A New Approach for Quantifying Radio-Biological Effects Using the Time Course of Mouse Leg Contracture

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A digitization approach to the time course of radiation-induced mouse leg contracture was proposed for quantifying the radiation effect on an individual living mouse. The shortening of the mouse leg length can be easily measured with a caliper/ruler to offer a very simple digitalized index of the radiation effect. Left hind legs of mice were irradiated with single dose of 32 Gy of 290 MeV carbon-ion beam using 0, 50, or 117 mm binary filter (BF). The right legs were used as a control. The lengths of both hind legs of the mice were measured using a digital caliper before irradiation and every week after irradiation. The degree of leg contracture, ΔS, at the time point t was estimated by subtraction of the left irradiated leg length from the right control leg length. Equation was fitted on the daily time course of ΔS and two parameters, ΔS_max and T_s, were estimated. ΔS=ΔS_max×(1−exp(−t/T_s)), where ΔS_max is the maximum degree of leg contracture, and T_s is time of leg contracture. The effect of carbon-ion irradiation on a living mouse was quantified by ΔS_max and T_s of the leg contracture, and then compared to that of X-rays. By 32 Gy irradiation, ΔS_max was largest for the BF117 experiment, followed by X-ray~BF50~BF0. T_s was shortest for the BF50 experiment, while other irradiation conditions give similar T_s. A logarithmic function was successfully repurposed for the evaluation of radio-biological response.

Key words digitization; carbon-ion beam; X-ray; relative biological effectiveness; high linear energy transfer; heavy-ion beam

The biological effects of radiation have traditionally been estimated by the ratio of surviving irradiated cells. However, the physiological conditions of tissues/organs are difficult to reconstruct in cultured cell systems. Therefore, animal experiments are necessary to understand the true effects of radiation on intact tissues, organs, or body conditions. A conventional metric to estimate radiation effects on the whole body is the 30-d survival rate of a group of mice, which requires 10 or more mice to be sacrificed to draw a survival profile. A survival rate can estimate the ratio at which a biologically significant event, i.e., a lethal action, will happen in a group of subjects; therefore, the survival rate could not estimate the degree of radiation-induced damage in an individual animal. Either the cell survival or the 30-d survival rate of mice can evaluate acute severe damage due to radiation, but this method is not appropriate for evaluating the late damage. Another method to estimate radiation effects on living animals is scoring the level of injury recognized by appearance, which requires an experienced evaluator, and the resulting score is not a quantitative value.

It was reported that single local irradiation to the flank of a mouse caused shortening the leg due to fibrosis of the tissues.1) The radiation-induced leg shortening could occur as a result of muscular fibrosis, muscular atrophy, and/or arthritic atrophy. Therefore, the radiation-induced leg shortening of mice can be considered an index of comprehensive tissue damage. The shortened leg length can be easily measured with a caliper/ruler and can offer a quite simple digitized index of the radiation effect. The degree of leg shortening can be a quantitative index of radiation effect versus an identical body. Several reports2–7) have utilized leg contracture to estimate radiation-induced late damage, with various analytical approaches.

The high linear energy transfer (LET) carbon-ion beam has been used for clinical cancer therapy since 1994 in Japan,8) and is becoming a common therapeutic technique around the world. High LET heavy particle (carbon-ion) beams induce higher relative biological effectiveness (RBE) compared to low LET photons (X-rays and/or gamma-rays).9,10) The high LET beams have a relatively low oxygen effect compared to low LET radiations,11) and generation of reactive oxygen species (ROS) by high LET beams is not considered a severe problem. However, several reports have drawn attention to the ROS generation by high LET beams.12–14) Therefore, generation of ROS in high LET beam therapy will become non-negligible as the clinical dose becomes higher in future low-fractionated high LET protocols. The mechanisms of the biological effects of high LET ion beams have been actively investigated,15–18) while concrete examinations of the relationships among ROS generation, LET, and RBE are in progress. One conceivable reason is the difficulty in quantitative digitization of the radio-biological effect.

In this paper, the time courses of leg shortening in mice after irradiation by X-ray or carbon-ion beam were measured, and a quantitative approach for analyzing radio-biological effects on the leg contracture was evaluated.

