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13. SUPPLEMENTARY NOTES

14. ABSTRACT This project is a pre-clinical study designed to determine if amifostine might be effective in preventing breast cancer initiation by medical exposures to ionizing radiation. The experiments will determine if amifostine is protective in a murine model of breast cancer and, if so, determine the optimum dose, route and timing for its administration. The first year's objective was to test a high dose of amifostine administered I.P. prior to irradiation for reduction of ductal dysplasia in an outgrowth assay. Between September 2002 and May 2003, twenty-two donor mice were irradiated and 412 mammary fat pads were transplanted with mammary epithelial cells from these irradiated donors. Of these, 306 were harvested and examined as whole mounts. No dysplasias were seen, but due to the low outgrowth frequency no conclusions could be drawn on the effectiveness of amifostine. In August 2003 the PI changed institutions and experienced a delay of nearly 2 years in having the grant transferred. The project resumed in August 2005 with the establishment of a new breeding colony. An additional 116 mice were transplanted (232 fat pads) and outgrowths prepared for histology. Once again, there were few outgrowths and it was decided to switch from ductal dysplasia to frank mammary tumors as the experimental endpoint. A new Statement of Work was submitted in January 2007 and approved mid-April 2007. 294 mice divided into three groups: irradiated, irradiated and amifostine treated and unirradiated, were set up on June 1, 2007 and are being followed for mammary tumor development. At the time of this report there is no difference in survival between the groups. Histopathological classification of tumors is in progress.

15. SUBJECT TERMS
Prevention of diagnostic and treatment induced long-term morbidity, Chemoprevention, Radiation-induced breast cancer

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INTRODUCTION:

Historical medical exposures to radiation have been found to increase breast cancer risk. Some currently used diagnostic radiology and cancer radiotherapy procedures deliver doses to mammary tissue in susceptible girls and women that are comparable to the historical exposures. The research supported through DAMD17-02-1-0460 is designed to determine if a chemoprotective agent, amifostine, can prevent radiation-induced ductal dysplasias that progress to mammary tumors. The research design takes advantage of an inbred mouse strain that is susceptible to radiation-induced mammary tumors.

BODY:

The original approved statement of work is:

Task 1. To determine if the frequency of radiation-induced ductal dysplasia in BALB/cBy J mice can be reduced by 400 mg/kg amifostine (Months 1-12):

a. Mice required: 20 BALB/cByJ donors and 400 CB6F1 recipients. The recipients are F1 hybrids of BALB/c and C57BL/6J that are a bit more robust than BALB/c and give lower background levels in the dysplasia assay.

DONORS	TREATMENT	RECIPIENTS
10 BALB/c	saline	200 CB6F1
10 BALB/c	amifostine	200 CB6F1

b. Methods: Assays on donor mice are set up weekly. In each assay a donor mouse will receive either saline or 400 mg/kg amifostine by intraperitoneal injection. Half an hour later the mouse will be irradiated to the whole body with 1 Gy in a ^{137}Cs small animal irradiator. After six weeks the donor will be sacrificed and the mammary epithelial cells will be harvested. The mammary epithelial cells will be transplanted into cleared mammary fat pads in 20 recipients (two fat pads per recipient). Ten weeks later the fat pads will be harvested from the recipients and processed as whole mounts. The whole mounts will be examined for ductal dysplasias and any suspected dysplasias will be confirmed by histological examination.

c. The results of these experiments will indicate if a high dose of amifostine can reduce the frequency of radiation-induced ductal dysplasias in a susceptible mouse strain.

d. Since amifostine has been shown to be active against radiation-induced tumorigenesis in other rodent models we anticipate that it will be effective in reducing radiation-induced ductal dysplasia in this system. However, if it is not we will screen other sensitive mouse strains (Weil et al. 2001) and other chemopreventive agents, including N-acetylcysteine and captopril .

Task 2. To determine if post-irradiation amifostine treatment can reduce the frequency of radiation-induced ductal dysplasia. If amifostine can be delivered post-irradiation it would be more acceptable as an agent to prevent radiotherapy-induced breast cancer, since it is less likely to provide tumor protection with this schedule of administration (Months 13-20):

a. Mice required: 15 donors and 300 recipients.

