Modeling Human Choroidal Melanoma Xenograft Growth in Immunocompromised Rodents to Assess Treatment Efficacy

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PURPOSE. To evaluate potential treatments of primary uveal melanoma in rodent xenograft models, it is necessary to track individual tumor growth during treatment. Previously, high-frequency ultrasound (HF-US) was used to measure tumor volume in nude rats for up to 2 weeks. This study tests the hypothesis that HF-US can be used to repeatedly measure tumor volume for at least a month in both nude rat and severe combined immunodeficiency (SCID) mouse xenograft models of human uveal melanoma, with the goal of modeling tumor growth to evaluate treatment efficacy.

METHODS. C918 human uveal melanoma spheroids were implanted in the choroids of six nude rats and six severe combined immunodeficiency mice. OCM-1 human uveal melanoma spheroids were implanted in six nude rats. Every 4–7 days thereafter for up to 5 weeks, HF-US images of the tumor-bearing eye were captured every 100 or 250 μm . Tumor areas were measured on each image and integrated to calculate volume. Tumor growth was modeled using a logistic curve, and parameters characterizing growth, including the time to reach a target volume (t_T), were evaluated as potential measures of treatment efficacy.

RESULTS. Tumor volume could be measured for up to 5 weeks in all models, and the logistic curve described the growth well. The parameter t_T was shown to be a suitable endpoint to evaluate treatments.

CONCLUSIONS. HF-US is a practical method to track uveal melanoma growth in the same nude rat or SCID mouse for up to a month. Such growth data can be used to evaluate treatments in these xenograft models. (*Invest Ophthalmol Vis Sci.* 2012;53:2693–2701) DOI:10.1167/iovs.11-9265

The annual incidence of intraocular melanoma in the western world is approximately six cases per million,¹ and it is the most common intraocular malignancy in adults, accounting for 70% of all adult primary eye cancers.² About 20% of patients with choroidal melanoma die of metastatic disease within 5 years of diagnosis.³

Treatment of primary uveal melanoma is primarily based on tumor size. In the past, medium-sized choroidal melanomas were typically treated by enucleation or radiotherapy²; but over the last few decades, there has been a trend to treat these tumors with radiation in an effort to spare the patient's affected eye.^{4,5} Although eye-sparing therapies like radiation are equivalent to enucleation in terms of long-term survival,⁶ these treatments do not always succeed in saving patient vision. Two large studies showed that only 55%–66% of all patients maintain a visual acuity better than 20/200 3 or 5 years after plaque radiotherapy.^{7,8} External proton beam therapy resulted in a loss of visual acuity in 56% of the patients and glaucoma in 29% of the patients.⁹ These findings highlight the need to investigate new methods to decrease the deleterious side effects of radiation therapy, while maintaining or enhancing its good local control of medium-sized primary uveal melanomas. One radiation optimization strategy is to identify and develop agents that can be used to lower the radiation dose necessary to kill the tumor, including cytotoxic drugs that kill tumor cells via other mechanisms and radiosensitizers that target radioresistant cells.¹⁰

Development of effective new combination therapies will require testing in animal models of primary uveal melanoma. Evaluation of anticancer therapies usually involves the performance of tumor growth delay studies, in which tumor size is monitored during a specific treatment.^{11,12} A tumor growth delay study of primary human uveal melanoma xenografts would require the ability to serially measure tumor volume in the same immunocompromised animal in a sterile environment over an extended period of time. As a preliminary step in the realization of such a model, a method was developed to repeatedly measure human orthotopic uveal melanoma volume in the eyes of nude rats for up to 2 weeks using high-frequency ultrasound (HF-US) under sterile conditions.¹³ Unfortunately, that length of time is insufficient to potentially evaluate the impact of treatments on tumor volume. The current study hypothesized that HF-US could be used to follow tumor growth in both nude rats and severe combined immunodeficiency (SCID) mice for approximately a month and that the data could be used to quantitatively model orthotopic tumor growth and develop parameters to determine the efficacy of tumor treatments.

MATERIALS AND METHODS

Animals

Male and female *WAG/RijHs-rnu* athymic rats and female Fox Chase SCID Beige mice (CB17.B6-*Prkdc^{scid}Lys^{tg}/Crl*) were used in this study, since these animals both permit the xenotransplantation of human tumor tissue into the choroid.^{13–15} The *WAG/RijHs-rnu* rats were bred and housed in the animal facility at Wayne State University. Fox Chase SCID Beige mice were purchased from Charles River Laboratories

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(Wilmington, MA) and housed in the same facility. All procedures were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by Wayne State University's Animal Investigation Committee.

Human Choroidal Melanoma Cell Lines

The human choroidal melanoma cell lines C918 and OCM-1 were used in this study. The OCM-1 cells were originally cultured from a human choroidal melanoma specimen in 1989.¹⁶ The C918 cell line was derived from a patient tumor at the University of Iowa in the 1990s.¹⁷ Both cell lines were maintained in RPMI medium + 10% fetal bovine serum + antibiotic.

