW exposure the animals were placed in a water-filled container and kept fixed in position by means of a plastic cocoon.

With in vitro pressure measurements, using a piezo-electric crystal transducer (Imotec) connected with an oscilloscope (Gould, DSO, 4072), the focal area was determined (area limited by pressures which are half the maximum pressure (P_{max} 50)). It appeared that there is a marked pressure fall in the lateral plane, indicating the importance of immobilizing the animal and positioning the tumor precisely [8, 14]. The diameter of the focal area in the lateral plane is only 6 mm, e.g. the pressure in the center of the focal area is 3.75 MPa, at the edge 24 MPa and 2 mm outside the focal area 7.5 MPa (18.4 kV).

After the desired tumor volume (60–70 mm^3) was reached, the tumor-bearing animals were randomly divided into a sham-treated control group, a group receiving shock waves, a group receiving BRMs or Adriamycin, and a group receiving both treatment modalities. Each group consisted of 6 or more animals.

The nude mice were anesthetized with ketamine hydrochloride (Ketalar, Parke-Davis) 150 mg/kg and received two, three or four sessions of 800 shock waves (18.4 kV) on days 0, 2, 4 and 6. As BRM we used interferon alpha (IFN alpha) and tumor necrosis factor alpha (TNF alpha). IFN alpha was given three times a week at a dose of 5.0 ng/g body weight and TNF-alpha, five times a week at 500 ng/g body weight, s.c. around the tumor during the study period. Adriamycin (Adriablastine, Pharmitalia) 5 mg/kg was administered once on day 0, i.p., just before the first HESW exposure.

Biological response modifiers

Human IFN alpha and TNF alpha, kindly supplied by Boehringer Ingelheim, Alkmaar, The Netherlands, were produced in *Escherichia coli* by recombinant DNA technology. The specific activity of IFN alpha was 3.2×10E8 units/mg protein. It was measured by inhibition of encephalomyocarditis (EMC) virus replication in A549 cells with reference to the National Institute of Health (NIH) leukocyte IFN alpha standard Go 23-901-527. The purity of IFN was >98% as determined by SDS polyacrylamide gel electrophoresis and the amount of endotoxin was less than 1.0 ng/mg protein for IFN alpha based on the limulus amebocyte lysate assay. The specific activity of TNF alpha determined in the presence of actinomycin-D was 6×10E7 units/mg protein (L-929 cytotoxicity assay). The purity was >99% as determined by SDS-polyacrylamide gel electrophoresis, and it contained 1.0 ng or less endotoxin/mg protein based on the limulus amebocyte lysate assay. The drugs were dissolved in the accessory dissolvent and diluted with unsupplemented RPMI medium (Gibco, Paisley, UK). After dilution, the drugs were stored in small aliquots at −80 °C until use.

Evaluation of tumor growth

Tumor volume was determined in vivo every other day by using a precision sliding caliper to measure the three dimensions, i.e. maximum diameter (L) and the perpendicular diameters (W, H); the volume was expressed as the tumor size index (TSI) calculated from the equation L×W×H×0.52. In this way, tumor growth patterns were evaluated by calculating the mean volume of the tumors in each group.

Statistical analysis

Per animal, loglinear regression over the first 2 weeks was used to estimate the growth rate (α), i.e. the number of times the tumor volume doubles per day (the doubling time equals $1/\alpha$). Treatment versus control differences in growth rate were then analyzed by two-sided *t*-tests.

For combined treatments, two treatments were considered partly additive if the decrease in growth rate resulting from the combined treatment was larger than the decreases resulting from either of the treatments alone. They were considered synergistic if the decrease

