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13. ABSTRACT (Maximum 200 Words)

This report describes the progress achieved during the second year of the project. We proposed to complete Task 2 and begin to implement Task 3 in the second year of the project. Task 2 (a and b) focuses on in vivo studies of ultrasound-induced penetration of real anti-cancer drugs in human MCF-7 breast tumors of nude mice. Tasks 2a and 2b are devoted to in vivo studies of ultrasound-induced penetration of real anti cancer drugs: 5-FU and Interleukin-2, respectively, in the breast tumors. We conducted these studies to implement the proposed tasks. Our data obtained with the use of histological evaluation of the tumors indicate that the ultrasound produces enhanced delivery of the anti-cancer drugs in the tumors. This results in dramatic tumor necrosis that was obtained when mice were injected with the anti-cancer drug (5-FU or Interleukin-2) and the tumors were irradiated by ultrasound, while minor tumor necrosis was noticed when the drug was used without irradiation. At present, according to our research schedule, we are performing the studies proposed in the Task 3: to study efficacy of breast cancer therapy with the use of ultrasound-enhanced delivery of anti-cancer drug 5-FU.

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INTRODUCTION

Breast cancer is a major health problem in the USA. Despite the recent progress in the development of promising anti-cancer chemo- and biotherapeutic agents, no breakthrough has been achieved in breast tumor therapy and therapy of tumors in other organs. The efficacy of anti-cancer drugs (especially most promising macromolecular agents) is limited due to their poor penetration through tumor capillary walls, interstitium, and cancer cell membranes [1, 2]. The objective of the proposed research is to develop and test a novel drug delivery technique utilizing interaction of ultrasound with exogenous microparticles that can selectively accumulate in tumors [3]. The interaction of ultrasound with the microparticles results in cavitation (formation, growth, and collapse of microbubbles) in tumors without damage to normal tissues. The microparticles serve as cavitation nuclei that substantially lower cavitation threshold selectively in tumors [4-8]. The ultrasound-induced cavitation perforates tumor blood vessel walls and cancer cell membranes and induces microconvection in the interstitium that enhances penetration of anti-cancer drugs in the cancer cells. The focus of this research project is to study ultrasound-enhanced delivery of model and real drugs in human breast tumors and perform breast cancer chemo- and biotherapy by using the ultrasound-enhanced drug delivery. The studies are performed *in vivo* in nude mice bearing human MCF-7 breast tumors. The second year of the project focuses on the studies of ultrasound-enhanced delivery of real anti-cancer drugs with low and high molecular weights (5-FU and Interleukinj-2, respectively) by using histological evaluation of irradiated and control tumors.

BODY

Materials and Methods

The studies were performed in athymic nude mice (average weight of 30 g). These studies were approved by the Institutional Animal Care and Use Committee (IACUC) of UTMB.

Suspensions of human breast MCF-7 cancer cells (15×10^6 per site) were injected s.c. in the dorso-scapular area on the left and right sides of each mouse. Experiments were initiated when tumors reached the size of 5 to 8 mm.

One tumor was irradiated by ultrasound for 10 minutes, while the other tumor served as control. Polystyrene nanoparticles (10% w/w in water, dia. = 100 nm) were used as cavitation nuclei. The nanoparticles were injected in the tail vein of nude mice one day prior to irradiation to allow extravasation of the particles in tumor blood vessels.

Anti-cancer drug 5-Fluorouracil (5-FU) was used to study tumor necrosis and in the cancer therapy experiments. 5-FU is a drug with low molecular weight (M.W. = 130). It is the only drug that is currently being used for colon cancer chemotherapy. The drug was injected i.p. prior to irradiation at the dose of 90 mg/kg which is the optimal doze for cancer therapy in nude mice. Interleukin-2 (MW = 15,500) will be used as a macromolecular anti-cancer drug in our studies. Interleukin-2 was injected at the dose 600,000 IU/kg in these studies.

Tumor necrosis was examined using standard H&E staining. Animals were euthanazed 72 hours after irradiation to assess necrosis induced by the drugs. Irradiated and control tumors were harvested and fixed in formalin. Thin slices of the tumors were analyzed histologically after standard staining with hematoxylin and eosin (H&E).

In Task 3, length and width of each tumor are measured with a caliper. Tumor volume, V, is calculated by the formula: $V = L \times W^2 / 2$, where L and W is the length and the width of the tumor, respectively. This formula is commonly used for the calculation of tumor volume in cancer therapy studies. At the end of Task 3 (26^{th} month of the project), the tumor volume will be plotted as a function of time (in days) after the first treatment (irradiation).

