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Radiation therapy may increase metastatic potential in alveolar rhabdomyosarcoma

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Abstract

Background—We previously determined that radiation could be safely administered using a mouse-flank *in vivo* model to both alveolar (Rh30) and embryonal (Rh18) rhabdomyosarcoma xenografts. Mice from both tumor lines in this experiment developed metastases, an event not previously described with these models. We sought to determine if radiation-induced changes in gene expression underlie an increase in the metastatic behavior of these tumor models.

Procedure—Parental Rh18 and Rh30 xenografts, as well as tumor that recurred locally after radiotherapy (Rh18RT and Rh30RT), were grown subcutaneously in the flanks of SCID mice and then subjected to either fractionated radiotherapy or survival surgery alone. Metastasis formation was monitored and recorded. Gene expression profiling was also performed on RNA extracted from parental, recurrent, and metastatic tissue of both tumor lines.

Results—Rh30 and Rh30RT xenografts demonstrated metastases only if they were exposed to fractionated radiotherapy, whereas Rh18 and Rh18RT xenografts experienced significantly fewer metastatic events when treated with fractionated radiotherapy compared to survival surgery alone. Mean time to metastasis formation was 40 days in the recurrent tumors and 73 days in the parental xenografts. Gene expression profiling noted clustering of Rh30 recurrent and metastatic tissue that was independent of the parental Rh30 tissue. Rh18RT xenografts lost radiosensitivity compared to parental Rh18.

Conclusion—Radiation therapy can significantly decrease the formation of metastases in radio-sensitive tumors (Rh18) and may induce a more pro-metastatic phenotype in radio-resistant lines (Rh30).

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Keywords

Soft tissue sarcoma; radiation therapy; rhabdomyosarcoma; cancer genetics; pediatric oncology

Introduction

A pilot and optimization radiation study for rhabdomyosarcoma involving alveolar (Rh30) and embryonal (Rh18) rhabdomyosarcoma xenografts of the Pediatric Preclinical Testing Program (PPTP)[1], as described by Kaplon et al.[2], demonstrated that clinically relevant radiation doses of 2 Gy per fraction up to a total of 40 Gy can be administered to mice with acceptable toxicities. During these experiments, some of the mice from each tumor line developed metastases. There have been numerous formally reported stage I trials of novel and standard compounds within the PPTP and the formation of distant metastases from subcutaneous flank xenografts during these projects had not been previously reported. Because the application of radiotherapy is new to the PPTP and has a direct effect on the genome, we theorized that radiotherapy might play a role in the metastasis formation by inducing or selecting for a more pro-metastatic phenotype, given its known direct mutagenic properties.

Orthotopic transplantation of human cancer xenografts in nude mice has been proven to provide an effective metastatic model [3]. Recently, Irons et al. were able to develop a metastatic model for basal MDA-MB-231 breast carcinoma cells by orthotopically injecting the cells into the mammary fat pads of mice [4]. A limitation with orthotopic transplantation is the ability to follow tumor growth. Heterotopic subcutaneous transplantation allows for measurement of tumor progression, but spontaneous metastases from subcutaneous human tumor xenograft implants are rare in the nude mouse model. Therefore, subcutaneous transplantation is rarely utilized when a metastatic model is indicated.

Cancers that relapse after radiotherapy are difficult to treat and patients have a poor prognosis. Evidence points to the irradiated tumor microenvironment as the likely source for the more aggressive phenotype [5]. Although the exact mechanisms remain unclear, angiogenesis, a hypoxic environment, stromal cell activation/differentiation, as well as recruitment of vasculogenic bone marrow derived cells have been described as contributing factors. Low doses of radiotherapy have been shown to induce VEGF expression in hypoxia-mimicking conditions and to activate vascular endothelial growth factor (VEGF) receptor 2, which promotes endothelial cell migration leading to metastasis formation [6]. Kaplon et al. described metastatic events in mice without spontaneous recurrence of local disease at the original xenograft site, suggesting that another mechanism other than an irradiated microenvironment contributed to formation of distant metastases [2]. We hypothesized that radiotherapy induced changes in genomic expression were an integral part of the metastatic process along with the altering of the tumor microenvironment.

In this report, we characterized the effects of fractionated radiotherapy on metastasis formation from subcutaneously transplanted Rh18 and Rh30 xenografts as well as recurrent Rh18 and Rh30 xenografts (labeled as Rh18RT and Rh30RT). Further, we performed gene expression profiling to assess the molecular changes potentially underlying the metastases.

