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Selective Targeting of Magnetic Albumin Microspheres Containing Low-dose Doxorubicin: Total Remission in Yoshida Sarcoma-bearing Rats

KENNETH J. WIDDER,*†‡ ROBERT M. MORRIS,§ GERALD A. POORE,§ DONALD P. HOWARD§ and ANDREW E. SENYEI||†

*Department of Pathology, Duke University Medical Center, Durham, NC 27710, U.S.A., §Lilly Research Laboratories, Eli Lilly & Co., Indianapolis, IN 46285, U.S.A. and ||Department of Obstetrics and Gynecology, University of California, Irvine, Orange, CA 92688, U.S.A.

Abstract—*Magnetically responsive albumin microspheres containing doxorubicin hydrochloride were selectively localized in Yoshida sarcoma tumors. Tumors were implanted subcutaneously in the tail of Holtzman rats and allowed to grow to at least* 200 mm^2 *size before initiation of experimental treatment. Drug-bearing microspheres at a dose level of either* 0.5 *or* 2.5 mg/kg *were infused proximal to the tumor via the ventral caudal artery. A bipolar permanent magnet was placed adjacent to the tumor during the infusion to effect localization. Control animals were treated with free doxorubicin infused intra-arterially at* 5.0 mg/kg *or* 0.5 mg/kg. *In other test groups animals received placebo microspheres localized in the tumor via influence of the external magnetic field, or drug-containing microspheres were infused without utilization of the magnet to effect localization. Of the* 22 *animals receiving magnetically localized doxorubicin microspheres* 17 *had total histological remission of the tumor. The remaining animals demonstrated marked tumor regression representing as much as* 500–600 mm^2 *decrease in tumor size. While no deaths or metastases occurred in the groups receiving localized drug, animals treated with free doxorubicin, placebo microspheres or non-localized doxorubicin microspheres exhibited a significant increase in tumor size with metastases and subsequent death in* 90–100% *of the animals. No significant differences were noted in tumor regression/remission data between the* 0.5 *and* 2.5 mg/kg *dose levels of magnetically localized doxorubicin spheres. These results represent a significant advance in targeted chemotherapy in that* 77% *of the animals in the magnetically localized doxorubicin microsphere treatment groups exhibited total remission after only one regimen of drug therapy.*

INTRODUCTION

SELECTIVE targeting of antineoplastic agents to known foci of tumor has been an ongoing challenge in cancer chemotherapy. Many different approaches have been taken with relatively little success. Two such approaches consist of utilizing biophysical modalities such as liposomes [1, 2] or immunologic methods such as coupling tumor-specific antibodies with chemotherapeutic agents [3, 4]. Recently Weinstein *et al.* [5, 6] and Yatvin *et al.* [7] have described increased local concentrations of drugs using temperature sensitive liposomes. However, to date, no experimental data describing significant regression or total remission of established solid tumors by targeting methods has been reported. The various other modalities for the targeting of drugs have recently been extensively reviewed and will not be discussed here [8].

Drug-containing magnetically responsive albumin microspheres were developed to allow a high degree of selective targeting *in vivo* by utilizing external magnetic fields [9, 10]. By

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†Present Address: Molecular Biosystems, Inc., 11180-A Roselle Street, San Diego, CA 92121, U.S.A.
‡To whom reprint requests should be addressed.

choosing appropriate magnetic field gradient products [11] we have demonstrated the ability to localize significant levels of injected microspheres both *in vitro* and *in vivo*. Moreover, the localized microspheres were shown to contain biologically active doxorubicin [12], which was retained in high concentration in the target tissue over an extended period of time [13]. The microspheres remained at the target site for at least 24 hr and ultrastructural examination revealed evidence for extravasation of the carrier [9]. This data suggested the possibility that drug-carrying magnetic microspheres could be used to create a depot for local sustained release of a drug in a confined body site.

Recently we reported the use of magnetic albumin microspheres containing doxorubicin in the treatment of an established solid tumor [14]. We now present further data demonstrating total remission in Yoshida sarcoma-bearing rats utilizing magnetic microspheres containing doxorubicin.

MATERIALS AND METHODS

Animal and tumor

The ascitic form of the Yoshida rat sarcoma was supplied by the Mason Research Institute (courtesy of Dr. Arthur Bogden) and was serially passaged intraperitoneally in female Holtzman rats (80–125 g). Animals used in the studies were inoculated with 3.5×10^8 cells subcutaneously in the lateral aspect of the tail approximately two-thirds the distance distal from the base of the tail.