Results

Typical results of the studies with the anti-cancer drug 5-FU are presented in Fig. 1. Histological examination of the breast MCF-7 tumor slices stained with H&E revealed: viable tumor cells with well-defined nuclei in control (non-irradiated) tumors of mice injected with 5-FU and nanoparticles (Fig. 1, left). Very minor necrosis was produced by 5-FU in non-irradiated tumors in small areas of the tumors of the same mice. Dramatic necrosis was produced in large areas in irradiated tumors when ultrasound was used in combination with 5-FU and nanoparticles (Fig.1, right).

Similar results were obtained in the experiments with Interleukin-2. Figure 2 shows histological examination of the breast MCF-7 tumors of mice injected with Interleukin-2 and nanoparticles. The control tumors have minor necrosis in small areas (Fig. 2, left). Strong necrosis was produced in large areas in irradiated tumors of the same mice when ultrasound was used in combination with 5-FU and nanoparticles (Fig.2, right).

KEY RESEARCH ACCOMPLISHMENTS

1. We studied ultrasound-enhanced delivery of real anti-cancer drugs (FU-5, an anti-cancer drug with low molecular weight, widely used for cancer therapy and Interleukin-2, a macromolecular anti-cancer drug) in human breast tumors of nude mice. Histological examination of the ultrasound-irradiated and control tumors indicated that: (a) minor necrosis produced by the anti-cancer drug in the control, non-irradiated tumors; and (b) irradiation of tumors by ultrasound performed with anti-cancer drug injections resulted in dramatic tumor necrosis and death of cancer cells in the tumors.
2. We initiated studies of efficacy of breast cancer therapy with the use of ultrasound-enhanced drug delivery of anti-cancer drug 5-FU. According to our project timetable, the studies will be completed in the third year of the project.

The results of these studies suggest that this novel drug delivery technique substantially improves delivery of anti-cancer drugs in the breast tumors and may improve efficacy of the breast cancer chemotherapy. Our studies planned for the third year of the project will demonstrate efficacy of breast cancer chemotherapy with the ultrasound-enhanced drug delivery with anti-cancer drugs 5-FU and Interleukin-2.

