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Biological Effectiveness of Fractionated Dose of Pions in Microscopic SCCVII Tumors: Comparison between Tumor Control Dose and Tumor Growth Time Assays

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The relative biological effectiveness (RBE) of fractionated pions for tumor growth time (TGT) assay changes with the endpoints, so it is essential to determine the RBE for tumor control dose (TCD) assay. For this purpose, the TCD_{50} of fractionated pions was compared with that of photons, and the RBEs for TGT and TCD assays were concurrently compared as a function of the effect level. A "convenient" RBE (cRBE) was substituted for the RBE when the comparison was made between similar fractionation schedules with different dose per fraction. SCCVII tumors (2×10^4 or 2×10^5 cells) were implanted into the feet of C3H mice and irradiated starting from 2 days after implantation at a total dose range of either 9.6–38.4 Gy pions (2.4–6.4 Gy per fraction) or 14.4–50.4 Gy photons (3.6–7.2 Gy per fraction) in 2–10 fractions over 5–6 days. The cRBE and the RBE at the iso-effective level of 30 days TGT were 1.53–1.60 for 2.4–4.8 Gy pions and 1.50 for 4-fractionated pions, respectively: there were only small differences within these schedules used. However, the cRBE values decreased from 1.60 to 1.15 with increasing TGT from 30 to 75 days. In contrast, the cRBE values for TCD_{50} increased from 1.08 to 1.40 (95% confidence limits [CL]; 1.18–1.63) with increasing evaluation time from 60 to 100 days: pions significantly inhibited late tumor appearance. The TCD_{50} at 100 days was 28.7 Gy (CL; 25.0–32.5 Gy) for pions and 40.3 Gy (CL; 36.3–44.2 Gy) for photons. In conclusion, the RBE for TCD_{50} was not predictable from the RBE for TGT assay. The cRBE value of 1.4 for microscopic tumor control was in close agreement with the reported values for skin reaction.

Key words: Pions — Relative biological effectiveness — Tumor control dose — Tumor growth time — SCCVII tumor

Pions have unique physical and biological characteristics. After passing as low linear energy transfer (LET) radiation into tissue, various kinds of radiation including high LET particles such as neutrons, alpha particles, and other fragments are formed (star formation) at the stopping point of the pion particles (Bragg peak) by the interaction with nuclei. As a result, high biological effectiveness is expected at the peak. The value of the relative biological effectiveness (RBE) for pions at the peak is around 1.2 for mouse skin reaction with a single dose.[1,2] When pions are irradiated in multi-fractions, the RBE increases with the number of radiation fractions. The RBE values for normal tissues are 1.3–1.7 in ten fractions.[1,2] For mouse tumors, the RBE values in ten fractions are 1.5–1.7 for tumor growth delay assay.[3,4] However, the RBE for growth delay assay decreases with increasing biological effectiveness.[4] Therefore it is essential to determine the RBE for tumor control assay. This has not been reported for fractionated pions. In this study, the RBE values for stopping pions were simultaneously compared between tumor growth time (TGT) and tumor control dose (TCD) assays.

To cure large tumors, large radiation doses are required because of the huge number of tumor cells, increased heterogeneity, and high proportions of hypoxic cells. Furthermore, in fractionated irradiation, larger doses are required because of repair and repopulation of tumor cells during irradiation intervals. Large doses damage normal tissues and could influence tumor response. Therefore, our goal was to use small numbers of tumor cells (microscopic tumors) to obtain a tumor control.

MATERIALS AND METHODS

Tumors and animals Female C3H/He mice (Charles River Inc., Quebec, Canada) aged 8–10 weeks were used throughout this study. They were kept in plastic cages on sawdust bedding with six animals per cage. Food and water were freely available at all times. SCCVII tumors were maintained by subcutaneous inoculation into the back of C3H/He mice. They were dissected and single

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tumor cells were obtained by mechanical and enzyme disaggregation as described previously.[5]

When various numbers of viable cells in an amount of 0.025 ml were implanted subcutaneously in the dorsum of mouse feet, the "tumor takes" (TD) and the tumor growth time during the microscopic phase were obtained as shown in Fig. 1. Of 12 mice implanted with 50 cells, 6 mice developed tumors: TD_{50} was 50 cells. When the median tumor growth time from the implantation to reach 100 mm^3 in volume was plotted against the number of implanted cells (Fig. 1), a linear relation was found. The relation was formulated as follows:

$$\text{Log}_{10}\,(\text{number of implanted cells}) = -0.55\,(\text{days after implantation}) + 6.65$$

On the assumption that the growth rate during the microscopic phase was stable, the mean volume doubling time was calculated to be 1.94 days.