To authenticate these cell lines,¹⁸ STR analysis was performed using the Promega PowerPlex 16 system (Promega US, Madison, WI) according to the manufacturer's protocol. Cell lines matched the genetic profile determined by Folberg et al.,¹⁸ verifying that these are the original cell lines.

Growth of Orthotopic Human Choroidal Melanoma Xenografts

C918 and OCM-1 tumor spheroids were grown in tissue culture as previously described.^{13,14} C918 spheroids were implanted into the suprachoroidal space of six rats,¹⁴ which were 86 ± 3 days old ($n = 6$) at the time of implantation. OCM-1 spheroids were implanted into the suprachoroidal space of six rats when they were 112 ± 15 days old ($n = 6$). To demonstrate that the growth of an orthotopic uveal melanoma could also be safely followed in a mouse model, C918 spheroids were also implanted into the suprachoroidal space of seven SCID mice when they were 45 ± 2 days old ($n = 7$). Rats and mice were anesthetized with a ketamine/xylazine mixture at a dose of 60/6 mg/kg IP or 108/8 mg/kg IP, respectively, and tumor spheroid implantation was performed under sterile conditions in a BSL-2 safety hood in the animal facility as detailed previously for nude rats.¹⁴

HF-US Imaging of Eyes and Calculation of Tumor Volume

A previous manuscript by study authors showed that tumor volume estimated with HF-US was highly correlated with the volume estimated from serial histological sections in the nude rat model of human uveal melanoma.¹³ Although HF-US cannot distinguish tumor from retina, which is very thin and cannot readily be separated from the sclera by HF-US in these animals, analysis of histological sections verified that this region was primarily composed of tumor.¹³ The tissue measured as tumor in the ultrasound image appeared as a hyperechoic (bright) region in the superior portion of the eye. Therefore, this same technique was used to estimate tumor volume in the current study.

HF-US imaging was performed under aseptic conditions in a BSL-2 hood in the procedure room at the animal facility as described previously.¹³ Animals were weighed before each imaging session. The rats bearing C918 tumors were imaged every 4 to 5 days ($n = 6$), while the rats bearing the OCM-1 tumors were imaged every 7 days ($n = 6$). Mice were imaged every 3 or 4 days ($n = 3$) or every 7 days ($n = 4$). Briefly, rats and mice were anesthetized with a ketamine/xylazine mixture at a dose of 60/6 mg/kg IP or 100/10 mg/kg IP, respectively. The high-resolution 35 MHz HF-US system for small animal research (Model MHF-1; E-Technologies, Inc., Bettendorf, IA) was used to obtain B-scan ultrasound image series of the right eye bearing the human choroidal melanoma xenograft.¹³ Images were obtained every 250 or 100 μm for the rats and mice, respectively. Usually three image series across the eye were recorded at each session.

Tumor volumes were calculated from the images by numerical integration of the tumor areas.¹³ For each imaging session, one to four series of images were analyzed, and the average volume was used as the tumor volume for that day.

Modeling Tumor Growth

The HF-US tumor volume data were fitted to a symmetric sigmoid logistic model, which is a standard model used to describe tumor or microorganism population growth.^{19,20} One form of the symmetric sigmoid logistic model is given by:

$$V = \frac{4g_M}{r_0(1 + e^{-r_0(t-\tau)})} \quad (1)$$

where V is tumor volume (mm^3); g_M is maximum growth rate (mm^3/day); r_0 is initial (maximum) rate of cell division (divisions/day); and τ is time at which the tumor is growing at its maximum rate (days). Equation 1 was fitted to all tumor volume data by optimizing the parameters g_M , r_0 , and τ using nonlinear least-squares regression (GraphPad Prism; GraphPad Software, Inc., La Jolla, CA).

The growth rate is obtained by differentiating equation 1 with respect to time:

$$\frac{dV}{dt} = \frac{4g_M}{(1 + e^{r_0(t-\tau)})^2} \quad (2)$$

Evaluation of Treatment Efficacy

Although other growth parameters can be monitored, a parameter commonly used in standard tumor growth delay studies is the time for a tumor to reach a specific volume or a specific relative volume.^{11,12} Treatment can either begin at a specific time after implantation or when the tumors have reached a specific volume range and is continued until the tumor reaches a "target" volume. At that time, the treatment has failed, and the animal is removed from the study. The difference between the time-to-reach the target volume for treated tumors and control tumors is the treatment-associated growth delay, which is a measure of treatment efficacy.

The time-to-reach a target volume, t_T , can be estimated in at least two ways. If a measured volume exceeds the target volume, t_T can be estimated by performing linear interpolation between the surrounding measurements. If the target volume is slightly larger than the final measured volume, t_T can be estimated by linear extrapolation. Alternatively, a model of tumor growth can be used to calculate the parameter. Equation 1 can be solved for t_T when $V = V_T$:

$$t_T = \tau - \left(\frac{1}{r_0}\right) \ln \left(\frac{4g_M - V_T r_0}{V_T r_0} \right) \quad (3)$$

where V_T is target tumor volume (mm^3) and t_T is time at which the tumor reaches the target volume (days).