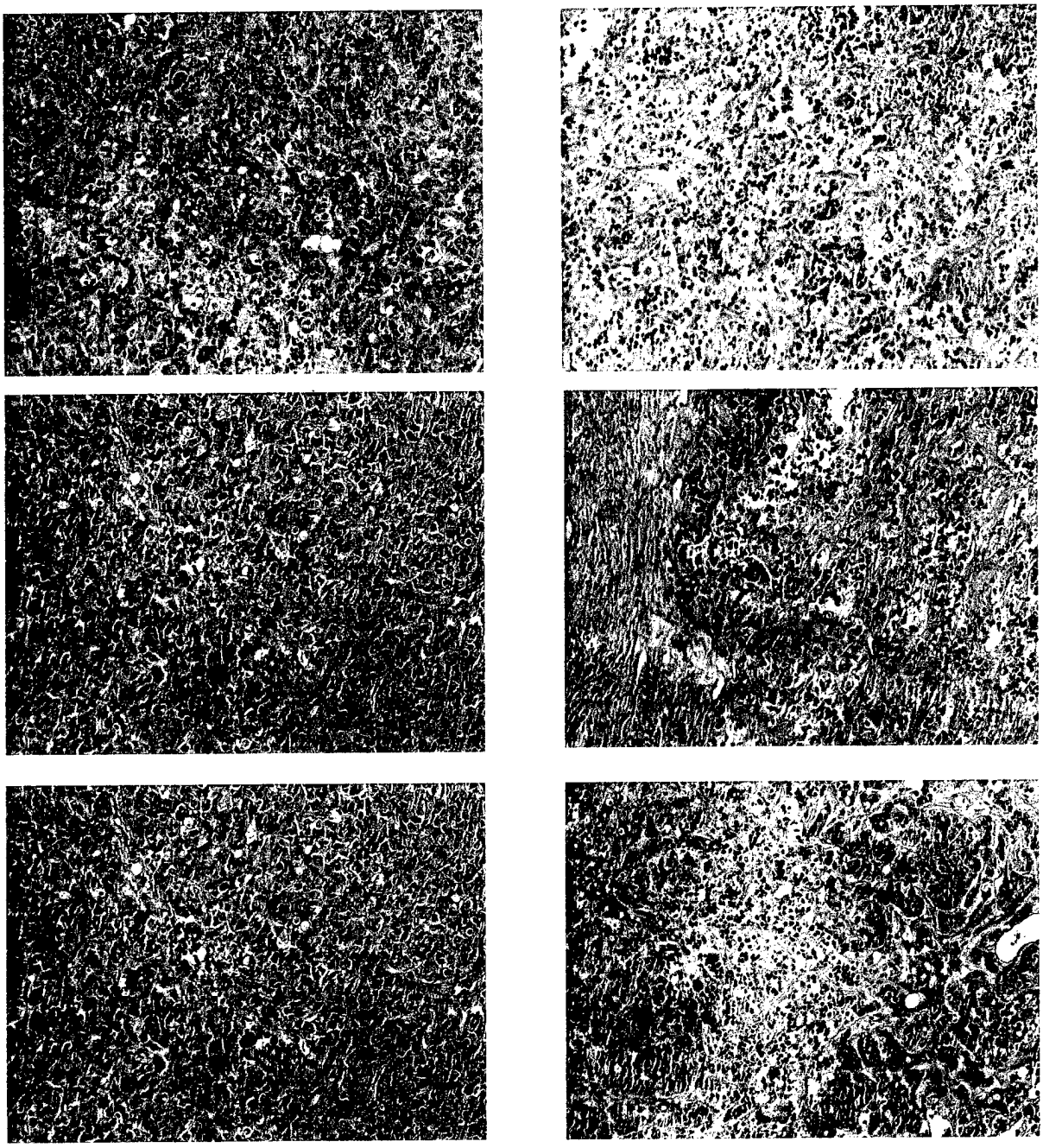

Figure 1. Microscopic histological examination of: (1) control (non-irradiated) MCF-7 breast tumors of nude mice injected with anti-cancer drug 5-FU and nanoparticles (left) and (b) irradiated breast tumor of the same mice (right).