Irradiation procedures For irradiation experiments, 2×10^5 (Exp. No. 1) and 2×10^4 (Exp. Nos. 2, 3) cells in 0.025 ml were injected subcutaneously in the feet. The tumor-bearing legs were irradiated at various doses with pions or photons starting from 2 days after implantation (Table I). The number of cells at the time of irradiation was estimated to be about two times the number of implanted cells from Fig. 1. The treatment period was fixed at 5 or 6 days for every fractionation schedule: 2 fractions with one 4-day interval, 3 fractions with two 1-day intervals, 4 fractions with a 1-day interval at the mid-period, and 5 or 6 fractions with no interval. Some treatment groups were irradiated twice a day with a split of 6 h. Irradiations with photons or pions were carried out on the same days. Mice were not anesthetized during

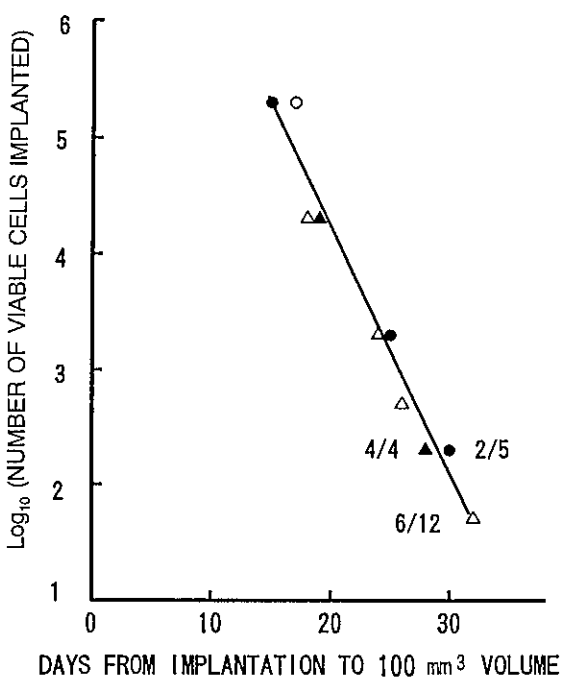

Fig. 1. Relationships between the implanted number of SCCVII tumor cells and the growth time. The median tumor growth times (X-axis) from implantation to reach 100 mm^3 in volume were plotted as a function of the number of implanted cells (Y-axis). Different symbols represent the data from separate experiments. The tumors appeared in all mice (5–10 mice), except for the fractions indicated in the graph: the number of tumors that appeared per the number of implanted tumors.

Table I. Treatment Schedule

Days	Treatments	(Exp. 1)	(Exp. 2)	(Exp. 3)
−2	Tumor cell implantation			
	Number of cells	2×10^5	2×10^4	2×10^4
0	Beginning of irradiation			
	Dose/fraction (Gy)			
	pions	2.4, 4.8	4.8	6.4
	photons	3.6, 7.2	7.2	6.4
	Fraction numbers	2–10	2–6	5–6
	Treatment period	5 (days)	5	6
14–120	Observation of tumor appearance			
	Measurement of tumor diameters			

