TUMOR GROWTH INHIBITION BY SHOCK-WAVE-ACTIVATED CELLULAR SENSITIVITY TO CHEMOTHERAPY

I.V.Mastikhin*, **V.S.Teslenko[#]**, **V.P.Nikolin⁻**, **N.G.Kolosova[±]**, **V.N.Gorchakov⁻**

*Physics Department, University of New Brunswick, CANADA, #Lavrentiev Institute of Hydrodynamics, _Institute of Cytology and Genetics, ±Institute of Physiology, _Institute of Lymphology, Novosibirsk, RUSSIA

mast@unb.ca

Abstract

Development of methods for efficient drug delivery to the cells is of essential importance for chemotherapy and gene therapy. The goal of this work was to apply acoustic shock waves (ASW) for localized drug delivery into tumor cells. It was shown that combination of ASW with cyclophosphan significantly enhances the cyclophoshan treatment efficiency, both *in vitro* and *in vivo*. This enhancement nonlinearly depends on the number of ASW pulses. To explain that result, the studies of bioeffects of ASW action at the cellular level were conducted. ASW significantly change structural and functional characteristics of the cells, the concentration of lipid peroxidation (LPO) products, membrane lipid viscosity, protein-lipid interactions, and the transmembrane electrical potential of the cells. The direct measurements showed an increase in drug concentration in tumor cells as a result of ASW-induced activation of the cellular transport. The evidence of ASW cavitation in tissues was visualized by MRI and histological analysis.

Introduction

Various physical factors (light, heat, ultrasound, etc) are used these days to solve the goal of efficient delivery of drugs to the cells to tissues and organs. This goal has to be achieved at two separate levels: the organ and tissue level where the delivery is achieved, for example, by changes in blood microcirculation; and the cell level where the cellular transport provides the consumption of the drug by the cells.

The acoustic transparency of the significant part of human body makes an idea of using acoustic waves for localized treatment or drug delivery activation very attractive. The acoustic waves can be focused with a good precision inside the body, providing localized, non-invasive, extracorporeal treatment. Both ultrasound and acoustic shock waves were successfully used for the purposes of tumor growth reduction [1-4] and DNA transfer [3,4].

However, a mechanism responsible for such reduction is not very clear. One might suggest that the destructive changes in tumor tissues after SW treatment are due to injury to the tumor vascular system: the changes of the capillary walls permeability may entail local increase in the drug concentration in the tumor tissue, that is, at the tissue level.

It is also possible that the mechanical and chemical action of ASW may directly affect structural and functional parameters of tumor cells i.e. produce changes at the cell level.

In this work, we studied the effects of combined action of chemotherapy and ASW on the tumors, both *in vitro* and *in vivo*. The significant inhibition of the tumor growth was observed. The non-linearity of the inhibition with respect to the number of ASW pulses correlated well with the observed non-linear changes of several functional and structural parameters of cell membranes, such as the concentration of lipid peroxidation (LPO) products, membrane lipid viscosity, protein-lipid interactions, and the transmembrane electrical potential of the cells. The changes at the tissue level were also observed by MRI and histological analysis, providing the evidence for cavitation processes in tissues.

The observed non-linear reaction of cell membranes to the ASW action is important for optimization of treatment parameters. The significant increase in chemotherapy efficiency at the cellular level can be used in developing strategies of localized drug delivery in biological and medical applications.

Methods

A portable "desktop" electromagnetic generator of ASW with acoustic lens focusing was designed and built in the Lavrentiev Institute of Hydrodynamics (Fig.1). The ranges of pulse durations is 0.5 – 5 μs, the pressure is the focus is 25-45MPa, discharge power 60MW. In our research, we used 0.5 μs pulses with 45 MPa pressure at focal distance of 60 mm.

We studied the sensitivity of mice Krebs-2 tumor cells to cyclophosphan (CF), a drug widely used in clinical chemotherapy, in combination with ASW treatment. For *in-vitro* experiments, a tumor cell suspension was subjected to 5-70 ASW pulses with 5

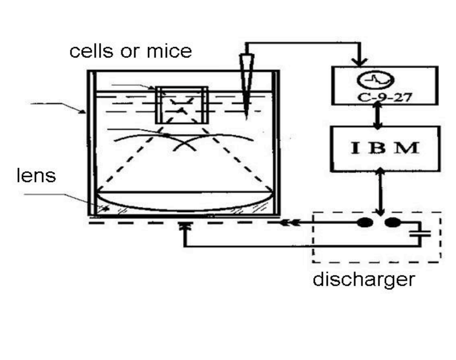


Figure 1. ASW generator

sec interval between the pulses. Then, the cells were inoculated in the mice feet pads. The mice received CF at 100mg/kg body weight intraperitoneal injection 30 minutes after inoculation. After 12 days, the mice were sacrificed to determine the mass of grown tumors. There were 11 groups of mice, 14-16 mice in each group: the control group that received neither CF nor ASW treatment; the group that received CF only; 4 groups that received 10,20,40 and 70 ASW pulses; 5 groups that received both CF and 10,20,40,60 and 70 ASW pulses .