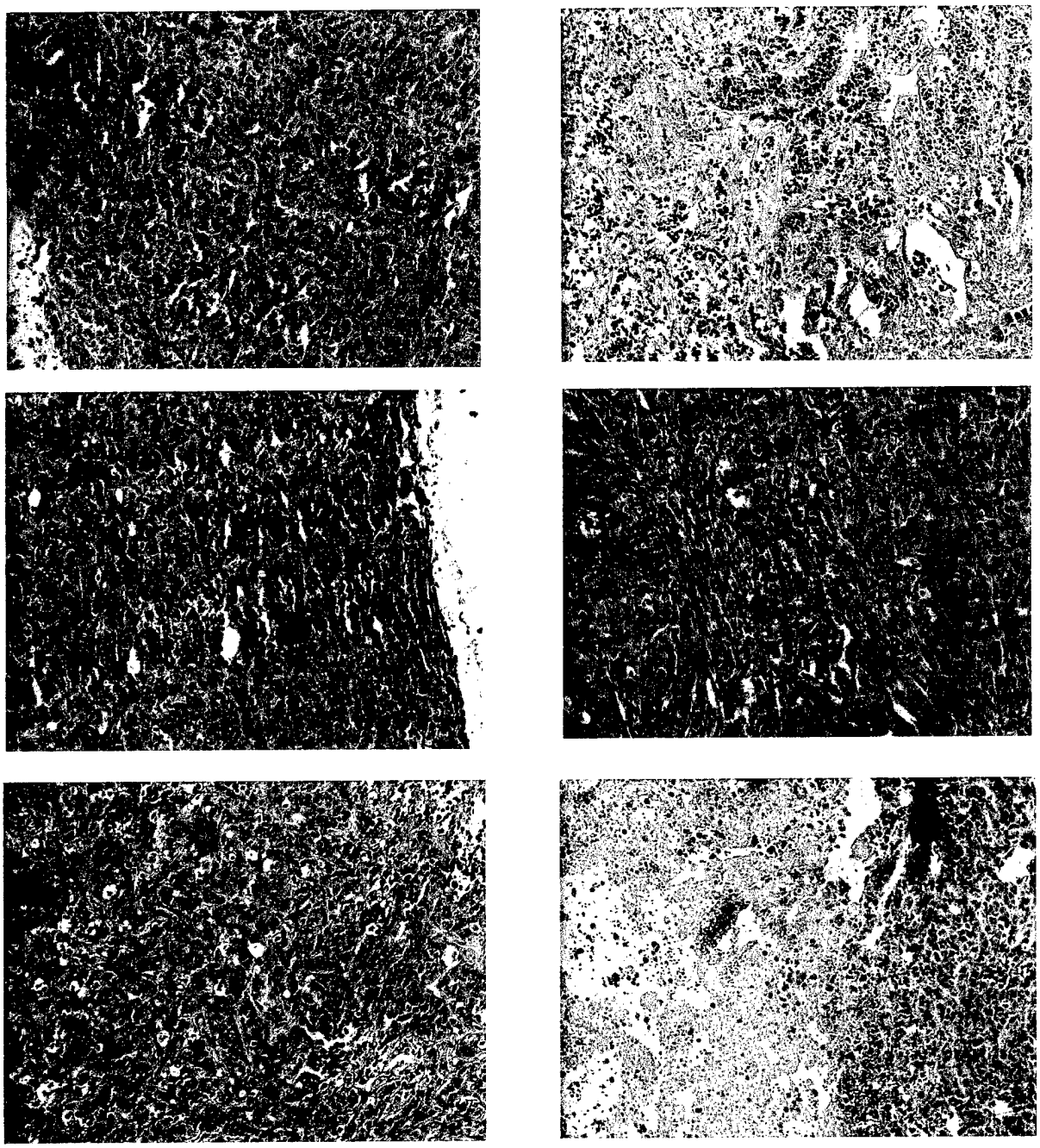

Figure 2. Microscopic histological examination of: (1) control (non-irradiated) MCF-7 breast tumors of nude mice injected with macromolecular anti-cancer drug Interleukin-2 and nanoparticles (left) and (b) irradiated breast tumor of the same mice (right).

REPORTABLE OUTCOMES

We have presented two oral talks at a highly competitive conference: the Second Joint Conference of the IEEE Engineering in Medicine and Biology Society and the Biomedical Engineering Society:

1. Larina I.V., Evers B.M., Bartels C., Ashitkov T.V., Larin K.V., Esenaliev R.O. Ultrasound-enhanced Drug Delivery for Efficient Cancer Therapy. 2nd Joint EMBS-BMES Conference (Houston - October 2002).
2. Ivanova Y., Evers B.M., Robert T., Ashitkov T.V., Esenaliev R.O. Nanoparticles and Ultrasound for Delivery of Model Macromolecular Anti-cancer Drugs in Tumors. 2nd Joint EMBS-BMES Conference (Houston - October 2002).

The results of the studies performed in the first year of the project were published in the Conference Proceedings [7, 8]:

1. Larina I.V., Evers B.M., Bartels C., Ashitkov T.V., Larin K.V., Esenaliev R.O. "Ultrasound-enhanced Drug Delivery for Efficient Cancer Therapy", in Proceedings of 2nd Joint EMBS-BMES conference, Houston, TX, October 23-26, 2002, pp. 492-493.
2. Ivanova Y., Evers B.M., Robert T., Ashitkov T.V., Esenaliev R.O. "Nanoparticles and Ultrasound for Delivery of Model Macromolecular Anti-cancer Drugs in Tumors", in Proceedings of 2nd Joint EMBS-BMES conference, Houston, TX, October 23-26, 2002, pp. 504-505.

The reprints of these publications are enclosed. The results of the studies performed in the first and second years of the project will be published in two articles that are in preparation for submission to two peer-reviewed journals.

CONCLUSIONS

The results of our studies performed in the second year of the project demonstrated that interaction of ultrasound with nanoparticles results in enhanced delivery of real anti-cancer drugs with low and high molecular weight in breast tumors. Histological examinations of tumors after irradiation with ultrasound in combination with anti-cancer drug and nanoparticles injections showed dramatic necrosis produced by ultrasound-enhanced delivery of the drugs in tumor tissue. These data suggest that interaction of ultrasound radiation with nanoparticles may substantially improve the efficacy of breast cancer chemotherapy.