Statistical Analysis

All data are expressed as mean $\pm$ SD. Since the data passed normality tests, all statistical comparisons were made using standard parametric tests. Animal weights during the study were compared using a repeated measures one-way ANOVA test. Significant differences ($P < 0.05$) were further analyzed using Bonferroni's multiple comparison tests to compare values between any two groups.

Differences between tumor growth parameters for C918 and OCM-1 tumors growing in rats were compared using the unpaired Student's t -test. A P value < 0.05 was considered statistically significant.

The appropriateness of the symmetric sigmoid logistic model to describe the volume data was evaluated by the runs test.²¹ A P value < 0.05 would indicate that the curve deviates systematically from the data. The goodness of fit was determined by evaluation of the r^2 and RMS error values. The validity and uniqueness of the parameter values were assessed by evaluating the 95% confidence intervals.

Sample sizes for hypothetical growth delay studies were determined using standard power analysis, assuming a power of 80% and a P value of 0.05.²²

FIGURE 1. *Top:* HF-US images from the same C918 tumor-bearing eye of a rat (Rat 61 in Fig. 2A) at different times following spheroid implantation. The superior portion of the globe is toward the top of the images, and the cornea is at the left. *Middle:* HF-US images from the same OCM-1 tumor-bearing eye of a rat (Rat 74 in Fig. 2B) at different times following spheroid implantation. The superior portion of the globe is toward the top of the images, and the cornea is at the left. *Bottom:* HF-US images from the same C918 tumor-bearing eye of a mouse (Mouse 5 in Fig. 2C) at different times following spheroid implantation. The superior portion of the globe is toward the right of the images, and the cornea is at the top. All images are from near the middle of the eye.

RESULTS

Modeling the Growth of C918 Xenografts in Nude Rats

As reported previously, the tumor could be visualized as a relatively hyperechoic region beneath the superior sclera that changed in size as the HF-US probe was moved nasally across the right eye.¹³ HF-US images of a C918 tumor xenograft growing in the same rat eye at different times after implantation are shown in the upper panels of Figure 1. These images were taken from approximately the center of the eye and qualitatively demonstrate the growth of the tumor over the course of 28 days. A small tumor was already present 5 days after implantation. By Day 19, it had grown enough to fill the vitreous and had pushed the lens noticeably anteriorly into the anterior chamber. By Day 28, it had grown anteriorly and had deformed the globe superiorly.

In each rat, a series of three scans were typically collected at each imaging session, with an average of 2.9 ± 0.3 series per session ($n = 37$ sessions). Tumor volume could be estimated from the series of HF-US images as demonstrated previously.¹³ There was variability in growth rates among the tumors (Fig. 2A). In particular, one tumor grew extremely slowly ($\blacktriangle$) and two tumors grew very rapidly ($\blacksquare$ and $\blacklozenge$). The variable growth patterns resulted in large standard deviations in tumor volume

at later measurement times. The average tumor volumes on Days 19 and 23 were $15.7 \pm 11.3 \text{ mm}^3$ and $34.6 \pm 21.4 \text{ mm}^3$, respectively ($n = 6$).

Anesthesia of the rats twice a week did not cause significant weight loss. There was a trend for the mean weight to increase, and repeated measures one-way ANOVA revealed a significant effect of time on body weight ($P = 0.011$). The rats initially weighed $164 \pm 9 \text{ g}$ ($n = 6$). Two rats transiently lost weight during the study, but by Day 28, the average weight was $169 \pm 7 \text{ g}$.

Fits of equation 1 to the volume data are shown by the curves in Figure 2A. The three circled points on Day 28 were not included in the fits, since the entire tumor could not be reliably imaged on that day (see Discussion). In three of the six cases, the fitted value of τ was greater than the time of the last measurement, t_{end} . For one of these fits, the value of τ was within three days of the last measurement day (i.e., $\tau \leq t_{\text{end}} + 3$). That fit yielded relatively tight 95% confidence intervals, while the parameters from the other two fits had very broad confidence intervals. In those two instances, there was a very shallow minimum sum-of-squares error, and a wide range of τ values resulted in fits with very similar errors. Based on these considerations, it was decided to accept the fitted parameters if the fitted τ was less than or equal to $t_{\text{end}} + 3$, and to set $\tau = t_{\text{end}}$ if the fitted τ was greater than $t_{\text{end}} + 3$. Table 1 shows that this model, given these restrictions, fitted all of the data well, and

FIGURE 2. Tumor volume determined by HF-US as a function of time after implantation for: (A) C918 cells growing in 6 nude rats, (B) OCM-1 cells growing in 6 nude rats, (C) C918 cells growing in 7 SCID mice. HF-US scans performed: (A) every 4 or 5 days, (B) every 7 days, (C) every 3 to 7 days. Each symbol represents a different animal. The curves are the fits of the symmetric sigmoid logistic model (equation 1) to the data. In panel (A), the three circled points on Day 28 were not included in the fits, since the entire tumor could not be imaged on that day.

runs tests showed that the data did not deviate systematically from the curve ($P > 0.64$). Values of r^2 ranged from 0.967–1.000, indicating that the model accounted for most of the variability in the data. The average RMS error was only 0.8 mm³. The relatively tight 95% confidence intervals of the parameters g_M and r_0 demonstrated that these parameters could be fitted uniquely.