For *in vivo* experiments, 2-month old gemale mice (SVA line) were inoculated with Krebs-2 tumor cells (2 mln cells) in their feet pads. After the tumor reached 0.4-0.5 cm in diameter, the mice were divided into six groups: (I) control group (16 mice); (II) 10 mice treated with 20 ASW pulses; (III) 18 mice received CF at 100mg/kg body weight intraperitoneal injection simultaneously with groups IV, V and VI; (IV) 9 mice were treated with 10 ASW pulses; (V) 9 mice were treated with 20 ASW pulses; 9 mice were treated with 40 ASW pulses. The mice of groups IV, V and VI received CF at 100mg/kg body weight intraperitoneal injection 30 min after the local treatment with ASW. The tumor weight was determined 7 days later.

To examine the hypothesis that ASW treatment could increase the cell membrane permeability to drugs, we used X-ray fluorescence analysis using a synchrotron irradiation source. Cis-Dichlorodiamine platinum(II) was used as another chemotherapeutic medicine. Krebs-2 cells were centrifuged, the pellet containing the cells was placed into 1ml polyethylene tubes with an addition of Hanks solution. After the tubes were subjected to 20 pulses of ASW in the focal zone of the acoustic lens, the 0.5mM/l solution of platinum was added, and cells were incubated at 37C for 30 min. Then, the cells were washed three times with physiological saline, centrifuged and burned after water evaporation. The content of Pt was measured at the RFA station of the Institute of Nuclear Physics (Siberian Division of RAS). Triplicate measurements revealed a increase by 29(1)% of platinum content in all samples treated by ASW.

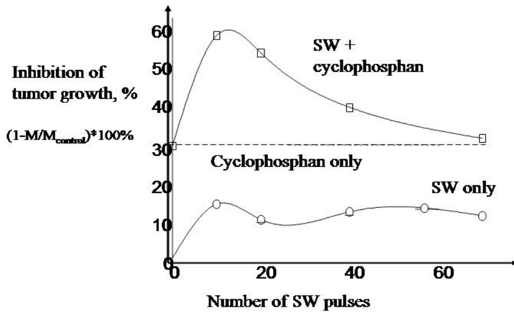
The changes in structural and functional characteristics of cell membranes induced by ASW were studied by measurements of concentration of LPO products, membrane lipid viscosity, protein-lipid interaction, and the cell transmembrane electrical ($\Delta\Psi$) [5]. The value of $\Delta\Psi$ is the sum of potentials on the plasma and mitochondrial membranes. For comparison with Krebs-2 cells, we also studied the effects of ASW on Wistar-rat thymocytes. The cells were washed, suspended in Hanks solution and treated as above The content of LPO products (conjugated dienes) was measured spectroscopically (by the lipid-extract absorption in the UV spectral region) 30 min after exposure. Lipid extracts were prepared in the presence of 0.001% ionol. The following fluorescence probes were used: 4-n-dimethylaminostyryl-1-methyl pyridinium cation (DSM; Zonde, Latvia) for measuring $\Delta\Psi$; pyrene (Serva, Germany) and 4-n-dimethylaminostyryl-1-methyl dodecylpyridinium (DSP-12; Zonde, Latvia) for measuring lipid viscosity. The lipid viscosity was calculated from the results of measurements by comparing the ratio of pyrene to DSP-12 in the membranes studied and in liposomes with known viscosity. The lipid-protein interaction in cell membranes was assessed from the efficiency of the non-radiating energy transfer from tryptophanyl residues of membrane proteins to hydrophobic pyrene localized in lipids. Fluorescence was measured using a Hitachi MPF-4 spectrofluorimeter (Japan) in a round cuvette (0.5 cm diameter) at room temperature and a cell concentration of $6x10^6$ ml^{-1}. Absorption spectra were measured using a Hitachi-556 spectrophotometer (Japan).

Results and Discussion

The results of the experiments with inoculated tumors where the tumor cells were treated by ASW *in vitro* are shown in Fig. 2.