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7. Larina I.V., Evers B.M., Bartels C., Ashitkov T.V., Larin K.V., Esenaliev R.O. "Ultrasound-enhanced Drug Delivery for Efficient Cancer Therapy", in Proceedings of 2nd Joint EMBS-BMES conference, Houston, TX, October 23-26, 2002, pp. 492-493.
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Appendices

Proceedings of the Second Joint EMBS/BMES Conference
Houston, TX, USA • October 23-26, 2002

Ultrasound-enhanced Drug Delivery for Efficient Cancer Therapy

I. V. Larina[1,2], B. M. Evers[3], C. Bartels[1,2], T. V. Ashitkov[1,2], K.V. Larin[1,2,4], R. O. Esenaliev[1,2,4,5]
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Abstract- **Poor penetration of anti-cancer drugs through tumor vasculature and cancer cell membrane as well as slow diffusion of the drugs in the interstitium limit efficacy of cancer chemo- and biotherapy. Recently we proposed to use ultrasound-induced cavitation (formation, growth, and collapse of microbubbles) to enhance anti-cancer drug delivery through these barriers. Cavitation can be selectively induced in tumors by using interaction of ultrasound with nanoparticles that lower cavitation threshold and can be accumulated in tumors. In this paper, we measured cavitation threshold in water suspensions of polymer (polystyrene) nanoparticles and studied efficacy of cancer therapy in nude mice with the use of this technique. Experiments were performed at different irradiation conditions and concentration and size of nanoparticles. In vivo studies were conducted in nude mice bearing human colon (KM20) tumors at optimum conditions found in the experiments in water suspensions. Our studies demonstrated that: (1) polystyrene nanoparticles decrease cavitation threshold in water; and (2) application of this drug delivery technique substantially improve the efficacy of cancer therapy in nude mice when ultrasound was used in combination with polymer nanoparticle injections. Our results suggest that the ultrasound-induced cavitation enhances drug delivery in tumors and may provide efficient cancer chemo- and biotherapy.**

Keywords – **nanoparticle, drug delivery, ultrasound**

I. INTRODUCTION

To reach cancer cells in a tumor, anti-cancer drugs have to penetrate through three physiological barriers: blood vessel wall, interstitial space, and cancer cell membrane [1, 2]. These barriers may substantially limit efficacy of cancer chemo- and biotherapeutic drugs (especially macromolecular agents) [1, 2]. To enhance delivery of anti-cancer drugs from blood into tumor cells, we proposed to use interaction of nanoparticles having certain optical and acoustic properties with laser or ultrasound radiation to selectively induce cavitation in tumors [3]. The nanoparticles can lower cavitation threshold in tumors and provide conditions for enhanced drug delivery in cancer cells without damage to normal tissues. Interaction of tensile pressure of ultrasound with nanoparticles (that can be accumulated in tumors due to passive or active delivery) produces cavitation in tumors. The ultrasound-induced cavitation can perforate tumor blood vessel walls and cancer cell membranes and produce microconvection in the interstitium. This may result in enhanced delivery of the anti-cancer drugs through the physiological barriers. We performed preliminary studies of laser- and ultrasound-induced cavitation in tissue phantoms, in vitro, and in vivo with the use of this technique that demonstrated encouraging results [4-6].

In this work, we measured cavitation threshold in water and suspensions of nanoparticles and studied influence of ultrasound-induced cavitation on efficacy of cancer therapy in nude mice with human colon tumors that are highly resistant to chemotherapy.

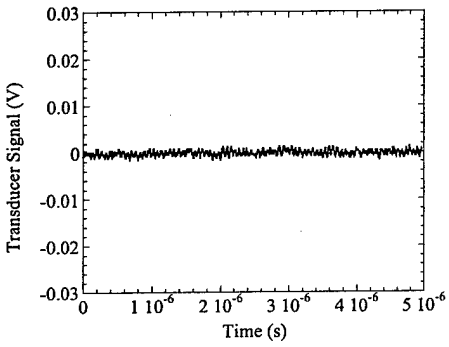
II. METHODOLOGY

Ultrasound generator operating at 20 kHz was used to produce cavitation in our experiments. Cavitation induced in suspensions of polystyrene nanoparticles (dia. = 100 or 280 nm) at different concentration was detected by a focused acoustic transducer in a water tank. Signals from the transducer were recorded by a scope, filtered by a high-pass filter to eliminate 20-kHz frequency, and processed by a computer. High-frequency signal induced by collapsing microbubbles was used to measure cavitation threshold. Water used in the experiment was distilled, boiled for 5 minutes, and left overnight to eliminate air bubbles that can lower cavitation threshold [5].