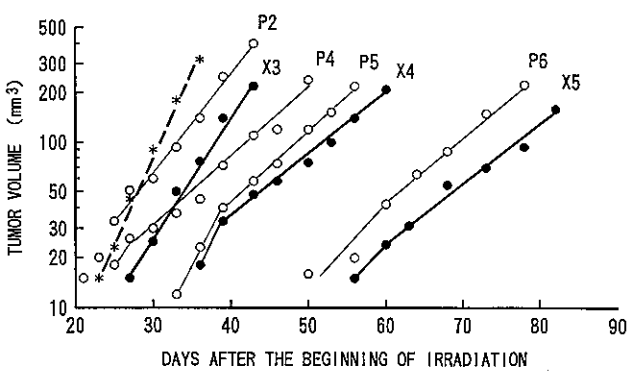

Fig. 2. Growth curves for irradiated SCCVII tumors. Tumor volumes of the 2nd largest tumor among 5 to 6 mice per group were plotted as a function of days after the beginning of irradiation. The tumors were irradiated with fractionated pions (○) or photons (●) starting from 2 days after implantation of 2×10^4 cells (Exp. 2). The numerals indicate the irradiation times with 4.8 Gy pions (P) or 7.2 Gy photons (X). Asterisks indicate tumor volumes for non-irradiated tumors after implantation of 50 tumor cells. TAT (days needed to reach 20 mm^3), VDT (days needed to grow from 100 to 200 mm^3), and TGT (days needed to grow from implantation to 200 mm^3) were obtained from the curves.

either of the irradiation procedures. Six mice per data point were irradiated.

For photon irradiation, 250 kVp X-ray irradiation (250 RT: Phillips Ltd., Eindhoven, Holland) was used at 15 mA with 0.5 mm Cu and 2 mm Al filtration. The dose rate was corrected with Victoreen dosimetry at 1.5 Gy/min. The mice were put into lead boxes, and the left tumor-bearing legs and the tails were pulled out through windows of the boxes. The tails were fixed with adhesive tape, but the legs were left free.