The combination of CF and ASW inhibits the tumor growth much better than CF or ASW only. The remarkable result is the non-linearity of the inhibition with respect to the number of ASW pulses, with optimum at 10-20 pulses, where the number of cells

directly killed by ASW is only 10-15% of the total amount[2]. Notice also the inefficiency of ASW alone to inhibit the tumor growth despite almost linear



dependence of killed cells on the number of pulses (see [2]). The *in-vitro* application of ASW excludes the effects associated with microcirculation and tissue damage, so the non-linearity suggests that ASW modify the structural and functional parameters of the cells.

The qualitatively same results were obtained for ASW treatment *in vivo* where the combination of CF and ASW again was more successful. In that case, both the cell and tissue effects have to be analyzed.

The response of $\Delta\Psi$ vs. number of pulses is shown in Fig.4. The transmembrane electrical potential is generated by the energy transformation and ion-homeostasis systems of cells. Therefore, changes in $\Delta\Psi$ can be regarded as an integral characteristic of these systems. Thirty minutes after exposure to 10-20 ASW pulses, the value of $\Delta\Psi$ increased both in Krebs-2 cells and in thymocytes.

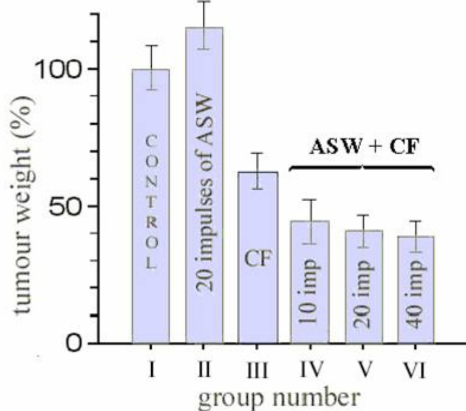


Figure 3. Tumor growth inhibition in vivo

Exposure to a larger number of pulses caused a $\Delta\Psi$ decrease. Experiments with thymocytes showed that, after 1 h of ASW treatment, hyperpolarization was replaced by a decrease in $\Delta\Psi$.

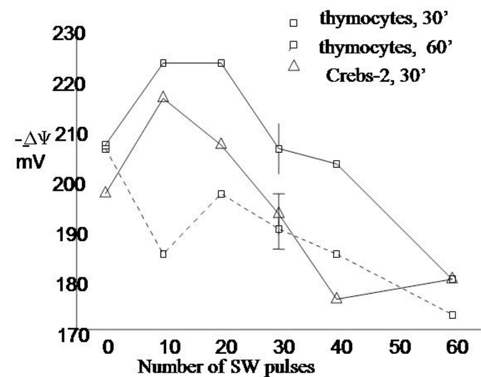


Figure 4. $\Delta\Psi$ of Krebs-2 cells in 30 min and thymocytes in 30 min and 1h after ASW

Figure 5. Concentration of LPO products

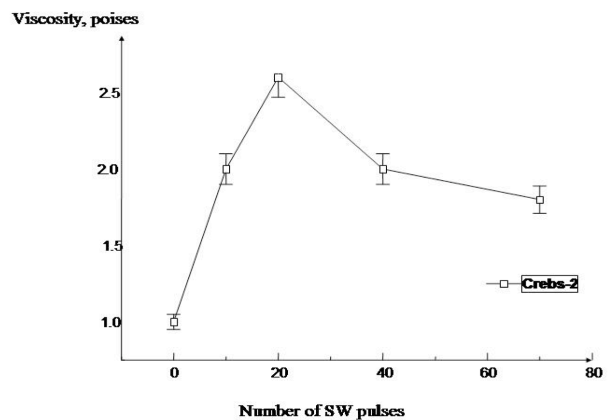


Figure 6. Membrane lipid viscosity

As shown in Fig.5, the activation of LPO was also dose-dependent. There was a correlation between the changes in the level of LPO products and in the membrane lipid viscosity (Fig.6). However, the lipid-

protein interaction disorders became more pronounced as the number of pulses increased [5].

All these data show the strong non-linearity in cellular response to ASW treatment that manifests itself both in the tumor tissue growth and in the cell membrane parameters. An observed activation of free radical processes can be produced by chemical effects of ASW in the focal zone. The transient cavitation in ASW can decompose peroxides and other unstable species always present in cells (about 1μM)[2] and thus activate free radical lipid peroxidation, causing changes in membrane properties and functions. However, there may be another, indirect mechanism of activation of free radical processes in cells. Mitochondria are the main source of oxygen-containing radicals in cells with normal functional activity. Activation of metabolic reactions may cause an increase in the rate of production of oxygen-containing radicals. The $\Delta\Psi$ increase induced by 10-20 ASW pulses can be regarded as evidence for activation of bioenergetic processes. It can be assumed that activation of energy transformation in cells increases the rate of active uptake of drugs by tumor cells exposed to ASW and also activates LPO. Exposure to a larger number of pulses significantly damages the membrane structure and causes inhibition rather than activation of uptake.