DONORS	AMIFOSTINE TREATMENT	RECIPIENTS
5 BALB/c	1/2 hour post-irradiation	100 CB6F1
5 BALB/c	2 hours post-irradiation	100 CB6F1
5 BALB/c	4 hours post-irradiation	100CB6F1

b. Methods: As described in Task 1 except amifostine will be injected ½, 2 and 4 hours following irradiation.
c. The results of these experiments will determine if amifostine can prevent ductal dysplasia if it is delivered after irradiation and, if so, provide a first indication of the how much later.
d. We anticipate a simple positive or negative result. However, if no protection is seen we may also assay a time as short as 5 minutes post-irradiation.

Task 3. To determine if lower doses of amifostine are effective in preventing radiation-induced ductal dysplasias. Reducing the dose would decrease side effects and alleviate concern about tumor protection if the drug is administered during tumor radiatherapy (Months 21-30):

a. Mice required: 15 BALB/c donors and 300 CB6F1 recipients.

DONORS	TREATMENT	RECIPIENTS
5 BALB/c	100 mg/kg amifostine	100 CB6F1
5 BALB/c	20 mg/kg amifostine	100 CB6F1
5 BALB/c	1 mg/kg amifostine	100 CB6F1

b. Methods: As described in *Task 1*, except decreasing doses of amifostine will be used.
c. The results of these experiments will determine the lowest dose of amifostine, down to 1 mg/kg, that will reduce the frequency of radiation-induced ductal dysplasia.
d. We do not anticipate problems with this task.

Task 4. Test oral administration of amifostine. Oral administration is easier than intravenous delivery in patients that are not receiving other intravenous medications. (Months 31-36)
a. Mice required: 5 donors 100 recipients.

b. Methods: As described in *Task 1*, except amifostine will be delivered by gavage.
c. The results of these experiments will determine if amifostine administered p.o. will reduce the frequency of radiation-induced ductal dysplasia.
d. If oral administration is ineffective we will examine other routes such as subcutaneous or intramuscular injection. We will also test compounds similar to amifostine but more suited to oral administration such as WR-3689, WR151327, N-acetylcysteine and captopril.

The first task is to determine if high dose amifostine administered I.P. prevents radiation-induced ductal dysplasia. Work was begun at M.D. Anderson Cancer Center and transferred to Colorado State University. Transferring the grant required approximately two years. During that time mice used in the project were maintained on the Principal Investigator's start up funds until they became too old to use. A summary of the work to date, broken down by work site, is provided below.

M.D. Anderson Cancer Center

Treatment	Donors	Fat pads transplanted	Outgrowths	Dysplasias
Amifostine	6	148	39	0
none	6	158	34	0

The table above reveals a technical shortcoming - a low percentage of transplanted fat pads have outgrowths. Ideally this number should be 70% but in these experiments it was only 24%. We have seen this before and other groups have also experienced similar reductions in outgrowth frequency. The decreased efficiencies have been transient and their cause(s) remain unknown. Although we had normal mammary epithelial cell yields from the donors and good cell viabilities we have, never the less, replaced all the reagents used in the assay.

Colorado State University

Treatment	Donors	Fat pads transplanted	Outgrowths	Dysplasias
Amifostine	14	92	22	5
none	22	140	40	6

The table above summarizing transplants at CSU also reveals fewer outgrowths than we would have liked. The difference between the amifostine treated group and untreated group is not significant but there are two major concerns. The first is that with this number of outgrowths there is little power (.079) to detect even a 2-fold difference in incidence between the groups assuming a 20% incidence in the untreated mice. The second is that the pathologist that screened the outgrowths is relatively inexperienced with this assay.

At this point it was decided to switch to tumorigenesis as the endpoint for testing the efficacy of amifostine. This endpoint takes longer to assay and requires more drug, but it circumvents the

technical obstacles we've encountered with the ductal dysplasia assay. We submitted a modified Statement of Work (below) that was approved in mid-April 2007.