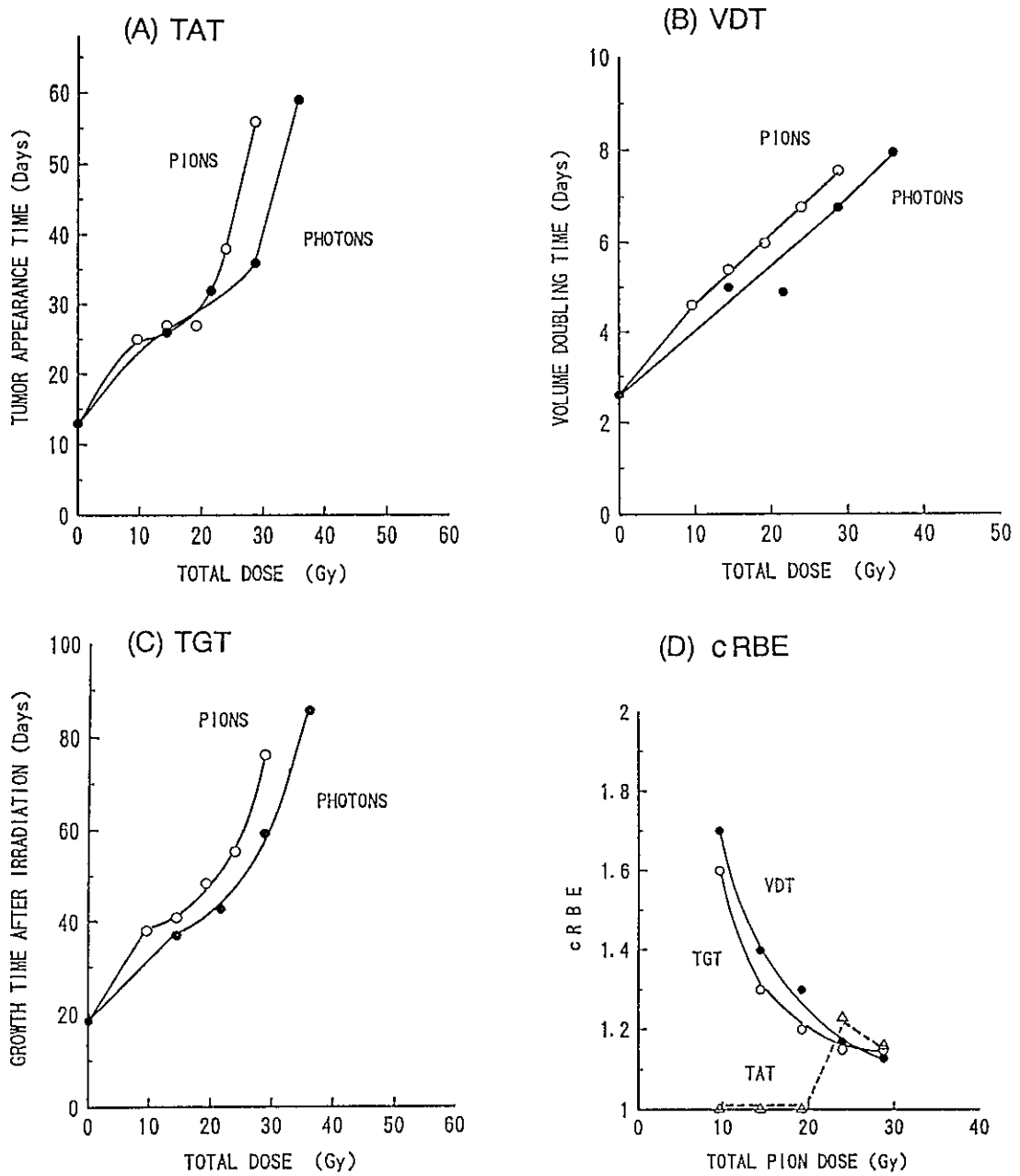

Fig. 3. Relationships between TAT, VDT, TGT, or cRBE and radiation dose. The tumors were irradiated as described in Fig. 2 (Exp. 2). The TAT (A), VDT (B), and TGT (C) for tumors irradiated with pions (○) or photons (●) were plotted as a function of total dose. The cRBEs for each assay were obtained by comparing two doses (photons/pions) at the iso-effect level with 2–6×4.8 Gy pions (D): the cRBEs for TGT (○), VDT (●), and TAT (△) were plotted as a function of pion doses.

Pions were produced at TRIUMF (Tri-university meson facility, Vancouver, Canada). The energy was about 100 MeV. The dose rate was 0.2 Gy/min on average. The mice were put into the lead boxes and the tails were fixed with adhesive tape. The tumor-bearing legs were encased in a solid plastic phantom designed for pion irradiation. They were positioned at the Bragg peak of pions with a field size of 2×5 cm^2. This methodology has been reported previously in detail.[6]

Assays After tumors became palpable, the tumor diameters were measured. The tumor volumes were calculated by use of the formula for a prolate ellipsoid: $(\pi/6) \times$ width $\times$ length $\times$ thickness. The tumor appearance time (TAT) is the latent time of tumors from the beginning of irradiation to reach a tumor volume of 20 mm^3. The volume doubling time (VDT) between 100 and 200 mm^3 was also observed. The TGT was defined as the growth time from the beginning of radiation to 200 mm^3 in volume. The median TGT (Exp. 1) was assessed for each treatment group. For concurrent observation of TGT and TCD_{50} (Exps. 2, 3), the 2nd longest TGT among 5–6 mice was assessed for TGT assay, because median TGT was not obtainable in the case of >50% tumor control level. The mice bearing tumors over 200 mm^3 were killed. The tumor control rate was observed by 120 days after implantation. No additional tumors appeared between 100 and 120 days. The TCD_{50} was assessed at 60, 80, and 100 days after irradiation. The ratio of the doses (photons/pions) giving the iso-effect was defined as a cRBE instead of the RBE, when the comparison was made between similar fractionation schedules (e.g. 5–6 fraction pions vs. 4–5 fraction photons) with different doses per fraction.