Microsphere preparation

Magnetic microspheres of 1 μm average diameter were prepared using methods previously described [10]. A phase emulsion polymerization process was used in which the aqueous phase consisted of human serum albumin, doxorubicin hydrochloride and magnetic iron oxide. One hundred and twenty-five milligrams of human serum albumin (Sigma Chemical), 16 mg of doxorubicin hydrochloride (Aldrich Chemical) and 36 mg of magnetite (Fe_3O_4, Ferrofluidics Corporation) were combined in 0.5 ml distilled water. Thirty milliliters of cottonseed oil (Sargent Welch) was added to the aqueous phase and an emulsion was formed by sonication with a Heat Systems (Model W185D) sonifier with a 1/4-inch probe for 1 min at 100 W. The resulting homogenate was added to 100 ml of continuously stirred cottonseed oil at 120–125°C contained in a flask immersed in a constant-temperature oil bath. After 10 min the suspension was removed from the oil bath and allowed to cool. Microspheres were washed four times with anhydrous diethyl ether and separated by centrifugation.

Placebo microspheres were prepared in an identical manner except doxorubicin was eliminated from the preparation.

Chemical analysis of doxorubicin

Total doxorubicin content entrapped in microspheres was determined by incubating microspheres for 12 hr at 4°C using 5% hydrochloric acid in ethanol (acid alcohol) as the release medium. The extraction tubes were centrifuged at 48,000 *g* for 15 min and the supernatants were analyzed for fluorescence at λ ex/em = 470/585 nm, using an Aminco SPF-125S spectrophotofluorometer (lower level of detection 250 ng/ml). The amount of unchanged doxorubicin after encapsulation has previously been reported and was determined by chromatographing the products released after 24 hr of incubation in PBS (4°C) on thin-layer silica gel developed in chloroform:methanol:acetic acid: water (60:20:24:6) [10, 12]. Migration of the parent compound, adriamycinol and aglycones was evaluated by comparing R_f values to known standards. The absolute concentration of each compound was determined by extracting specific areas of the chromatogram with acid alcohol and determining the relative fluorescence of each band as described above.

Tumor sensitivity to doxorubicin

Initially the sensitivity of the Yoshida sarcoma to doxorubicin was assessed by inoculating female Holtzman rats with 2.9×10^7 cells in the axillary region. Drug was administered intravenously at 0.5 mg/kg at 1, 5 and 9 days post-inoculation of the tumor. A control group was injected with sterile physiological saline. Response to treatment was assessed by weighing animals and by measuring tumor in two dimensions with a micrometer. Tumor size is stated as mm^2, being a product of maximum tumor width multiplied by tumor length.

Treatment studies with microspheres

Female Holtzman rats were inoculated 6–8 days prior to experiments with 3.5×10^8 cells subcutaneously into the lateral aspect of the tail. By the time of treatment tumors were not only visible and measurable, but on occasion the skin overlying the tumor was necrotic. Tumor size at the time of experiments averaged at least 200 mm^2; however, much larger tumors were also used in an effort to randomize size and to treat both large and small tumors. At the time of treatment animal weights ranged from 185 to 250 g. All animals were palpated for the presence of metastases prior

to acceptance for treatment to attempt to eliminate those with obvious metastatic nodules. Experiments were performed in a double blind manner. Animals were randomly chosen and color coded by investigators who treated the animals and recorded the mode of therapy. Another independent group of investigators assessed tumor growth/regression and weight change.

Animals were anesthetized with methoxyfluorane (Dow Chemical) and the ventral caudal artery was exposed near the base of the tail. A polyethylene catheter (Clay Adams PE10) was inserted to a point 2 cm proximal to the tumor. In those groups in which magnetic localization was used a permanent bipolar adjustable gap magnet (Edmund Scientific) with a field strength of 5500 Oe was positioned so that the tumor was in contact with one of the pole faces. All doses were infused in 1 ml of normal saline containing 0.1% Tween 80 (polyoxyethylene sorbitan monooleate, Sigma Chemical) into the ventral caudal artery via a constant flow syringe pump (Sage Model 341) at 0.25 ml/min. Following infusion, 1 ml of normal saline was infused at the same rate to clear the tubing of the full dose. In those groups in which there was dose localization the magnet was retained in position for 30 min. Following dosing, the catheter was removed and the skin overlying the cut-down site was sutured. Animals were kept for 30 days post-treatment or until death and monitored for weight change, tumor size and metastases. Surviving animals were killed 30 days after treatment. Organs and original tumor sites were examined both grossly and microscopically for evidence of tumor or distant metastases.