MATERIALS AND METHODS

Animals Seven-week-old female C3H/He Slc mice were supplied by Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). Animals were housed five per cage in climate-controlled (23±1°C and 55±5% humidity), circadian rhythm-adjusted...
(12-h light–dark cycle) rooms and were allowed food and water ad libitum. Mice were used for experiments after a habituation period of 1 week. Experiments were carried out in compliance with and approved by the Animal Use Committee of the National Institute of Radiological Sciences.

X-ray Irradiation X-ray irradiation was performed using PANTAK 320S (Shimadzu, Kyoto, Japan). The effective energy was 80 keV under the following conditions: X-ray tube voltage 200 kV, X-ray tube current 20 mA, and thickness and materials of the pre-filter 0.5 mm copper and 0.5 mm aluminum. The dose rate of X-ray irradiation was 1.7–1.8 Gy/min when the distance between the X-ray tube and the sample was 400 mm. Mice were anesthetized by intraperitoneal (i.p.) injection of pentobarbital. Three or four mice were fixed on a special acrylic plate using adhesive tape. The position of the left hind leg of each mouse was carefully adjusted to the 2 cm slit made by a pair of lead plates. A single radiation dose of 32 Gy was delivered to the left hind leg of each mouse.

Carbon-Ion Beam Irradiation The left hind legs of female C3H mice were irradiated by 290 MeV/nucleon carbon-ion beam at the Heavy Ion Medical Accelerator in Chiba (HIMAC, National Institute of Radiological Sciences, Chiba, Japan). Mice were anesthetized by i.p. injection of pentobarbital, fixed on a special Lucite plate using adhesive tape, and then set on the beam track. The focused 10 cm diameter carbon beam was again collimated by a 2 cm slit, and delivered to the left hind legs of mice. The other body parts of the mice were adequately blocked from irradiation. A single dose of 16–45 Gy was given with 6 cm depth spread-out Bragg peak (SOBP) protocol. Experiments were repeated using several binary filter (BF) thicknesses, i.e., 0, 50, or 117 mm. The experiment using 0 mm BF (BF0) supposed an irradiation at the end of beam line. The experiment using 50 mm BF (BF50) supposed an irradiation at 5 cm short of SOBP. The experiment using 117 mm BF (BF117) has supposed an irradiation at the middle of SOBP. Expected LET obtained by BF0, BF50, or BF117 were around 10, 20, or 50 keV/µm, respectively. The dose rates were 3.6, 3.7, or 4.7 Gy/min for BF0, BF50, or BF117, respectively. Figure 1 shows a schematic drawing of the geometry for carbon-ion beam irradiation.

Measurement of Leg Length Lengths of legs were measured every week after irradiation by the X-ray or carbon-ion beam. Mice were anesthetized by isoflurane and fixed on a special jig.1) The lengths of both hind legs of the mice were measured using a digital caliper. The non-irradiated right leg served as the control.

Statistical Analysis Statistical differences were estimated using the TTEST function in Microsoft Excel XP. Suitable ‘tail’ and ‘type’ for the TTEST function were selected as follows. The ‘tail’ was 2 (two-tailed distribution) for stability tests, because the difference between the two independent experiments was simply compared in this study. The ‘type’ was 2 (equal variance) or 3 (unequal variance), which was selected according to data variances, and the Student’s or Welch’s t-test analysis was performed according to the ‘type,’ respectively. Grades of significance were estimated by $p < 0.05$, $p < 0.01$, or $p < 0.001$.

RESULTS AND DISCUSSION A single radiation dose by 16 Gy carbon-ion beam did not induce leg shortening within 220 d after irradiation in either BF50 or BF117 (data not shown). Therefore, this leg shortening effect has a threshold dose of around 16 Gy. This does not mean that no effect occurred below 16 Gy; just that any effect was difficult to digitize. Single dose of 24, 32, or 45 Gy caused leg shortening, and the degree of leg-shortening became larger with increasing dose (Fig. 2). The experiment with 24 Gy irradiation seemed to show a short delay before shortening commenced (data not shown). The experiment with 45 Gy irradiation caused skin problems in some mice (data not shown).
Subsequent experiments were therefore performed at 32 Gy.