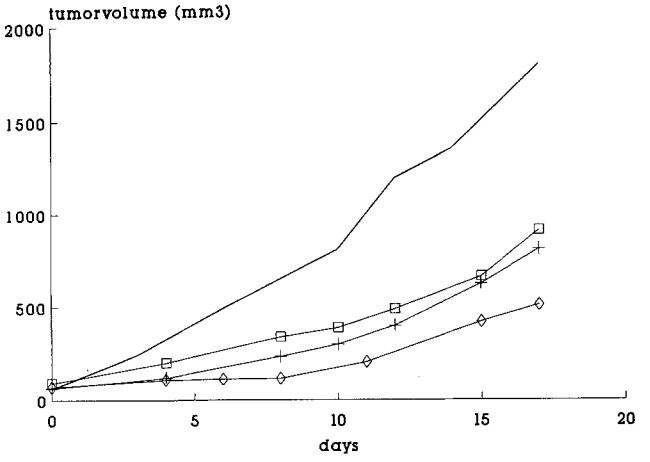

Fig. 2. Tumor volume after four exposures (every 48 h) to 800 HESW (18.4 kV, 37.,5 MPa) combined with Adriamycin 5 mg/kg i.p. on day 0 in the NU-1 human kidney tumor. ———, Control; -□□□-, Adriamycin; -×××-, HESW; -◊-◊-◊-, HESW + Adriamycin

Table 1. Estimated growht rates (α), standard error of the mean (SEM) and *P*-values: Loglinear regression calculation after treatment of the NU-1 human renal cancer xenograft with HESW, Adriamycin, BRMs or a combination of these

Treatment	First 10 days after treatment			Figure in text
	α	SEM	*P*	
Control	0.28	0.01	–	2
4 × 800 HESW	0.21	0.03	0.01	
Adriamycin	0.21	0.01	0.002	
Combination	0.15	0.01	0.0001	
Control	0.29	0.01	–	3
4 × 800	0.21	0.03	0.01	
BRM alone	0.20	0.03	0.01	
Combination	-0.02	0.03	0.0001	
Control	0.28	0.01	–	4
BRM alone	0.20	0.03	0.01	
BRM + 2 × HESW	0.12	0.02	0.01	
BRM + 3 × HESW	0.04	0.08	0.001	
BRM + 4 × HESW	-0.02	0.03	0.0001	

for the combined treatment was larger than the sum of the decreases for the treatments given individually (one-sided *t*-tests, each $P<0.05$).

Histology

Treated and untreated animals were sacrificed at different intervals after HESW exposure. The tumor and surrounding tissues were fixed in formalin 4% and embedded in paraffin, and 4-μm sections were stained with hematoxylin and eosin.

Results

Temporary growth delay

For HESW exposure the animals are placed in the water-filled container and kept in position in a specially designed polyethylene tube. At the site of the limb of the animal a 1.5 × 1.5 cm hole in the tube allows the shock waves to reach the tumor while the animal is protected by the tube against shock wave exposure at other sites. There was no animal mortality from the HESW treatment alone or from the combined treatments in these series.

Exposure to HESW mostly results in a temporary delay in tumor growth [9, 10]. After cessation of therapy the tumor starts growing again with the same doubling time. From earlier studies we know that the in vivo antitumor effect depends not only on the number of shock waves administered per session, but also on the number of shock wave sessions, the initial tumor burden and the tumor model used [9]. We now studied the influence of different sessions of 800 shock waves combined with Adriamycin or IFN alpha plus TNF alpha on the NU-1 human kidney cancer xenograft. Therapy was started when the tumors reached a volume of 60–70 mm^3.

HESW and Adriamycin

In vitro and in vivo studies have shown an additional cytotoxic effect when HESW were combined with cytotoxic drugs [6, 8–10]. Since HESW alone can only cause a temporary growth delay, additional treatment is needed for a longer and more definitive suppression of tumor growth. Combination with another suboptimal treatment or a drug given in a suboptimal dose might thus result in a more complete effect.

Repeated sessions of 800 HESW (18.4 kV), every 48 h on the human kidney tumor NU-1 implanted in the nude mouse caused a significant decrease in tumor growth. Four sessions of 800 shock waves caused lengthering of the lag-phase by approximately 12 days, i.e. a suppression of tumor growth for four doubling times (Fig. 2). The growth rate of the tumor ($\alpha = 0.28$) temporary decreased to 0.21 after 4 sessions of 800 shock waves (Table 1). Approximately 14 days after the first HESW exposure the tumor had ragained its original growth rate.

When a single dose of 5 mg/kg Adriamycin was administered i.p. on day 0 and the tumor was treated with four sessions of 800 HESW every 48 h an additive decrease in tumor growth, resulting in a growth rate of 0.15, was seen (Fig. 2, Table 1). The combination treatment was not only more effective ($\alpha = 0.15$); its effect was permanent. The tumor did not regain its original growth rate after 14 days, as was the case after the monotherapy (HESW or Adriamycin).