The histological analysis of tumor tissues exposed to ASW showed cavities with a diameter of tens microns in the focal zone that, we think, were produced by cavitation in tissues. Cavitation in the focal zone generates expanding bubbles. However,

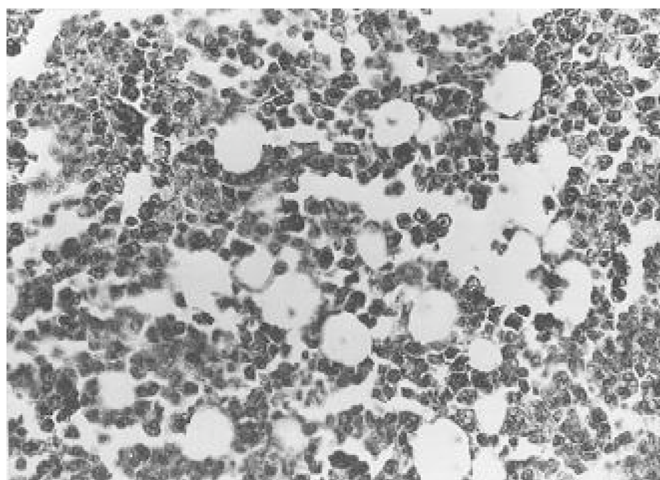


Figure 7. Cavities in tissue after 10 ASW pulses.

their growth is arrested by high viscosity and large energy dissipation in the tissue; then intercellular fluid fills the cavities. Similar effects were reported earlier for ultrasound [6] where such formations were called as “pseudobubbles”, because they were only traces left by real gaseous bubbles. The increased tissue heterogeneity was also detected by MRI [7].

Conclusion

The significant increase in efficiency of CF when combined with shock wave treatment can be explained by an activation of cellular transport. That was demonstrated both by direct measurements of cell Pt uptake and by observed changes in cell transmembrane electric potential. Such activation is also accompanied by LPO activation and changes in membrane structure. Non-linearity of tumor growth inhibition can be explained by increasing damage to the membrane structure by ASW.

The use of cell suspensions permitted studies of cellular response to ASW treatment at cell level and eliminated possible tissue effects. When one studies effects of ASW on biological objects, taking the ASW-cell interaction into account is necessary.

The observed non-linear reaction of cell membranes to the ASW action is important for optimization of treatment parameters. The significant increase in chemotherapy efficiency at the cellular level can be used in developing strategies of localized drug delivery in biological and medical applications. A further research will require investigation of mechanisms of ASW action on cell membrane permeability and determination of optimal ASW configurations.

Acknowledgements

Authors thank V.A.Maier for building the ASW generator and E.L.Zelentsov for RFA measurements of Pt content. This work was supported by ISF and RFBR, #96-02-19329 and #00-02-17992.

References

[1] F. Prat, A.Sibille, C.Luccioni, D.Pansu, J.Y.Chapelon, J.Beaumatin, T.Ponchon, D.Cathignol. “Increased chemocytotoxicity to colon-cancer cells by shock wave-induced cavitation”, Gastroenterology, vol. 106(4), pp.937-944, 1994.

[2] I.V.Mastikhin, V.P.Nikolin, V.S.Teslenko, E.L.Zelentsov, V.A.Maier, R.I.Salganik, S.I.Dikalov. “Treatment with shock waves increases the sensitivity of tumor cells to cyclophosphamide”, Doklady Biophysics vol.340-342, pp.14-16, 1995.

[3] D.L.Miller, J.Song. “Lithotripter shock waves with cavitation nucleation agents produce tumor growth reduction and gene transfer in vivo”, Ultrasound in Medicine and Biology, vol.28(10), pp.1343-1348, 2002.

[4] D.L.Miller, J.Song. “Tumor growth reduction and DNA transfer by cavitation-enhanced high-intensity

[5] V.S.Teslenko, N.G.Kolosova, I.V.Mastikhin, V.P.Nikolin. “Changes in structural and functional characteristics of cell membranes induced by acoustic pulses”, Doklady Biological Sciences, vol. 369, pp.618-620, 1999.

[6] O.Hug, R.Pape, Strahlentherapie, vol.94, 79-99, 1954.

[7] I.V.Mastikhin, V.S.Teslenko, V.N.Gorchakov, V.P.Nikolin, N.G.Kolosova. “Application of NMR-tomography and histological analysis to study cavitation processes induced by ultrasonic shock waves in biological objects”, Bulletin of Experimental Biology and Medicine, vol.6, pp. 653-655, 1999.