MATERIALS AND METHODS

Xenograft lines and mice

Two rhabdomyosarcoma (RMS) tumor lines previously verified as harboring wild type tp53 tumor suppressor protein, Rh18 and Rh30, were obtained from the PPTP. Rh30 cells are known to express the fusion transcription factor PAX3-FOXO1 whereas the Rh18 do not [7]. The fusion transcription factor was confirmed by RTPCR and the Rh18 line was confirmed to have MDM2 amplification (not shown). The two tumor lines were propagated and subcutaneously implanted into mouse flanks. These RMS xenografts were selected because they had not been exposed to cytotoxic agents before being harvested from patients and subcutaneously implanted into mice. CB17SC SCID $-/-$ female mice (Taconic Farms, Germantown, NY) were implanted with one of the tumor lines subcutaneously in the left flank. All mice were maintained under barrier conditions and experiments were conducted using protocols and conditions approved by the institutional animal care and use committee of The Ohio State University (IACUC protocol 2010A00000192 [effective 3-year approval period: 12/28/2010–12/28/2013]).

Treatment groups

Parental Rh18 and Rh30 xenograft lines were implanted into the mouse flank, had radiotherapy applied, and were followed for metastasis formation. Eight Rh18 and five Rh30 bearing mice underwent survival surgeries alone provide local control to the flank, while the remaining mice received radiotherapy alone. Lastly, locally recurrent tumors following radiation therapy (20-30 Gy), labeled Rh18RT and Rh30RT, were implanted into new SCID mice and subjected to radiotherapy or survival surgery alone to evaluate metastatic propensity. After implantation, all xenografts were allowed to grow until reaching approximately one cubic centimeter in volume before initiating their respective therapies. The rate of metastases formation per tumor line, number of metastases per mouse, time to metastasis appearance, dose of radiation per cubic centimeter of tumor, and location of metastases were recorded for each group.

Radiotherapy and survival surgery technique

In the irradiation group, mice received daily 2 Gy fractionated doses to total 20-30 Gy to the flank tumor, while shielding the host, using the apparatus and technique previously described by Kaplon et al. [2]. Although one group of five mice did tolerate 40 Gy in the previous study, the rest received doses ranging from 20-30 Gy and developed metastatic events, providing the rationale for the chosen dose. Groups of up to five mice were anesthetized at a time with 5% isoflurane and 4 L/min O₂ in an induction chamber. The mice were removed one at a time from the induction chamber and placed in individual units within the radiotherapy treatment chamber. A single 2 Gy fraction dose was applied to the xenograft and the mice were removed and allowed to recover under sterile conditions. Total time from induction to recovery from anesthesia was approximately 10 minutes. Mice received radiation (2Gy) daily for 5 days/week until a total dose of 20-30 Gy was achieved. Mice not receiving radiation did not undergo sham anesthesia to minimize the risk of harm to the animals. For the survival surgery alone control groups, mice were anesthetized as previously described and then moved to a nose cone where anesthesia was maintained with

1-2% isoflurane with oxygen at 1 L/min and the tumor was resected under sterile conditions. The mice were observed for tumor recurrence and subjected to repeated flank survival surgeries as needed to provide local control. All mice were observed for 120 days after their initial treatment unless metastatic disease resulted in early removal or if the primary tumor met early removal criteria by growing four fold in size larger than initial treatment tumor volume.

Metastasis verification, xenograft harvesting, nucleic acid extraction, and Affymetrix gene expression profiling

Rh18 and Rh30 parental xenografts, Rh18RT and Rh30RT recurrent tumor tissue after radiotherapy from previous studies, and metastatic tumor tissue were harvested, flash frozen, and stored in liquid nitrogen. The metastases harvested after mice were sacrificed were verified as tissue of human origin by the detection of human LDH. Portions of each sample were then subjected to RNA extraction for comparative analysis. The Qiagen miRNeasy mini kit (Valencia, CA; product no. 217004) was utilized to extract total RNA from each sample. Equivalent quantities of untreated xenograft, recurrent tumor tissue, and metastatic tumor tissue samples from each tumor line were combined into their own respective pooled samples. Every sample was quantified and qualified using the NanoDrop 2000 UV-Vis Spectrophotometer prior to and after sample pooling. Gene expression profiling was then performed on each collected pooled sample using Affymetrix U133 Plus 2.0 in order to illustrate the messenger RNA (mRNA) genomic similarities and differences between the samples.