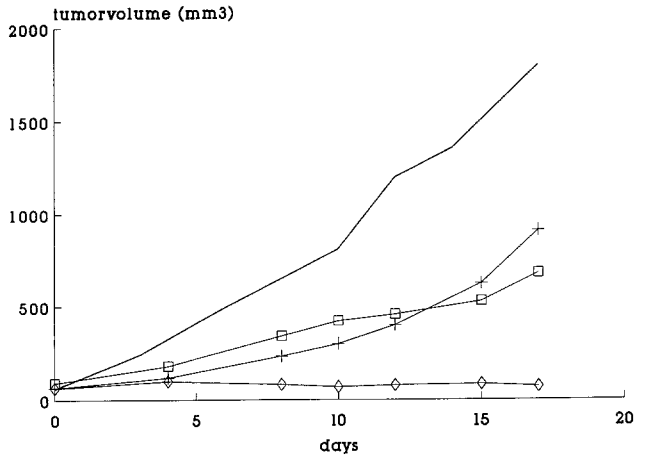

Fig. 3. Tumor volume after four exposures (every 48 h) to 800 HESW (18.4 kV, 37.,5 MPa) combined with Interferon-alpha and tumor necrosis factor alpha in the NU-1 human kidney tumor. ———, Control; -□□□-, BRMs; -×××-, HESW; -◇◇◇-, HESW + BRM

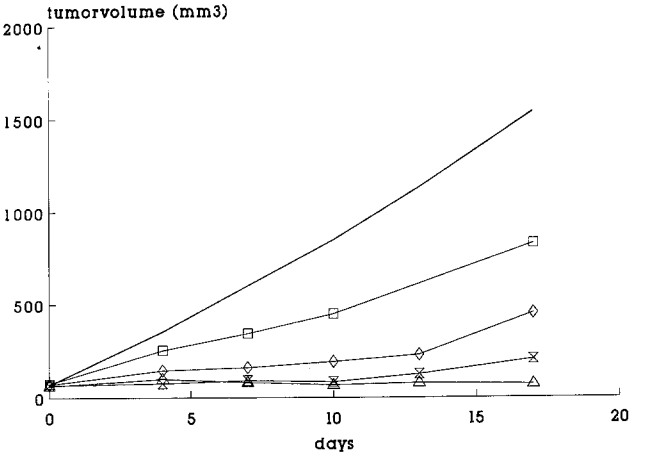

Fig. 4. Tumor volume after two, three or four exposures (every 48 h) to 800 HESW (18.4 kV, 37.,5 MPa) combined with Interferon-alpha and tumor necrosis factor alpha for the NU-1 human kidney tumor. ———, Control; -□□□-, BRMs alone; -◇◇◇-, 2×HESW + BRM; -⧖⧖⧖-, 3×HESW + BRM; -△△△-, 4×HESW + BRM

HESW with BRMs

When the NU-1 tumor was exposed to IFN alpha and TNF alpha alone the growth rate (α) decreased to 0.20. The combination of four sessions of HESW and BRM treatment resulted in a prolonged decrease in tumor growth (Fig. 3). In fact, there was a complete cessation (and even slight regression) of tumor growth in the 7 animals exposed to this combined treatment, resulting in a growth rate of −0.02 (Table 1). Thus, this combination proved to have a highly synergistic antitumor effect.

Two or three sessions of HESW and BRMs

This highly synergistic antiproliferative effect of the combination of four sessions of 800 HESW and BRM treatment led us to study the antiproliferative effect of the combination of fewer sessions of 800 HESW with BRMs. When the same BRM treatment was combined with two or three sessions of 800 HESW the effect on tumor growth was less pronounced. The antitumor effect after treatment with a fixed dose of BRM and two, three or four sessions of 800 HESW depended on the number of shock wave sessions (Fig. 4). There was no significant difference in tumor growth in the first 14 days of treatment with three of four sessions of HESW with BRMs (Table 1). However, at day 17 the efficacy of four HESW sessions was superior to that of three. We observed two complete regressions of the tumor, both in animals treated with the combination of four sessions of HESW and BRM. Regression of these tumors was complete on days 10 and 15 after the start of treatment. No complete regressions were seen after combined treatment with two or three sessions of HESW.