When the model parameters were used to calculate the time-to-reach 20 mm³, $t_{T=20}$, the average value was 21.8 ± 5.8 days ($n = 6$). The interpolation method yielded a value of 21.5 ± 5.8 days, which was similar to, but statistically less than, the value calculated from the model fits ($P = 0.004$).

Modeling the Growth of OCM-1 Xenografts in Nude Rats

Six rats were implanted with OCM-1 spheroids and treated twice daily with artificial tears. In this study, tumors were only imaged once a week. HF-US images of an OCM-1 xenograft growing in the same rat eye at different times after implantation are shown in the middle panels of Figure 1. These images from the center of the eye qualitatively demonstrate the growth of the tumor over the course of 28 days. A small tumor was present a week after implantation, but it grew dramatically between Days 14 and 21. By Day 28, it had enlarged the globe and pushed the lens anteriorly.

As before, a series of three scans was typically collected at each imaging session, with an average of 3.0 ± 0.2 series per session ($n = 26$ sessions). In one rat (115), HF-US images could not be obtained on Day 28 due to technical difficulties. Again, there was variability in growth rates among the tumors (Fig. 2B). There was a trend for the mean body weight to increase, and repeated measures one-way ANOVA revealed a significant effect of time on body weight ($P = 0.010$). The weights on Days 21 and 28 were significantly greater than the starting weight on Day 0 ($P < 0.05$). They initially weighed 256 ± 36 g (mean $\pm$ SD, $n = 6$); and at the end of the study (Day 28 or 35), the rats' average weight was 264 ± 39 g.

The symmetric sigmoid logistic model also fitted these data reasonably well (Fig. 2B), although in half of the cases, τ had to be set equal to t_{end} (Table 2). Runs tests again showed that there was no reason to doubt the appropriateness of the model ($P > 0.80$). Values of r^2 ranged from 0.992–1.000, and the average RMS error was 0.7 mm³. The time-to-reach a volume of 20 mm³ was approximately 28 days, whether the values were calculated using the model parameters or linear interpolation ($P = 0.235$, Table 2).

Comparison of the Growth of C918 and OCM-1 Xenografts

Qualitatively, initial growth of the C918 and OCM-1 tumors within the first week after implantation was very similar (Figs. 2A, 2B). Thereafter, the C918 tumors appeared to grow more rapidly than the OCM-1 tumors, with five of six C918 tumors reaching 25 mm³ or more by 23 days (Fig. 2A). Most of the OCM-1 tumors did not get this large until 30–35 days (Fig. 2B). Quantitatively, all three model parameters were significantly different between the two tumor cell lines (Table 2). The maximum tumor growth rate, g_M , and the proliferation rate, r_0 , of the C918 cells were both greater than the values for the OCM-1 cells ($P = 0.002$ and $P = 0.023$, respectively). Correspondingly, the time to reach the maximum growth rate, τ , was significantly less for the C918 cells ($P = 0.032$). Similarly, the C918 tumors reached the target volume of 20 mm³ about six days sooner than the OCM-1 tumors, regardless of which method was used to estimate $t_{T=20}$ ($P < 0.05$, Table 2).

TABLE 1. Fitted Parameters of the Logistic Model (equation 1) Used to Fit the C918 Tumor Volume Data from the Nude Rat Study (Fig. 2A)

Rat	<i>n</i>	g_M (mm ³ /day)	r_0 (divisions/day)	τ (days)	r^2	RMS Error (mm ³)	Modeled $t_{T=20}$ (days)	Interpolated $t_{T=20}$ (days)
52	6	6.48 <i>4.30–8.65</i>	0.393 <i>0.286–0.501</i>	23.0 <i>set to t_{end}</i>	0.989	1.51	20.9	20.7
53	8	2.20 <i>1.37–3.03</i>	0.216 <i>0.156–0.277</i>	33.0 <i>set to t_{end}</i>	0.967	1.37	32.8	32.6
54	7	8.26 <i>7.55–8.98</i>	0.301 <i>0.254–0.348</i>	26.4 <i>24.7–28.1</i>	1.000	0.54	21.4	21.1
59	6	11.11 <i>10.36–11.85</i>	0.284 <i>0.272–0.295</i>	23.8 <i>23.1–24.5</i>	1.000	0.13	17.0	16.4
60	6	4.54 <i>4.11–4.98</i>	0.299 <i>0.234–0.363</i>	19.7 <i>18.0–21.4</i>	0.999	0.60	17.3	16.9
61	7	4.57 <i>3.80–5.34</i>	0.244 <i>0.168–0.321</i>	25.7 <i>21.9–29.6</i>	0.998	0.94	21.6	21.4
Mean $\pm$ SD		6.19 $\pm$ 3.16	0.289 $\pm$ 0.061	25.3 $\pm$ 4.5	0.992 $\pm$ 0.013	0.84 $\pm$ 0.53	21.8 $\pm$ 5.8	21.5 $\pm$ 5.8