Athymic nude mice were injected s.c. with suspensions of human colon KM20 cancer cells (2×10^6 per site) on the left and right sides of the dorso-scapular area. We injected polystyrene nanoparticle suspensions (10% w/w in water, dia. = 100 nm, 150 μL) in the tail vein when tumors reached the size of 5 to 10 mm. We administered 5-FU (an anti-cancer drug currently being used for colon cancer therapy in patients and in nude mouse studies) i.p. at the dose of 90 mg/kg one day after the nanoparticles injections and irradiated one tumor by ultrasound with parameters found in the previous experiment. Length and width of control and irradiated tumors were measured with a caliper and used for tumor volume calculations. The tumor volume was presented as a function of time (in days) after the first treatment (irradiation) to assess efficacy of cancer therapy.

III. RESULTS AND DISCUSSION

Figure 1 shows typical acoustic transducer signals recorded below (top) and above (bottom) the cavitation threshold in a suspension of nanoparticles with the diameter of 100 nm and concentration of 0.1%. The transducer signals have no peaks without cavitation. If cavitation is induced, the transducer signals have multiple peaks with the duration of the order of 1 μs. These peaks are induced by the collapsing microbubbles. Integrated transducer signal was used to characterize level of cavitation in the sample. The integrated signal was plotted as a function of generated



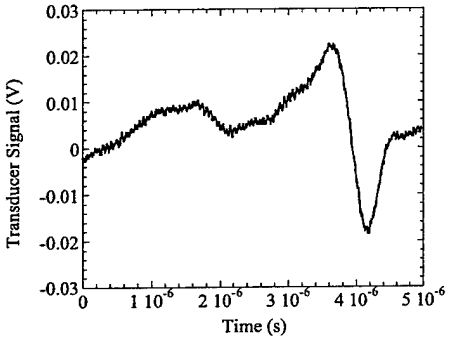


Fig. 1. Typical transducer signals recorded from a suspension of nanoparticles below cavitation threshold (top) and above cavitation threshold (bottom).

ultrasound pressure amplitude to measure the cavitation threshold.

It was found that the threshold is dependent on nanoparticle size and concentration. The lowest threshold measured at our irradiation conditions (3.5 bar) was obtained for nanoparticles with the concentration of 0.1% and diameter of 100 nm.

The nanoparticles with the diameter of 100 nm were used in the in vivo studies to produce cavitation in the tumors. Complete regression of irradiated tumors was obtained after one or two treatments when ultrasound was applied after injection of nanoparticles and 5-FU (Fig. 2). Arrows indicate days of treatment. The volume of the control tumors increased despite of 5-FU injections.

This response is typical for colon tumors due to their high resistance to chemotherapy. Combination of ultrasound with nanoparticle and 5-FU injections produced enhanced delivery of 5-FU into cancer cells as well as damage to tumor blood vessels in the irradiated tumors. This resulted in death of all cancer cells in the tumors.

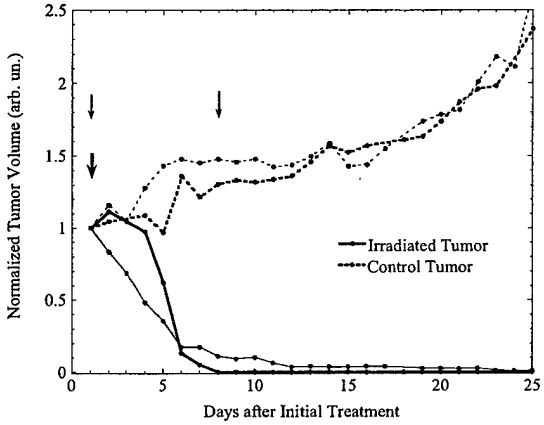


Fig. 2. Complete regression of tumors after irradiation by ultrasound in combination of nanoparticles and 5-FU injections.

III. CONCLUSION

Our studies demonstrated that: (1) polymer nanoparticles decrease cavitation threshold and can be used as cavitation nuclei for the drug delivery technique and (2) complete regression of tumors can be obtained by using drug delivery with ultrasound-induced cavitation.