Histology

During shock wave exposure petechiae appeared; they were visible after 10–30 shock waves. Macroscopical examination immediately afterwards showed hemorrhage in and around the tumor. In general, besides hemorrhage, H & E-stained paraffin slides showed vasodilatation and some neutrophilic migration. After 2 days more pyknotic tumor cells were obvious (Fig. 1B). Four days after the fourth session of 800 HESW vasodilatation and neutrophilic migration were still apparent. Also, macrophages were seen in necrotic parts besides fibroblast proliferation, and in-growth especially at the tumor capsule. These findings suggest shock wave-induced tissue damage with subsequent inflammation reactions and a wound-healing process.

Histological examinations after combination therapy with HESW and Adriamycin showed no essential difference from the findings after HESW exposure alone.

BRM treatment alone resulted in a pronounced necrosis and leukocyte infiltration. Tumors treated with the combination therapy showed extensive necrotic parts, surrounded by areas with marked vasodilatation, leukocyte migration and infiltration, and fibrin clots within the capillaries (Fig. 1C, D). These findigs suggest that the tissue damage with subsequent inflammation reactions induced by the shock waves are modulated or rather enhanced by the BRMs, resulting in a necrotic tumor.

Discussion

In several in vivo experiments with electrohydraulically generated shock waves a delay in tumor growth rate was seen after exposure to HESW [9, 10, 11, 12]. This effect depended on the number of HESW [10]. From our studies

we concluded that the effects of in vivo HESW also depend largely on the tumor line used and on the initial tumor volume [9]. Repeated HESW can suppress tumor growth for as long as the shock waves are administered. Finally, cytotoxic drugs can potentiate the effects of HESW [3, 9, 10, 14].

IFN alpha appeared to be a potential active antitumor agent in patients with renal cell cancer [4, 7] and TNF also showed cytostatic and cytolitic effects on human renal cancer cell lines in vitro [5]. In vitro studies with the combination of IFN alpha and TNF alpha on human kidney cancer xenografts showed a synergistic antiproliferative effect [1]. After transplantation of a human kidney tumor in athymic nude mice the non-T-cell mediated effects of BRMs can be studied. In vivo studies of human kidney cancer xenografts with TNF and IFN, performed in our own laboratory, showed the optimum therapeutic dosage schemes [2].

Little is yet known about the exact mode of action of shock waves on tumor cells. In vitro no cell cycle-specific effect could be found [8], but in vivo different mechanisms of action can be considered. There is probably direct non-specific damage to the cells, since tumor necrosis and hemorrhage are the main microscopic findings. Also, damage to the vascularization of the tumor, and especially the small capillaries, may explain the temporal growth delay after HESW [9]. We could not completely destroy a tumor by shock waves alone. This may be due to the fact that the focal area of the Lithostar is rather small (P_{max} 50 is 6 mm large) and that the pressure exerted in the center of the tumor is probably considerably lower than the maximum pressure measured in vitro (37.5 MPa).

The additional antiproliferative effect of cytotoxic drugs is intriguing. It is possible that the local concentration of the chemotherapeutic agent in the tumor increases due to vasodilation and damage of the vessels of the tumor. Histological examination of the tumors after shock wave administration alone shows shock-wave-induced tissue damage with subsequent inflammation reactions and a wound-healing process. After combined treatment with Adriamycin no histological explanation for the additional antitumor effect can be seen. Tumors treated with the combination of HESW and BRMs show extensive necrotic parts, surrounded by areas with marked vasodilatation, leukocyte migration and infiltration, and fibrin clots within the capillaries, suggesting that the tissue damage with subsequent inflammation reactions induced by the shock waves are modulated, or rather enhanced, by the BRMs, resulting in a necrotic tumor. In the seven animals treated with the combined treatment four sessions of HESW and BRMs, we observed complete regression of the tumor in two animals. The other animals were sacrified for histological tumor examination while the tumor was in partial regression or stabilized. The mean tumor volume on day 17 (end of treatment) was 72 mm^3 for the animals treated with the combination, 678 mm^3 after BRM alone, 908 mm^3 after HESW alone and 1794 mm^3 in the control group. In the animals treated with the combination of 2 or 3 sessions of HESW and BRM we never found complete disappearance of the tumor; nor was the combination of HESW and Adriamycin followed by complete regression in any of the animals in this study.