Animals involved in the study received only a single dose treatment 6–8 days post-inoculation. In all, seven groups of 10–12 animals each were utilized to compare various modes of treatment:

Group A: untreated controls in which no drug was administered;

Group B: 0.5 mg/kg of doxorubicin as solution administered via the ventral caudal artery (i.a.);

Group C: 5 mg/kg of doxorubicin as solution administered i.a.;

Group D: placebo microspheres administered i.a. with magnetic localization;

Group E: 0.5 mg/kg of doxorubicin as microspheres administered i.a. *without* magnetic localization;

Group F: 0.5 mg/kg of doxorubicin as microspheres administered i.a. with magnetic localization;

Group G: 2.5 mg/kg of doxorubicin as microspheres administered i.a. with magnetic localization.

RESULTS

Sensitivity of tumor to doxorubicin

Results of administering free doxorubicin to animals bearing axillary implants of the Yoshida sarcoma are shown in Table 1. Significantly, only when animals were given doxorubicin on a 5 mg/kg multiple dose regimen (days 1, 5 and 9) was there any regression of tumor. Tumor size was 82% smaller in this group as compared to saline control animals at day 12; however, doxorubicin toxicity was evident as determined from weight loss and the general appearance of the animals. No significant increase in survival time was noted for the 5 mg/kg treatment group over the saline controls or the low dose (0.5 mg/kg) doxorubicin group. The data from the 0.5 mg/kg doxorubicin group indicates that such a level even in a multiple dose regimen was ineffective against the Yoshida sarcoma.

Treatment of animals with microspheres

Results of single dose therapy with various modes of doxorubicin dosage are presented in Tables 2 and 3. All tumor and weight data are expressed as the average value, with the range indicated after the value. Tumor size increased in all groups except those in which doxorubicin microspheres were magnetically localized. In those cases where tumor growth was 50% or less, this generally occurred where the animal died of extensive metastases, thus resulting in a shortened survival time. The two groups in which doxorubicin was administered as a solution showed significant tumor growth, including the 5 mg/kg level, which was noted to be mildly effective in the multiple dose regimen in the initial tumor sensitivity studies. In contrast, when microspheres containing either 0.5 or 2.5 mg/kg doxorubicin were infused in the presence of the magnet adjacent to the tumor there was an overall 90–91% decrease in tumor size.

Of equal significance is the fact that in all but the magnetically localized sphere groups 90–100% of the animals in the other groups were dead by day 30 because of widespread distant metastases. These deaths, especially in certain animals with short survival times, have the effect of depressing the values in tumor growth data. In contrast, no deaths or metastases occurred in the localized sphere groups. Moreover, 77% of the animals treated with magnetically localized doxorubicin had complete histologic tumor remission, while the remainder exhibited significant tumor regression.

No remissions or regressions were noted in any other groups. There was one animal in both the doxorubicin solution group and in the group receiving doxorubicin microspheres without magnet that exhibited only 14% or less tumor

Table 1. The effect of intravenous doxorubicin administered 1, 5 and 9 days post-subcutaneous inoculation of the Yoshida sarcoma

Day		Saline control	Adriamycin (0.5 mg/kg)	Adriamycin (5 mg/kg)
6	Tumor size (mm^2)	584 (341–732)	751 (530–940)*	98 (66–140)
6	Weight change (g)	+23.3	+17.8	-12.0
6	Survival	5/5	5/5	5/5
12	Tumor size (mm^2)	570 (495–644)	780*	100*
12	Weight change (g)	+29.3	+15.8	-18.0
12	Survival	2/5	1/5	1/5
	Average day of death	9.2	9.6	10.2

All five animals in each group received an inoculation of 2.9×10^7 cells into the axillary region. Animals were treated via tail vein injection on days 1, 5 and 9 post-inoculation with 1 ml of doxorubicin at a dosage of 5 mg/kg or 0.5 mg/kg. Five animals received normal saline. Tumors were measured in two dimensions with calipers. These two measurements were used to compute area and the values reported represent the average tumor size (area: mm^2) of the five animals in the group. Numbers in parentheses represent range of values. Change in weight represents the average change of the five animals in each group.

*No tumor size range—only one survivor.