Revised Statement of Work

Task 1. To determine if the frequency of radiation-induced mammary tumors can be reduced by 400 mg/kg amifostine, 300 female BALB/cByJ mice at 10 weeks of age will be treated with amifostine and irradiated. The experimental groups are:

Group	Group size	Treatment	Irradiation
1	100	none	none
2	100	none	0.5 Gy
3	100	amifostine	0.5 Gy

Each mouse in group 3 will receive 400 mg/kg amifostine by intraperitoneal injection. Half an hour later the mice in groups 2 and 3 will be irradiated to the whole body with 0.5 Gy in a ^{137}Cs irradiator.

The experiment outlined above was set up on 6/1/07, except that only 94 mice were available for Group 1. The mice have now been followed for just under 600 days. As of January 20, 2009, thirty-five mice in Group 1, forty-eight mice in Group 2, and fifty-three mice in Group 3 have died or been euthanized because they were moribund. These mice were necropsied and tissues were collected for histopathological classification of tumors. The necropsy results (not yet supported by histopathology) suggest most of the tumors are likely lung tumors, mammary tumors and ovarian tumors. Radiation-induced lung tumors, ovarian tumors and acute myeloid leukemias have been observed by others in irradiated BALB/cByJ mice. They may become a source of competing mortality in this study, which is a weakness of using the tumorigenesis endpoint. However, statistical methods are available to deal with competing morbidity and it does provide us with the opportunity assess the effects of amifostine on other tumor types that are relevant to second cancers occurring in radiotherapy patients. At this time there is no significant difference between the groups by Kaplan-Meier Survival analysis (Figure 1).

Since radiation-induced tumors often have a long latency period we intend to monitor the mice until they are 800 days of age and confirm the classification of the tumors by histopathology. The extended monitoring period may allow a sufficient number of tumors to arise for statistically significant differences in the groups to be detected.

KEY RESEARCH ACCOMPLISHMENTS:

- 538 mammary fat pads have been transplanted with mammary epithelial cells from irradiated donors
- All transplanted fat pads glands have been harvested, prepared as whole mounts and screened for dysplastic outgrowths
- A 294 mouse experiment has been set up that uses mammary tumor development as a endpoint to assess the efficacy of amifostine in preventing radiation-induced mammary tumors

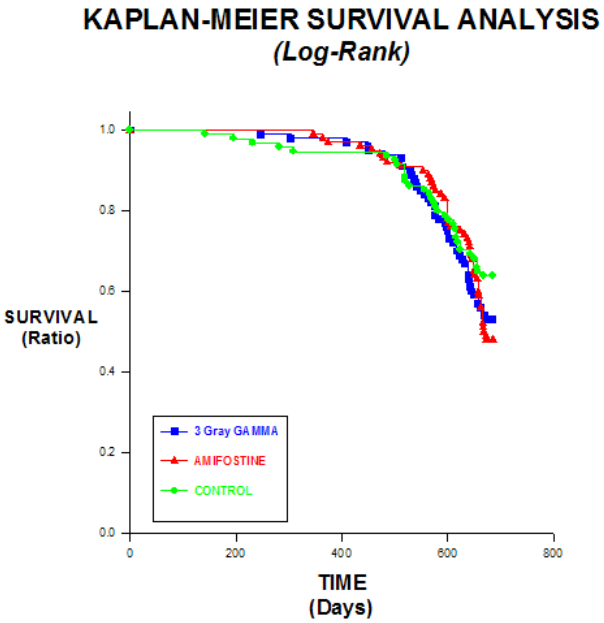

Figure 1. P = 0.212 for the log rank statistic for the survival curves.

REPORTABLE OUTCOMES:

None.

CONCLUSIONS:

The ductal dysplasia assay has not generated a sufficient number of outgrowths to determine the efficacy of amifostine in reducing that surrogate endpoint for radiation-induced mammary tumors. An experiment has been set up that uses actual tumorigenesis rather than ductal dysplasia as the endpoint. The mice used in this experiment will be monitored to 800 days of age.

REFERENCES:

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APPENDICES:

None.

PUBLICATIONS AND ABSTRACTS:

None.

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