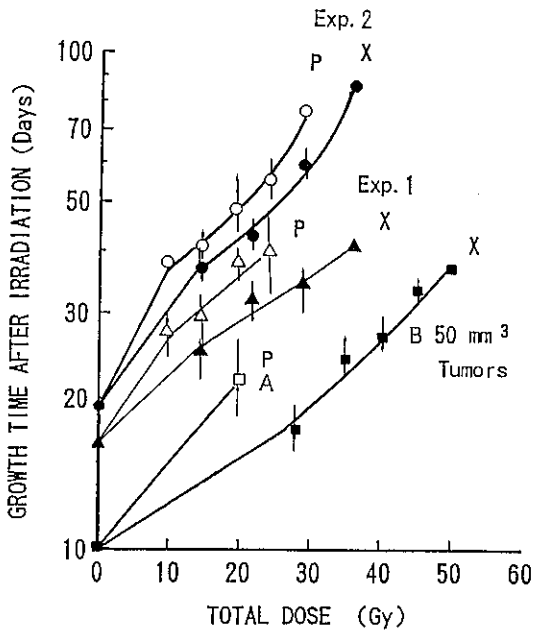

Fig. 4. Relationships between TGTs and total doses. Three paired TGT curves of pions (P; open symbols) and photons (X; closed symbols) were drawn as a function of total dose. The tumors were irradiated starting from 2 days after implantation of 2×10^4 cells (Exp. 2; circles) or 2×10^5 cells (Exp. 1; triangles), or from the time of 50 mm^3 in mean tumor volume (unpublished data; squares). The dose-fractionation schedules were 2–6×4.8 Gy pions (○, △), 2–5×7.2 Gy photons (●, ▲), 10×2.0 Gy pions (□), and 10×2.8–5.0 Gy photons (■). The points and bars indicate the mean TGTs (A, B) or the 2nd longest TGTs (Exps. 1 and 2) and SD of the mean TGTs, respectively.

RESULTS

Fig. 2 shows the growth curves for photon- or pion-irradiated tumors in Exp. 2. The curves were shifted to the right with a shallower slope as the radiation dose was increased. The TATs increased weakly depending on total dose at low doses and remarkably at high doses (Fig. 3A). The VDTs were prolonged linearly with increasing doses (Fig. 3B). The TGTs increased depending on total doses, especially at high doses (Fig. 3C). The cRBEs of pions for each assay were obtained as a function of pion dose (Fig. 3D). The cRBE values for TGT and VDT were close to each other and decreased with increasing doses. However, the cRBE values for TAT were low at low doses.

Fig. 4 shows three paired curves of the TGT for Exp. 1, Exp. 2, and our previous study, in which 50 mm^3 tumors were irradiated in 10 fractions. The TGTs increased with increasing doses and decreasing number of cells at a given time of irradiation. The doses needed to give the iso-effective TGT were obtained from the curves, and the cRBEs were calculated as summarized in Table II. The cRBE values for 30–40 days TGT varied from 1.44 to 1.60 between pions (2.4 or 4.8 Gy per fraction) and photons (3.6 or 7.2 Gy per fraction) with similar numbers of fractions. The RBE value of 4-fractionated pions was 1.5 at 30 days TGT (Fig. 5): there were only small differences among cRBE and RBE values. However, the evaluated effect level remarkably influenced the cRBE in Exp. 2 (Table II): the cRBE values decreased from 1.60 to 1.15 with increasing TGT from 30 to 75 days. Fig. 6 shows the cRBE changes as a function of the effect level.

Tumors were controlled at a total dose of 24–38.4 Gy pions (5–6×4.8–6.4 Gy/fraction) or 32–50.4 Gy photons (5–7×6.4–7.2 Gy/fraction) (Fig. 7). The TCD_{50} at 100 days after irradiation was calculated to be 28.7 Gy (confidence limits [CL]; 25.0–32.5 Gy) for pions and 40.3 Gy (CL; 36.3–44.2 Gy) for photons by fitting a

Table II. Pion or Photon Doses Giving the Iso-effect and the cRBEs of Fractionated Pions