Table 2. Effect of magnetic microspheres containing doxorubicin on growth of Yoshida tumor and animal weight

Group:	A	B	C	D	E	F	G
	Untreated control	Doxorubicin as solution, 0.5 mg/kg i.a.†	Doxorubicin as solution, 5 mg/kg i.a.	Placebo microspheres with magnet	Microspheres bearing 0.5 mg/mg doxorubicin; no magnet	Microspheres bearing 0.5 mg/kg doxorubicin with magnet	Microspheres bearing 2.5 mg/kg doxorubicin with magnet
Initial tumor size (mm^2)*	423 (140–728)	459 (220–840)	496 (300–900)	254 (88–370)	502 (260–900)	413 (230–594)	226 (105–350)
Final tumor size (mm^2)	932 (400–1425)	701 (363–1440)	859 (600–1350)	594 (300–1870)	1076 (500–1800)	38 (0–272)	22 (0–180)
Tumor change (%)	+120 (+52–+217)	+53 (+4–+1401)	+73 (+11–+221)	+149 (+30–+766)	+114 (+14–+362)	-91 (-37–100)	-90 (-44–100)
Initial weight (g)	239 (220–270)	230 (195–290)	216 (200–245)	200 (185–235)	228 (206–255)	231 (185–280)	210 (185–235)
Final weight (g)	294 (265–320)	249 (237–289)	237 (186–291)	247 (220–270)	252 (200–272)	263 (225–305)	252 (230–285)
Weight change (g)	+45	+19	+21	+47	+24	+32	+42

Animals received an inoculum of 3.5×10^8 cells subcutaneously into the lateral portion of the tail, 6–8 days prior to experimental use. Animals were color coded and selected for use in a double blind fashion. Animals received only single dose therapy regardless of the experimental group. The data is reported as the average result with the range indicated in brackets. Tumor data is expressed as area (mm^2).

*Tumors were measured in two dimensions with calipers and these two values were used to compute tumor size (area: mm^2).

†Final data reported represents mean value at day 30 (test completion) or at death.

Table 3. Effect of magnetic microspheres containing doxorubicin on growth of Yoshida tumor and animal weight

Group:	A	B	C	D	E	F	G
	Untreated control	Doxorubicin as solution, 0.5 mg/kg i.a.*	Doxorubicin as solution, 5 mg/kg i.a.	Placebo microspheres with magnet	Microspheres bearing 0.5 mg/mg doxorubicin; no magnet	Microspheres bearing 0.5 mg/kg doxorubicin with magnet	Microspheres bearing 2.5 mg/kg doxorubicin with magnet
Deaths (%)	90	100	100	100	100	0	0
Regressions (%)	0	0	0	0	0	100	100
Total remissions (%)	0	0	0	0	0	75	80
Metastases (%)	100	100	100	100	100	0	0

The experiment was carried out to day 30 post-treatment. Values reported represent data on day 30. The majority of metastases appeared in the inguinal region 14–16 days after inoculation.

*Final data reported represents mean value at day 30 (test completion) or at death.

growth. All three of these animals had very short survival times due to metastases.

Comparison of the data from the two magnetically localized doxorubicin treatment groups indicates no significant differences in the beneficial effects derived from a dose level of 2.5 vs 0.5 mg/kg. The data shows an approximate 90% decrease in average tumor size for both dose levels, with all animals exhibiting significant tumor regression. Nine of twelve animals at the 0.5 mg/kg dose had complete histologic tumor remission, while eight of ten animals exhibited a similar response to the five-fold higher localization dose.

DISCUSSION

Results from this study demonstrate the ability to achieve a significant number of total histologic remissions (77%) in tumor-bearing animals by targeting microspheres containing doxorubicin specifically to the tumor mass. Such results were achieved only by one administration of the microspheres at doses as low as 10% of the systemic multiple regimen dose. The multiple dose regimen of 5 mg/kg was also noted to be complicated by toxicity not seen with the targeted therapy. Other single dose modes of therapy with doxorubicin were noted to be totally ineffective against the Yoshida sarcoma with the vast majority of animals showing significant growth of tumor, distant metastases and subsequent death. Of interest also is the fact that none of the animals in the magnetically localized sphere group exhibited distant metastases when killed 30 days after treatment.

Currently, we do not know the lower level of sensitivity of the delivery system. In this and an earlier study [14] we demonstrated 90–100% regression, with a single magnetically localized doxorubicin dose of 0.5 mg/kg. Elevation of this dose five-fold did not significantly improve the already dramatic tumor regression effects noted with the lower dose. By using magnetic localization we are significantly concentrating the drug in a confined region, thereby creating extremely high localized drug levels [13]. Thus it seems entirely possible that dramatic regression/remission might still be possible with localized microsphere doses lower than those used in this study.

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