n = number of points (measurement days); g_M = maximum growth rate (mm³/day); r_0 = maximum rate of cell division (divisions/day); τ = time at which the rate of growth is maximum (days); t_{end} = time point at which last volume was recorded for a given tumor (days); and $t_{T=20}$ = time-to-reach a volume of 20 mm³ (days). If the fitted value of τ was greater than $t_{end} + 3$, then τ was set equal to t_{end} . Italicized numbers indicate the 95% confidence intervals.

Modeling the Growth of C918 Xenografts in SCID Mice

C918 tumors were also visible in the mouse eye as hyperechoic regions and could be visualized across the eye. HF-US images of a C918 tumor growing in the same mouse eye at various times after implantation are shown in the bottom panels of Figure 1. A small tumor was visible on Days 7 and 14, but it was not until Day 21 that the tumor thickened and spread along the superior portion of the eye. By Day 28, the tumor had further thickened and spread into the anterior chamber. By Day 35, it had filled much of the globe.

The mouse eyes were imaged once or twice a week, and an average of 2.5 $\pm$ 1.0 image series per imaging session were obtained (*n* = 42 sessions). As in the rat model, measurement and integration of the measured hyperechoic regions in each image series resulted in an estimate of tumor volume. At each subsequent session, the HF-US images revealed evidence of tumor growth, since the size of the hyperechoic region within

the eye increased (Fig. 1, bottom panel). As in the rats, there was some variability in growth rates among the tumors (Fig. 2C), and there was an overall trend for the mean weight to increase. Repeated measures one-way ANOVA revealed a significant effect of time on body weight ($P = 0.041$), but subsequent testing revealed no significant differences between any specific time points. The mice initially weighed 17.5 $\pm$ 2.2 g (*n* = 7), and at the end of the study (Day 26–35), the average weight was 17.9 $\pm$ 1.4 g.

Equation 1 described the growth well (Fig. 2C), except for Days 17 and 21 in Mouse 23 ($\square$). Nevertheless, runs tests showed that the curves did not deviate systematically from the data ($P > 0.58$). The parameters of the fits are shown in Table 3. In five of the seven cases, τ had to be set equal to t_{end} . Values of r^2 ranged from 0.982–1.000, and the average RMS error was less than 0.3 mm³. The time to reach a volume of 5 mm³ was calculated from the model parameters and had a mean value of 26.7 $\pm$ 3.2 days (Table 3). When the value was determined by

TABLE 2. Fitted Parameters of the Logistic Model (equation 1) Used to Fit the OCM-1 Tumor Volume Data from the Nude Rat Study (Fig. 2B)

Rat	<i>n</i>	g_M (mm ³ /day)	r_0 (divisions/day)	τ (days)	r^2	RMS Error (mm ³)	Modeled $t_{T=20}$ (days)	Interpolated $t_{T=20}$ (days)
74	5	3.47 <i>2.22–4.72</i>	0.202 <i>0.158–0.246</i>	29.0 <i>23.7–34.3</i>	1.000	0.18	24.5	24.1
79	5	2.86 <i>1.91–3.81</i>	0.180 <i>0.134–0.226</i>	28.0 <i>set to t_{end}</i>	0.992	1.37	23.7	23.6
90	5	1.61 <i>0.95–2.26</i>	0.198 <i>0.135–0.261</i>	28.0 <i>set to t_{end}</i>	0.988	0.85	30.4	30.3 ^a
104	6	2.50 <i>2.01–2.99</i>	0.185 <i>0.168–0.202</i>	37.1 <i>34.4–39.9</i>	1.000	0.08	34.2	34.1
113	6	3.56 <i>3.16–4.01</i>	0.172 <i>0.156–0.187</i>	35.0 <i>set to t_{end}</i>	0.998	0.75	28.3	28.5
115	6	1.55 <i>0.79–2.31</i>	0.130 <i>0.010–0.249</i>	30.5 <i>6.5–54.4</i>	0.997	0.90	27.9	27.6
Mean $\pm$ SD		2.60 $\pm$ 0.88	0.178 $\pm$ 0.026	31.3 $\pm$ 3.9	0.996 $\pm$ 0.005	0.69 $\pm$ 0.49	28.2 $\pm$ 3.9	28.0 $\pm$ 3.9
<i>t</i> -test <i>P</i> value OCM-1 vs. C918		0.023	0.002	0.032	0.537	0.605	0.049	0.047

Italicized numbers indicate the 95% confidence intervals. Parameters are defined in the legend to Table 1. a: value extrapolated, since volume was only 16.5 mm³ on final measurement day. The *P* value reports the significance of the Student's unpaired *t*-test to compare the parameter values between the OCM-1 and C918 (Table 1) tumors.