Exp. No.	Assay	Level (days)	Pion (Gy×times)	Dose (A) (Gy)	Photon (Gy×times)	Dose (B) (Gy)	cRBE (B/A)
0[a]	TGT	22.5	2.0×10	20.0	2.8–5.0×10	33.0	1.65[b]
1	TGT	30	2.4×4–6	14.5	3.6×4–8	22.0	1.53
		35	2.4×6–8	21.5	3.6×6–8	31.0	1.44
		30	2.4–4.8×4	14.0	3.6–7.2×4	21.0	1.50[b]
		30	4.8×2–3	12.5	7.2×2–3	20.0	1.60
		35	4.8×3–4	17.0	7.2×3–4	26.5	1.56
		40	4.8×4–5	22.0	7.2×4–5	33.0	1.50
2	TGT	40	4.8×2–3	13.0	7.2×2–3	19.0	1.46
		50	4.8×4–5	21.0	7.2×3–4	25.5	1.21
		60	4.8×5–6	25.0	7.2×4–5	29.0	1.16
		75	4.8×5–6	27.5	7.2×4–5	31.5	1.15
2,3	TCD_{50}	60	4.8,6.4×5–6	27.8	6.4,7.2×5–6	30.0	1.08
		80	4.8,6.4×5–6	28.7	6.4,7.2×5–6	36.0	1.25
		100	4.8,6.4×5–6	28.7	6.4,7.2×5–6	40.3	1.40

a) Previous study.
b) RBE value.

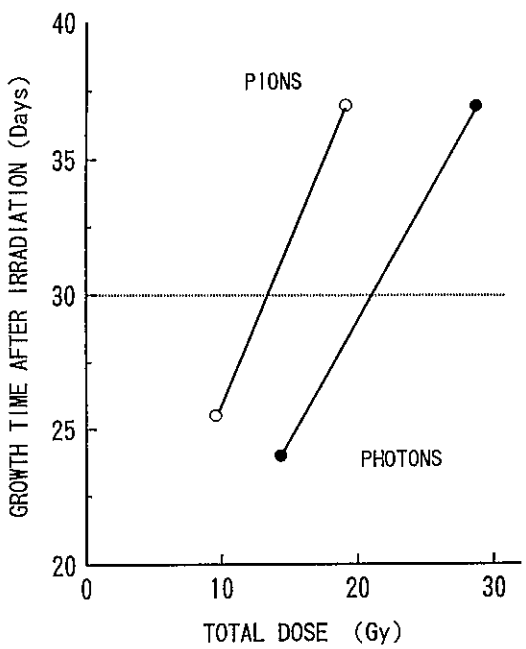

Fig. 5. Dose-TGT curves for 4-fractionated pions and photons. The tumors were irradiated with 4-fractionated pions (open circles) or photons (closed circles) in Exp. 1. The TGTs were plotted as a function of total dose. The RBE at 30 days TGT was calculated to be 1.5.

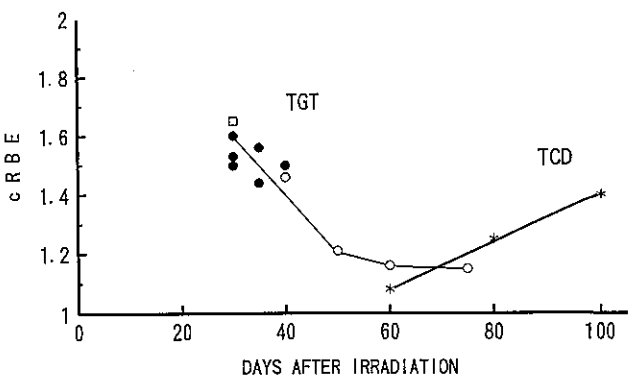

Fig. 6. Relationships between cRBEs and assay levels. The cRBE values for TGT and TCD_{50} were plotted as a function of TGT days and evaluated at various days after irradiation, respectively. Details of the dose-fractionation are summarized in Table II. The marks indicate the cRBEs for TGT in Exp. 1 (●), Exp. 2 (○), and unpublished data (□), and the cRBE for TCD_{50} (*) in Exps. 2 and 3.

logistic model. Table III shows the number of controlled or uncontrolled tumors at 60–100 days. Nine of 22 mice (41%) developed tumors between 60 and 100 days for photons, whereas only one mouse (8%) did so for pions: there was a significant difference ($P<0.05$) with the chi-square test. The cRBE values for TCD_{50} increased with prolongation of the assay period: 1.08 at 60 days; 1.25 at 80 days; 1.40 (CL; 1.18–1.63) at 100 days (Fig. 6).