ACKNOWLEDGMENT

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Nanoparticles and Ultrasound for Delivery of Model Macromolecular Anti-cancer Drugs in Tumors

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Abstract- **Penetration of macromolecular anti-cancer agents from blood into tumor cells is poor due to the physiological barriers: tumor capillary wall, interstitium, and cancer cell membrane. We proposed to use laser- or ultrasound-induced cavitation to enhance anti-cancer drug delivery through these barriers. Interaction of ultrasound with exogenous nanoparticles with certain acoustic properties may provide cavitation selectively in tumors and, therefore, may provide safe and efficient delivery of anti-cancer drugs in cancer cells without damage to normal tissues. In this paper, we studied enhanced delivery of model macromolecular anti-cancer drugs with ultrasound-induced cavitation in mice bearing human colon (KM20) and breast (MCF-7) tumors. Fluorescent rhodamine-dextrans of different molecular weight (10, 70, and 2,000 kDa) served as model drugs simulating antisense oligonucleotides, antibodies, and genes, respectively. Immunohistochemical staining of tumor vasculature with CD31 was used to visualize tumor blood vessels. Our studies demonstrated enhanced penetration of the drugs from blood vessels into tumor interstitium when ultrasound was applied in combination with polymer nanoparticle injections. Our results suggest that this drug delivery technique can potentially be used for efficient cancer chemo- and biotherapy.**

Keywords – **drug delivery, cancer therapy, nanoparticle, ultrasound**

I. INTRODUCTION

Efficacy and safety of cancer chemo- and biotherapy are limited, in part, due to poor penetration of anti-cancer agents from blood into tumor cells through the physiological barriers: blood vessel wall, interstitial space, and cancer cell membrane [1, 2]. Many promising macromolecular agents such as antisense oligonucleotides, antibodies, and genes have high molecular weight (of the order of 10, 100, and 1,000 kDa, respectively) and cannot reach cancer cells in tumors. We proposed to use laser- and ultrasound-induced cavitation to enhance delivery of anti-cancer agents from blood into tumor cells through these physiological barriers [3]. This technique utilizes interaction of exogenous nanoparticles with laser or ultrasonic radiation. The interaction of the nanoparticles with laser or ultrasonic radiation may produce cavitation. The ultrasound cavitation is produced by tensile pressure of ultrasonic waves and the nanoparticles serve as cavitation nuclei that lower cavitation threshold [4-6]. Nanoparticles can selectively be delivered to tumor capillaries by passive (due to increased leakage of tumor vasculature) or active (by using antibodies directed against tumor vasculature) delivery. The ultrasound-induced cavitation can perforate tumor blood vessel walls and cancer cell membranes and produce microconvection in the interstitium. This may result in enhanced delivery of the anti-cancer drugs in tumor cells.

In this paper, we studied delivery of model macromolecular anti-cancer agents with different molecular weight in human tumors of nude mice by using ultrasound-induced cavitation.

II. METHODOLOGY

We used athymic nude mice (average weight of 30 g) in this study. Suspensions of human colon KM20 or breast MCF-7 cancer cells (2×10^6 or 15×10^6 per site, respectively) were injected s.c. in the dorso-scapular area on the left and right sides of each mouse. Experiments were initiated when tumors reached the size of 5 to 10 mm (usually 3 or 5 weeks after the injection for colon or breast tumors, respectively). One tumor was irradiated by 20-kHz ultrasound for 10 minutes by pulses with duration of 0.1 s and repetition rate of 2 Hz, while the other tumor served as control. Ultrasound pressure amplitude measured by a calibrated hydrophone was 4 bar. Ultrasound with these parameters provides efficient cavitation in tissue phantoms with polystyrene nanoparticles [5]. Polystyrene nanoparticles (10% w/w in water, dia. = 100 nm) were used as cavitation nuclei. The nanoparticles were injected in the tail vein of nude mice one day prior to irradiation to allow extravasation of the particles in tumor blood vessels. Rhodamine-dextrans with molecular weights of 10, 70, 2,000 kDa were used to simulate anti-cancer agents: antisense oligonucleotides, antibodies, and genes, respectively. Thin (5-μm) sections of the irradiated and control tumors were studied with a fluorescence microscope to visualize rhodamine-dextrans. After fluorescence studies immunohistochemical staining of these sections with CD31 was performed to visualize tumor blood vessels. Same areas of irradiated and control tumors were found with the microscope under regular illumination and photographed for comparison with the fluorescence data.

III. RESULTS AND DISCUSSION

Figure 1 shows regular (top) and fluorescent (bottom) microscopy of a control tumor. The mouse had two KM20 tumors and was injected with the nanopartilces and rhodamide-dextran with the molecular weight of 2,000 kDa. Tumor blood vessels stained with CD31 are visible as dark areas. Fluorescence microscopy shows distribution of the rhodamide-dextran. Comparison of the two pictures indicates that the rhodamine-dextran is confined within the tumor vasculature and does not penetrate into the tumor interstitium.