TABLE 3. Fitted Parameters of the Logistic Model (equation 1) Used to Fit the C918 Tumor Volume Data from the SCID Mouse Study (Fig. 2C)

Mouse	<i>n</i>	g_M (mm ³ /day)	r_0 (divisions/day)	τ (days)	r^2	RMS Error (mm ³)	Modeled $t_{T=5}$ (days)	Interpolated $t_{T=5}$ (days)
3	5	1.10 <i>0.83-1.38</i>	0.261 <i>0.123-0.399</i>	25.9 <i>19.5-32.4</i>	1.000	0.13	22.6	22.3
5	6	1.84 <i>1.73-1.94</i>	0.332 <i>0.314-0.349</i>	35.0 <i>set to t_{end}</i>	1.000	0.08	31.3	30.3
8	5	5.24 <i>4.57-5.90</i>	0.498 <i>0.437-0.559</i>	28.0 <i>set to t_{end}</i>	1.000	0.16	24.0	22.3
9	6	3.27 <i>2.25-4.29</i>	0.517 <i>0.378-0.656</i>	32.0 <i>set to t_{end}</i>	0.992	0.49	29.3	28.9
19	9	4.89 <i>4.63-5.15</i>	0.695 <i>0.663-0.727</i>	31.0 <i>set to t_{end}</i>	1.000	0.11	28.8	28.5
21	7	1.08 <i>0.80-1.35</i>	0.791 <i>0.498-1.084</i>	23.3 <i>22.6-24.1</i>	0.997	0.14	26.4	26.2 ^a
23	8	9.56 <i>6.30-12.82</i>	1.031 <i>0.728-1.335</i>	26.0 <i>set to t_{end}</i>	0.982	0.91	24.2	24.1
Mean $\pm$ SD		3.85 $\pm$ 3.03	0.589 $\pm$ 0.269	28.8 $\pm$ 4.1	0.996 $\pm$ 0.007	0.29 $\pm$ 0.31	26.7 $\pm$ 3.2	26.1 $\pm$ 3.3

Italicized numbers indicate the 95% confidence intervals. Parameters are defined in the legend to Table 1. $t_{T=5}$ = time-to-reach a volume of 5 mm³ (days). a: value extrapolated, since volume was only 4.84 mm³ on final measurement day.

linear interpolation or extrapolation, it was slightly but significantly lower at 26.1 $\pm$ 3.3 days ($P = 0.045$).

Design of Tumor Growth Delay Studies to Evaluate Treatments

Using the growth data and modeling results, the number of animals that would be needed to detect a treatment-induced change in any of the growth parameters for the study's three uveal melanoma xenograft models can be determined using power analysis²² (Fig. 3). There are four different parameters that could be affected by a therapy: g_M , r_0 , τ , and t_T . In the following calculations, the model t_T values were used, but results using the interpolated t_T values would be very similar.

For the rat models, the least sensitive parameter to evaluate would be the maximum growth rate, since 15 rats would be required to detect even a 54% or 37% change in this parameter for the C918 and OCM-1 models, respectively (Figs. 3A, 3B). The most sensitive parameter for either model is the time at which this maximum rate is reached, τ . For example, 10 rats per group would be sufficient to detect a 23% or 17% increase in τ in the C918 or OCM-1 models, respectively. The parameter r_0 and the time it takes for the tumor to reach a target volume of 20 mm³, $t_{T=20}$, could also be useful test parameters. Eleven rats would be sufficient to detect changes of 26% and 19% in r_0 for the C918 and OCM-1 models, respectively. For the C918 tumor, 12 rats would be sufficient to detect a 32% treatment-induced difference in $t_{T=20}$ (Fig. 3A). Since the average $t_{T=20}$ value is 22 days (Table 1), 12 rats would be sufficient to detect a growth delay of about 1 week, from 22 to 29 days. For the OCM-1 tumor, 10 rats would be sufficient to detect an 18% change in $t_{T=20}$ (Fig. 3B). Since the average $t_{T=20}$ value is ~28 days (Table 2), 10 rats would be sufficient to detect a growth delay of 5 days from 28 to 33 days.

For the C918 mouse model, the least sensitive parameter to evaluate would be g_M (Fig. 3C). Unlike the rat models, the parameter r_0 would be a relatively poor choice to evaluate treatment efficacy for the mouse model. The most sensitive parameters would be τ and the time it takes for the tumor to reach 5 mm³, $t_{T=5}$. For both parameters, 10 mice would be sufficient to detect as small a change as 17%. Since the average τ for the mouse tumors is 28.5 days (Table 3), 10 mice could detect a treatment-induced increase in τ of only about 5 days.

Similarly, 10 mice would be enough to detect a change in $t_{T=5}$ of only 4 days.