DISCUSSION

A remarkable change in cRBE was noted in this study. Although the RBE depends on dose-fractionation schedules,[1-4] the cRBE change was small (1.5–1.6 at 30 days TGT) within a small range of dose per fraction (3.6–7.2 Gy photons) in several fractions. The cRBE was not very different from the RBE in these fractionation schedules:

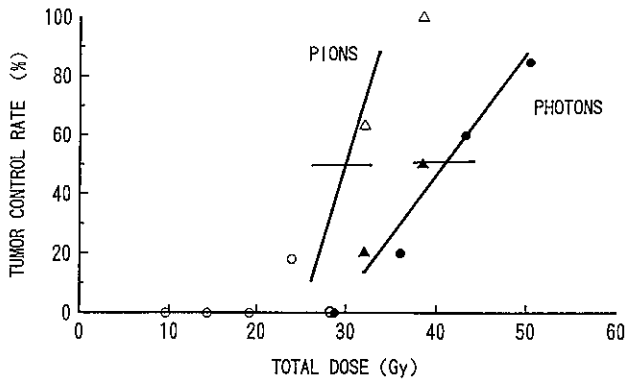

Fig. 7. Dose-cure curves for pions or photons. The tumor control rates for fractionated pions (open symbols) or photons (closed symbols) were plotted as a function of total doses. The slopes and 95% confidence limits of TCD_{50} (bars) were calculated by fitting a logistic model. Exp. 2; (○, ●) and Exp. 3; (△, ▲).

Table III. The Number of Controlled Tumors

	No. of controlled tumors at			No. of tumors appearing during 60–100 days
	60	80	100 days	
Photons	22	16	13	9
Pions	12	11	11	1

RBE of 1.5 vs. cRBE of 1.5–1.6 at 30 days TGT. In tumor growth delay assay by Takai *et al.*,[4] the RBE of fractionated pions changed from 1.4 to 1.65 as the photon dose was changed from 6 to 3 Gy per fraction. Rather than fractionation schedules, it was clear that the evaluated effective level markedly influenced the cRBE values (1.60–1.15 at 30–75 days TGT).

The cRBE change depending on TGT was analyzed. The TGTs are composed of the TAT and VDT. The VDT correlated linearly to total dose, and the growth delay was only several days at a cure level (Fig. 3B). The prolongation of VDT has been known as the tumor bed effect (TBE). The cRBE for VDT strongly influenced the cRBE for TGT (Fig. 3D). The TAT revealed small prolongations at low doses, but remarkable prolongations (above 40 days) at tumor-curative doses (Fig. 3A). When microscopic tumors composed of 4×10^4 cells were irradiated with a curative dose of 28.8 Gy pions (TCD_{50}), the prolongation of TAT was theoretically about 10 days: the required time from implantation of TD_{50} (50 cells) to develop palpable tumors in 50% of mice (Fig. 1). Therefore, the significant TAT prolongation at high doses (Fig. 3A) should be explained not only in terms of the number of viable cells, but also other mechanisms that produced further prolongation. Also, the TAT at low doses did not influence the cRBE for TGT (Fig. 3D). Thus, it was considered that the cRBE for TGT is influenced by complex phenomena that did not necessarily correlate to the dose or the number of viable cells.

Although the cRBE at 60 days TGT was nearly coincident with the cRBE at 60 days TCD_{50}, the cRBE for TCD_{50} increased with increasing evaluation time (Fig. 6) because of late appearance of tumors irradiated with photons (Table III). The late appearance was also observed during 40–60 days in TAT (Fig. 3A). Pions might kill photon-resistant cells in an indolent or a hypoxic condition. It has been considered that pions have a large killing effect against hypoxic cells.[7,8] The presence of hypoxic cells in microscopic tumors has been suggested, based on a study using a hypoxic radiosensitizer.[9]

When the cRBE value of 1.4 (CL; 1.18–1.63) for microscopic tumor control is compared with the RBE values for skin reaction,[1,2] it seems that fractionated pions have no therapeutic benefit. We have also observed simultaneously cRBEs of 1.2 to 1.6 for foot skin reaction in this study (data not shown). It is considered that radiobiological effectiveness for microscopic tumors is not different from that for normal skin. If large fractions of hypoxic cells are included in the tumors, the RBE of pions might be higher.