A dose-response relation could be seen after exposure of tumors to increasing number of shock wave sessions together with BRMs (Fig. 4). The shock waves are administered in the first 6 days (4 sessions at intervals of 48 h), but the dose (HESW)-related antitumor effect is more pronounced after 17 days. This suggests that the prolonged antitumor effect after a higher number of shock wave sessions is due to a more pronounced inflammatory effect of HESW on the tumor in the early days, resulting in an enhanved effect on the BRMs. Also, debulking of the tumor by the repeated shock waves in the first 6 days may render the tumor more susceptible to the cytotoxic effects of the BRMs administered on a constant basis.

We conclude that the combination of two suboptimal treatments (HESW and BRMs) may bring about a complete cessation of tumor growth for a prolonged period. In some cases the combined treatment completely destroyed the tumor. Further studies under different technical circumstances are needed to confirm these results in other tumor models.

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References

1. Beniers AJMC, Peelen WP, Hendriks BT, Schalken JA, Romijn JC, Debruyne FMJ (1988) In vitro antiproliferative efficancy of interferon-alpha, -gamma and tumor necrosis factor on two human renal tumor xenografts. Urol Res 16:309
2. Beniers AJMC, Moorselaar RJA van, Peelen WP, Hendrikx BT, Schalken JA, Debruyne FMJ (1990) Differential sensitivity of human renal cell carcinoma xenografts for Interferon-alpha, -gamma and Tumor Necrosis Factor alpha. (Submitted for publication)
3. Berens ME, Welander CE, Griffin AS, McCullogh DL (1987) HESW inhibit tumor cell proliferation and potentiate efficacy of cytotoxic agents. J Urol 137:228 A
4. Buzaid AC, Robertone A, Kisala C, Salmon CE (1987) Phase II study of interferon alpha-2a, recombinant (Roferon-A) in metastatic renal cell carcinoma. J Clin Oncol 5:1083
5. Heicappell R, Naito S, Ichinose Y, Creasey AA, Lin S, Fidler IJ (1987) Cytostatic and cytolytic effects of human recombinant tumor necrosis factor on human renal cell carcinoma cell lines derived from a single surgical specimen. J Immunol 138:1634
6. Lee K, O'Donnell R, Smith P, Cockett ATK (1988) High energy shock waves (HESW) enhance antitumor activity of cisplatin (DDP) in murine bladder cancer, MBT-2. J Urol 139:326 A
7. Muss HB (1987) Interferon therapy for renal cell carcinoma. Semin Oncol [Suppl 2] 14:36
8. Oosterhof GON, Smits GAHJ, Ruyter JE de, Moorselaar RJA van, Schalken JA, Debruyne FMJ (1989) The in vitro effect of electromagnetically generated shock waves (Lithostar) on the Dunning R3327 PAT-2 rat prostatic cancer cell-line. Urol Res 17:13

9. Oosterhof GON, Smits GAHJ, Ruyter JE de, Schalken JA, Debruyne FMJ (1990) In vivo effects of high energy shock waves on urological tumors; an evaluation of treatment modalities. J Urol 144:785
10. Randazzo RF, Chaussy CG, Fuchs GJ, Bhuta SM, Lovrekovich H, Kernion JB de (1988) The in vitro and in vivo effects of extracorporal shock waves on malignant cells. Urol Res 16:419
11. Russo P, Heston WDW, Fair WR (1985) Suppression of in vitro and in vivo tumor growth by high energy shock waves. Surg Forum 36:645
12. Russo P, Stephenson RA, Mies C, Heston WDW, Melamed MR, Fair WR (1986) High energy shock waves suppress tumor growth in vitro and in vivo. J Urol 135:626–628
13. Russo P, Mies C, Huryk R, Heston WDW, Fair WR (1987) Histopathologic and ultrastructural correlates of tumor growth suppression by high energy shock waves. J Urol 137:338–341
14. Smits GAHJ, Oosterhof GON, Ruyter JE de, Schalken JA, Debruyne FMJ (in press) Cytotoxic effects of high energy shock waves in different in vitro models: influence of the experimental set-up. J Urol

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