DISCUSSION

Serial Volume Measurements of Human Choroidal Melanoma Xenografts

Previously HF-US was used to monitor uveal melanoma volume in nude rats for 2 weeks.¹³ In the current study, tumor volume was tracked reliably for up to 4 weeks in most cases. The current study is the first to present a full series of HF-US images of an orthotopic human choroidal melanoma xenograft in the same animal at different time points for as long as a month.

In the C918 growth study in rats, tumor volume could not be reliably measured on the final day in three animals (circled points in Fig. 2A). When the tumors became very large (>40 mm³), imaging the entire tumor was not possible at times, because of the inability of HF-US to penetrate more than 3 or 4 mm into the eye or the tumor tissue. This was particularly a problem when the tumors grew inferiorly toward the optic disc or grew outwardly through the sclera and became very thick (>3 or 4 mm). Although this problem can lead to significant underestimation of volume in some very large tumors, this should not be an issue in most studies, since tumors do not usually need to be tracked beyond a volume of 40 mm³.

Repeated imaging under ketamine/xylazine anesthesia resulted in no significant weight loss in the nude rats, and some rats even showed an increase in body weight, which would be expected for these young adults. This is an important finding, since an imaging protocol requiring repeated anesthesia could result in weight loss. Typically, a 15% or 20% loss in body weight requires an animal to be removed from a study on ethical grounds, so any significant weight loss could jeopardize a growth delay study. The current results indicate that full growth curves can be obtained using an imaging schedule of one or two sessions per week. Therefore, the nude rat model of human uveal melanoma could be useful in future tumor growth delay studies to evaluate treatments.

We have now extended the measurement of tumor volume using HF-US to orthotopic xenografts in the mouse eye. Recently, Zhang and colleagues used HF-US to image mouse B16LS9 cutaneous melanoma orthografts growing in the eyes

of C57BL6 mice and demonstrated that the area of hyperechoic regions was correlated with a histologically measured tumor area.²³ Tumor volumes were not estimated in that study. Although it was not initially known whether uveal melanoma growth could be monitored for an extended period when they were growing in the much smaller mouse eye, tumor volume could be measured for more than 3 weeks. This permitted the measurement of full tumor growth curves in a time frame that did not require more than one or two imaging sessions per week. This was an important finding, since more frequent imaging under anesthesia could have possibly led to weight loss or even increased mortality. Using the imaging schedule reported here, the mice showed no significant weight loss. Thus, the SCID mouse human uveal melanoma xenograft model can also be used in future studies to evaluate potential treatments for primary uveal melanoma.

Variability in Tumor Growth

There was large variability in tumor growth within each of the three groups. There are many factors that can contribute to this variability. Perhaps the most obvious is the initial tumor load (i.e., how many tumor cells are implanted). Intuitively, the more tumor cells present initially, the faster the tumor should grow. To minimize this effect, the same number of tumor cells in suspension are typically injected into each animal. However, in some cases, this method results in a low tumor take (i.e., only a small fraction of the animals develop tumors). When that occurs, tumor pieces from a donor animal are usually implanted,²⁴ even though there is no ability to control the number of viable cells. This technique is similar to the implantation of spheroids used in our uveal melanoma models. As noted in an earlier manuscript, spheroids are implanted to maximize cell concentration and to avoid leakage associated with the injection of cell suspensions.¹⁴ Interestingly, there is always large interanimal variability in tumor growth whether tumors are initiated by injecting a specific number of cultured cells,²⁵ by implanting small tumor pieces,²⁴ or by implanting spheroids, suggesting that other factors play an even more significant role in determining tumor growth. Possible factors could include the distribution of cells after injection, the relative location of the cells within the tissue, the time at which any vascularization occurs, and inherent differences in the animals themselves. Regardless of the causes, this inherent variability in tumor xenograft growth makes longitudinal measurement of individual tumor volume a necessity.

Modeling the Growth of Human Choroidal Melanoma Xenografts

The growth of all tumors, except for one of the tumors in the mouse eye, was very well described by the logistic model. The ability to functionally characterize tumor growth has several advantages. First, it permits the comparison of growth-associated model parameters between different tumor types or between tumors in different treatment groups. For example, using the fitted parameters, this study was able to demonstrate that the C918 tumors grew more rapidly than the OCM-1 tumors in nude rats. In an earlier study, in which the growth of the two tumors using maximum tumor areas determined from histology was compared, no difference was apparent.¹⁴ The faster growth of the C918 tumors is consistent with the finding that C918 cells have a higher proliferation rate in vitro than OCM-1 cells.²⁶ Second, the modeling also permits the calculation of tumor growth rate at any time point (equation 2). For example, in Rat 54 (Fig. 2A) the C918 tumor was growing at a rate of 0.76 mm³/day on Day 14; but by Day 23, it was growing 8.5 times faster, at a rate of 6.4 mm³/day. This in

FIGURE 3. The theoretical number of rats or mice necessary to detect a given percentage change in the value of growth parameters for: (A) C918 rat model, (B) OCM-1 rat model, (C) C918 mouse model. g_M , solid gray line; r_0 , dotted black line; τ , dashed black line; $t_{T=20}$ or $t_{T=5}$, solid black line. Curves were calculated using standard power analysis, assuming a power of 80% and a P value of 0.05.

vivo evaluation of growth rate can be useful in evaluating treatment effects, and it could also be correlated with other in vivo measures of tumor function.