This experimental method using microscopic tumors would be useful for preclinical study, because the tumor control was obtained by using relatively low doses of radiation in multi-fractionated schedules, which were close to the clinical schedule at TRIUMF: total dose of 30 Gy in 10 fractions. A total dose of 32 Gy pions in 6 fractions could control the tumor in more than half the mice bearing microscopic SCCVII tumors. To control macroscopic tumors, large doses are required: for example, TCD_{50} of 113 Gy photons for 6 mm SCCVII tumors.[10]

In conclusion, the cRBE value of fractionated pions for microscopic tumors was 1.4 (CL; 1.18–1.63) for TCD_{50} at 100 days after irradiation. The values were not significantly different from the reported RBE values for skin reactions. The RBE for tumor control could not be predicted from the RBE for tumor growth assay.

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REFERENCES

1) Chaplin, D. J., Douglas, B. G., Grulkey, W., Skarsgard, L. D. and Lam, G. K. Y. The response of mouse epidermis to fractionated dose of π mesons. *Int. J. Radiat. Oncol. Biol. Phys.*, **13**, 1199–1208 (1987).
2) Douglas, B. G., Grulkey, W., Chaplin, D. J., Lam, G. K. Y. and Skarsgard, L. D. Pion and pig skin: preclinical evaluation of RBE for early and late damage. *Int. J. Radiat. Oncol. Biol. Phys.*, **12**, 221–229 (1986).
3) Ogawa, Y., Goodman, G. B., Chaplin, D. J., Grulkey, W. and Lam, G. K. Y. The response of mouse tumors to fractionated doses of pions: determination of therapeutic gain factor. *Oncology*, **48**, 81–87 (1991).
4) Takai, Y., Goodman, G. B., Chaplin, D. J., Grulkey, W. and Lam, G. K. Y. The response of murine B-16 melanoma to fractionated doses of pions. *Int. J. Radiat. Oncol. Biol. Phys.*, **23**, 573–578 (1992).
5) Olive, P. L., Chaplin, D. J. and Durand, R. E. Pharmacokinetics, binding and distribution of Hoechst 33342 in spheroids and murine tumours. *Br. J. Cancer*, **52**, 739–746 (1985).
6) Lam, G. K. Y., Henkelman, R. M., Douglas, B. G. and Evans, C. J. Dose dependence of pion RBE values for mouse foot skin reactions. *Int. J. Radiat. Oncol. Biol. Phys.*, 7, 1689–1694 (1981).
7) Hall, E. J. and Astor, M. The oxygen enhancement ratio for negative pi mesons. *Int. J. Radiat. Oncol. Biol. Phys.*, **5**, 55–60 (1979).
8) Sakamoto, K., Okada, S., Lam, G. K. L., Henkelman, R. M. and Skarsgard, L. D. The comparative survival of colonogenic cells of a murine epithelioma irradiated *in vivo* with 250 kVp x rays, ^{60}Co gamma rays, or negative pions produced by the cyclotron at TRIUMF. *Radiology*, **113**, 501–505 (1979).
9) Ohizumi, Y., Tamai, H., Maezawa, H. and Mori, T. Tumor control dose for microscopic Lewis lung tumors and hypoxic radiosensitizer, RP-170. *J. Jpn. Soc. Ther. Radiol. Oncol.*, **6**, 217–223 (1994).
10) Suit, H. D., Sedlacek, R. and Thames, H. D. Radiation dose-response assays of tumor control. *In* "Rodent Tumor Models in Experimental Cancer Therapy," ed. R. F. Kallman, pp. 138–148 (1987). Pergamon Press, New York.