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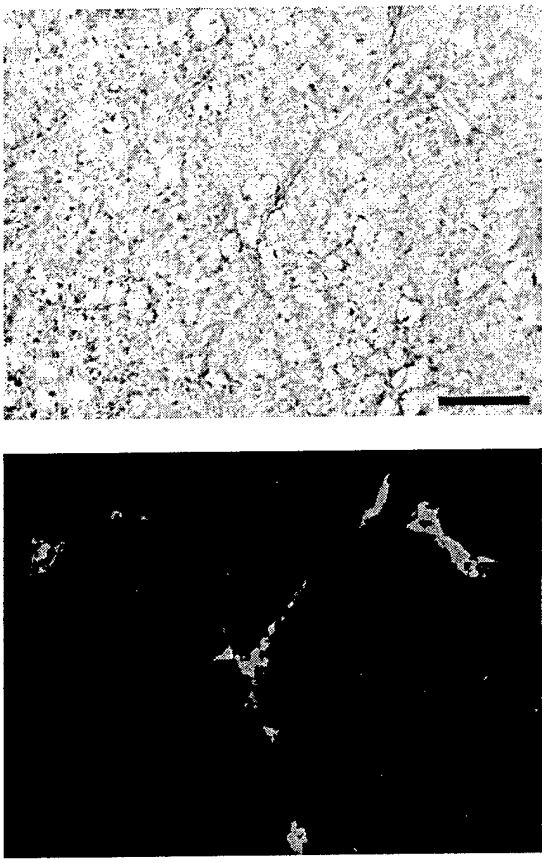

Fig. 1. Immunohistostaining of blood vessels of the control tumor (top) and distribution of the model macromolecular anti-cancer drug (M.W. =2,000,000) in the tumor (bottom). Bar = 100 μm.

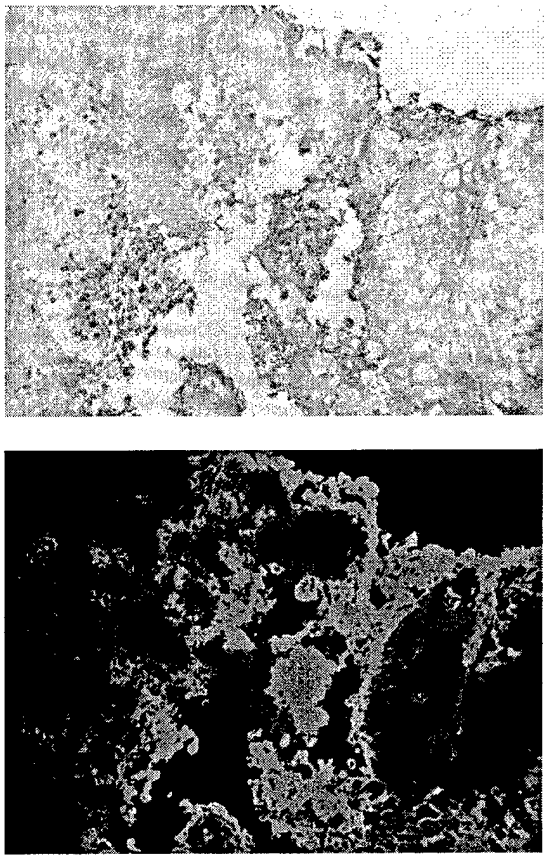

Fig. 2. Immunohistostaining of blood vessels of the irradiated tumor of the same mouse (top) and distribution of the model macromolecular anti-cancer drug (M.W. =2,000,000) in the tumor (bottom).

Regular and fluorescent microscopy of the irradiated tumor of the same mouse is presented in Fig. 2 (top and bottom, respectively). One can see that tumor vessels are damaged by the ultrasound-induced cavitation and rhodamine-dextran penetrated in the interstitium. Similar results were obtained for rhodamine-dextrans with the other molecular weights.

III. CONCLUSION

Our studies demonstrated that interaction of ultrasound with nanoparticles results in: (1) damage to tumor blood vessels and (2) enhanced delivery of macromolecular anti-cancer drugs in the tumor interstitium. This technique may be used for delivery of macromolecular anti-cancer drugs in tumor cells.

ACKNOWLEDGMENT

The authors would like to thank the Department of Defense Breast Cancer Research Program (grant #DAMD17-01-1-0416), Texas Advanced Technology Program (grant #004952-0088-2001), and John Sealy Memorial Endowment Fund for support of these studies.

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