Design of Tumor Growth Delay Studies to Evaluate Treatments

A major purpose of this project was to assist in the design of future tumor growth delay studies to evaluate the efficacy of a given treatment. The first step in such a study would be to determine an initial tumor volume at which treatment would begin. This is necessary, since there is no true tumor on the day of implantation, whether the tumor is initiated by injection of an equal number of tumor cells in suspension,²⁴ implantation of a small tumor piece from a donor animal,²⁵ or injection of tumor spheroids. To account for differences in initial tumor load, it is best to start treatment after a tumor has formed, and the size of the tumors can be estimated. The initial tumor volume can be set in one of two ways. First, one can select an initial volume range and start treatment when each individual tumor falls into that range. In that scenario, treatment would begin on different days after implantation, but all tumors are initially in a defined size range. Unfortunately, this method requires frequent volume measurement and is not practical when tumors can only be measured twice a week, as in the uveal melanoma models. An alternative method is to set a given day after implantation as the start of treatment, and the initial volume is the tumor volume on that day. This does not require frequent volume measurement, but could result in larger variability in initial volume. In the uveal melanoma models, it seems reasonable to start treatment on Day 7 or 10, when solid tumors are present, but they are not yet in the rapid growth phase (Fig. 2). Once a starting point is determined, the tumors in any experimental cohort need to be sorted in order of volume and then alternately assigned to the control or treatment groups, resulting in an equal distribution of tumor sizes in the groups. There can be no significant differences in initial volumes among the experimental groups at the start of treatment.

Another prerequisite for a tumor growth delay study is the availability of a useful test parameter that can be evaluated as a measure of tumor growth. Once treatment begins, tumor volume is measured until the treatment has “failed,” (i.e., the tumor reaches a previously determined endpoint at which time it is removed from the study). The treatment efficacy is then evaluated by comparing the test parameter between the control and treatment groups. Possible test parameters include tumor size on a given day after the start of treatment, the time to reach a given volume or relative volume (t_T), or model parameters that are indicative of tumor growth.

In a previous manuscript involving the nude rat model, tumor growth was monitored by obtaining a single histological measurement of maximum tumor area in each rat at the end of a given period of tumor growth.¹⁴ Although tumor growth was estimated with this test parameter, there was large variability at any given time point. For example, the average maximum area of C918 xenografts after three weeks of growth was $3.38 \pm 2.63 \text{ mm}^2$ ($n = 5$).¹⁴ Given this variability, a study to detect a treatment-induced 50% change in maximum tumor area (from 3.38 to 1.69 mm^2) would require 40 rats in each group.²² In the current study, C918 tumor volume in the nude rat on Day 23 was $34.6 \pm 21.4 \text{ mm}^3$ ($n = 6$). A study to detect a treatment-induced 50% change in tumor volume (from 34.6 to 17.3 mm^3) would require 26 rats in each group.²² Clearly, tumor size on a given day could not be feasibly used to evaluate treatment efficacy in our xenograft models.

A power analysis using the four tumor growth-related parameters characterized in the current study revealed that the

parameter τ might be the most promising test parameter to compare tumor growth (Fig. 3). However, since τ is sometimes fixed at the last time point in order to reliably fit the data, its use as a test parameter is questionable, especially if a significant number of the fits involve setting $\tau = t_{\text{end}}$. Given this consideration, the next best test parameter across all three models is t_T , since 10 to 12 animals per group would be sufficient to detect a treatment-associated growth delay of a week or even less. Although in the mouse and rat C918 models, the t_T values determined from the model were significantly greater than the values determined from interpolation, the mean differences were 0.6 days or less. Therefore, both methods give similar results and either could be used to estimate t_T , as long as that method is used consistently. Thus, determination of t_T from longitudinal tracking of uveal melanoma xenograft volume using HF-US is a feasible method to evaluate treatment efficacy in both the nude rat and SCID mouse models.

Summary

For the first time, the tumor volume of an orthotopic human choroidal melanoma xenograft in an animal model was tracked for up to a month or more using HF-US. In the nude rat and SCID mouse models, tumor growth was variable, indicating the importance of serial monitoring of tumor volume in the same animal. A logistic model described the growth data well, and several model parameters are appropriate to compare tumor growth when evaluating treatment efficacy. This technique should be useful to perform standard tumor growth delay studies in these models and will permit the impact of various treatments of primary choroidal melanoma to be better evaluated using fewer numbers of animals. Since the C918 and OCM-1 cells form tumors in the lungs when they are present systemically (data not shown), these current models cannot be used to evaluate metastatic hepatic disease. However, once a human uveal melanoma cell line that naturally metastasizes to the liver is found or developed, this model could also be used to determine the impact of primary tumor treatments on metastatic spread.

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