Statistical analysis

Statistical analysis of the differences in metastatic rates between the irradiation group and survival surgery alone groups amongst the Rh18, Rh30, Rh18RT, and Rh30RT were performed using Fisher's exact testing. P values <0.05 were considered to indicate statistical significance. Statistical analysis volumetric change between parental xenografts and xenografts previously treated with radiotherapy was performed using a one-tailed student t-test, with p values of <0.05 indicative of statistical significance.

Results

This study included 68 mice with parental Rh18 xenografts and 43 mice with parental Rh30 xenografts. The frequency of metastases and sites are provided in Table 1. Sixty mice bearing Rh18 xenografts received irradiation of clinically relevant doses between 20-30 Gy. Three of the 60 experienced metastases for a rate of approximately 5%. Eight mice transplanted with an Rh18 xenograft were subjected to survival surgeries alone to serve as treatment controls for the groups receiving radiotherapy. Three of eight Rh18 (38%) developed metastases. The radiation dose per cc of tumor did not affect metastasis formation, as metastases were seen in a wide array of doses.

In the Rh18 irradiated xenograft group that experienced metastases, the three mice received 19.6, 29.2, and 62.8 Gy per cc of tumor. The two mice with smaller radiation doses developed an intra-abdominal metastasis at 60 days and 118 days after treatment began,

respectively. The mouse that received 62.8Gy/cc first metastasized 43 days after treatment began to the left flank, just above the original xenograft. This mouse experienced four other metastases: on day 71 to the left inner flank fold, day 78 subcutaneously to the left flank above the primary site for implantation, and day 92 to the left axilla as well as the caudal portion of the intra-abdominal cavity. In the Rh18 survival surgery alone mice, three developed intra-abdominal metastases on days 55, 64, and 92 after their initial surgery. The difference in metastasis rate was statistically significant between these two treatment groups ($p=0.02$).

There were 15 mice transplanted with Rh18RT xenografts, of which nine received irradiation and six were subjected to survival surgery alone. All of the Rh18RT xenografts in the survival surgery group developed metastases. The metastases were detected on days 22, 26, 33, 34, 46, and 50. The most common sites of metastasis were intra-abdominal and the axilla on the ipsilateral side of the flank xenograft implant. Two mice experienced multiple metastatic episodes. The Rh18RT irradiation group experienced a 44% metastasis rate; these mice received 39.5, 54, 66.1, and 94.8 Gy per cc of tumor, respectively, and had intra-abdominal and ipsilateral flank fold lesions. The variance in metastasis rate in these treatment groups was statistically significant ($p=0.0440$).

Thirty eight mice transplanted with parental Rh30 xenografts received irradiation and five were subjected to survival surgery alone. The Rh30 irradiated xenograft group had two mice develop metastases, for a rate of approximately 5%. These mice received 42 and 48.9 Gy per cc of tumor. The metastases were first noted on day 85 and day 63, respectively, and were both located in the intra-abdominal cavity. None of the Rh30 survival surgery alone mice experienced metastases. The difference in metastasis rate between these treatment groups was not statistically significant.

There were 14 mice transplanted with Rh30RT xenografts, nine of which received irradiation and five were subjected to survival surgery alone. Two of the mice exposed to radiation developed metastatic lesions (22%), whereas none in the survival surgery alone group developed metastases. The irradiated mice received 20.6 and 55.3 Gy per cc of tumor. The metastases were noted on day 84 and day 23, respectively, and were both intra-abdominal lesions. The difference in metastasis rate between the irradiated group and survival surgery alone group was not statistically significant.

When examining the Rh18RT and Rh30RT xenografts, the mean time to appearance of metastases was approximately half of the parental lines (40 versus 73 days). Also, the Rh18RT xenografts were radio-resistant compared to parental Rh18 xenograft ($p=0.001$), while the Rh30RT xenograft maintained radiosensitivity seen in the parental Rh30 xenograft ($p=0.29$) (Figure 1).

Expression Profiles

Messenger RNA extraction was completed on pooled samples of parental untreated tumor, recurrent tumor, and metastatic tumor tissue for both xenografts lines. Unsupervised hierarchical clustering of the most varied gene expression (Figure 2) revealed that untreated Rh18 and Rh18RT clustered together along with untreated Rh30. It also revealed the

clustering of metastatic Rh30, Rh30RT, and metastatic Rh18. We were unable to identify any specific genes or gene pathways responsible for the clustering.

Discussion

Our hypothesis was that irradiation influences metastasis formation. Here, we show an increased propensity for radiotherapy to induce metastasis formation in some cancer models. Our data suggest that metastases and recurrent tumors following radiotherapy share underlying molecular mechanisms.

The hierarchical clustering of the expressed mRNA did reveal that the variability in the Rh18 tumor line for the parental tissue and the recurrent/metastatic tissue was not substantial (Figure 2). The Rh18RT group had a 100% metastasis rate in the survival surgery alone group which was greater than the Rh18 group at 38%. This difference suggests that the radiotherapy may have induced changes in gene expression of Rh18 cells to increase metastatic proclivity. Radiotherapy still decreased the metastatic inclination in the Rh18RT line, even though the tumors were less sensitive to treatment. The clustering did reveal a significant difference in the mRNA expression from the parental Rh30 xenograft and the Rh30 metastasis and Rh30 recurrent samples. The Rh30 parental sample was isolated from the other 2 samples. The metastatic and the recurrent Rh30 samples clustered together, suggesting shared molecular characteristics between the two samples. These findings suggest that the radiotherapy could have induced a change in gene expression in the tumor cells which increased its tendency to metastasize.

The Rh18RT and Rh30RT samples also metastasized sooner than Rh18 and Rh30 parental tissue, with an average time of 40 days post-implant compared to 73 days. In some cases, the primary tumor implant had not even reached appropriate volume for treatment before the metastases occurred. As the previously treated tumors were implanted into naïve SCID mice that had not been subjected to radiation, the microenvironment should not have been directly affected. However, tumor cells create the microenvironment by secreting cytokines and chemokines which may be different in these previously irradiated cells. Thus, the prior radiation these xenografts had received may have induced changes leading to either an increased predisposition to metastasize or to create a microenvironment conducive to metastasis formation. The Rh30 and Rh30RT xenograft lines did not show statistical significance between the mice treated with radiotherapy and those that received survival surgery alone. However, the mRNA gene expression data and the recurrent tumor's ability to metastasize sooner do suggest that radiotherapy might lead to a more aggressive phenotype.

We demonstrated that radiotherapy can significantly decrease the formation of metastases in radiosensitive tumors like Rh18 and in the Rh18RT line that is not radiosensitive. In more radio-resistant lines (Rh30), radiation may induce or select for a more pro-metastatic phenotype. This could be contributing the 30% of rhabdomyosarcoma patients that relapse or progress despite advancements in therapy [8]. Given our limited study in only two cancer models, we are unable to determine the generalizability of our findings. Further validation studies will be required to determine how prevalent the phenomenon occurs. It is interesting to note, however, that survival after relapse in rhabdomyosarcoma is known to be poor with

an overall reported 17% five year survival rate from first recurrence, and the 80% of patients with recurrent disease who are not in the prognostically favorable categories have an overall five year survival of approximately 10%. Also, patients with distant relapses have a much poorer overall survival than those with local or regional recurrences [8]. Our findings may explain, in part, clinical causes of relapse following radiotherapy. Whether or not the omission of radiotherapy in selected cases achieving complete response with chemotherapy alone would decrease late metastatic recurrence would need to be tested in further studies and subsequent clinical trials.

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Figure 1.
Radiosensitivity of parental and recurrent Rh18 and Rh30 tumor lines

Figure 2.
Unsupervised hierarchical clustering of mRNA gene expression from parental, recurrent, and metastatic tissue of Rh18 and Rh30 tumor lines.

Table 1

Metastasis frequency and location per xenograft line

		Tumor Line				Total - All Lines
		Rh18	Rh30	Rh18RT	Rh30RT	
Total Mice	Radiotherapy Group	60	38	9	9	116
	Survival Surgery Group	8	5	6	5	24
	All Groups	68	43	15	14	140
Total Mice With Metastasis	Radiotherapy Group	3	2	4	2	11
	Survival Surgery Group	3	0	6	0	9
	All Groups	6	2	10	2	20
Total Meta static Occurrences **		10	2	14	2	28
Met Locations	Axillary	1	-	3	-	4
	Flank fold	1	-	2	-	3
	Intra-abdominal (lower)	3	2	3	2	10
	Intra-abdominal (upper)	3	-	3	-	6
	Sub-Q body other than axillary or flank fold	1	-	-	-	1

** Some mice had multiple occurrences at the same location. In those cases, the location was counted once for that tumor line.