Figure 3 shows the time course of lengths of un-irradiated and irradiated legs. The carbon beam irradiations were conducted on the same day with the exactly the same experimental conditions except for BF thickness. Average values and standard deviation (S.D.) of three mice irradiated at the same time were plotted. The leg shortening was more severe at higher LET, when an identical dose was given (Fig. 3). X-Irradiation caused similar leg shortening to BF50 irradiation by carbon beam.

Leg contracture is a result of a reduction in the knee joint angle.6,19) X-ray radiographs showed that the lengths of bones were not affected by radiation.19) The irradiated leg grows, just like the non-irradiated leg, but simultaneously contracts. To account for growth of the non-irradiated leg, the difference in length between the control leg and the irradiated leg, ΔS(t), was obtained for each mouse at each time point as shown in Eq. 1.

$$\Delta S(t) = L_{\text{Cont}}(t) - L_{\text{Irrad}}(t)$$  \hspace{1cm} (1)

$t$: measurement time point (days after irradiation). $L_{\text{Cont}}(t)$: length of un-irradiated control leg (mm) at time point $t$. $L_{\text{Irrad}}(t)$: length of irradiated leg (mm) at time point $t$.

Figure 4 shows plots of averaged $\Delta S(t)$ among 3 mice irradiated at the same time, which are from the data shown in Fig. 3. The $\Delta S(t)$ values showed rapid increase for a few months (<100d) after irradiation, and then gradually approached a maximum.

Here, if the leg-shortening was assumed to be a logarithmic function, increasing values of $\Delta S(t)$ were expressed by Eq. 2.

$$\Delta S(t) = \Delta S_{\text{max}} \times (1 - e^{-t/T_s})$$  \hspace{1cm} (2)

$\Delta S_{\text{max}}$: prediction of maximum value of $\Delta S(t)$ (mm). $T_s$: time (days) required to show 63.2% (1−e⁻¹) shortening.

This is an exact substitution of the $T_1$ relaxation used in magnetic resonance. If so, the reciprocal of $T_s$, 1/$T_s$, is a rate constant of leg shortening. Using Eq. 2, $\Delta S(t)$ were simulated, and then best fits to the experimental values were sought. After incrementing the $T_s$ value, the correlation coefficient, $R^2$, was maximized, and then $\Delta S_{\text{max}}$ was adjusted to minimize the difference between experimental and simulated values. Figure 5 shows examples of fitted simulated $\Delta S(t)$ on the corresponding experimental data, which are shown in Fig. 4.

The average of $\Delta S(t)$ for the plateau region, $\Delta S_{p}$, was
calculated as an index of the degree of leg shortening. The time-window of 120–300 d was uniformly defined as the plateau region. \( \Delta S_p \) could be calculated for each mouse. Figure 6 shows a comparison of \( \Delta S_p \) among X-ray and the various LET carbon-ion beams. X-ray and BF50 showed similar degrees of \( \Delta S_p \). Among the carbon-ion beam irradiations, \( \Delta S_p \) increased with increasing LET.

Parameters \( \Delta S_{\text{max}} \) and \( T_s \) could be also estimated for each mouse. Figure 7 shows a comparison of \( \Delta S_{\text{max}} \) and \( T_s \) among irradiations. \( \Delta S_{\text{max}} \) showed the same trend as \( \Delta S_p \) as shown in Fig. 6. The carbon-ion beam experiment BF0 showed significantly short \( T_s \) around 30 d, while the other irradiation conditions gave similar \( T_s \) around 40 d.

In general, relative biological effectiveness (RBE) is larger for high LET particle radiation compared to that of low-LET...
The degree of leg contracture observed in this paper, i.e., $\Delta S_{\text{max}}$, was in order of BF117>X-ray>BF50>BF0 irradiations. A radio-biological effect estimated by cell or animal survival rates usually depends on LET, but the leg contracture, which is complicated result of biological responses, might not always be dependent on LET.

The effect of SOBP carbon-ion beam irradiation with BF0, i.e., raw beam, was weaker but showed a quicker response than X-rays, when the same dose was given. SOBP carbon-ion beam irradiation with BF50 showed a similar effect as X-rays, while the irradiation with BF117 showed a stronger effect than to X-rays, when the same dose was given. When considering single carbon-ion beam irradiation of a large solid subject, the SOBP region focused at the target tissue can give a larger effect than the same dose of X-rays. However, the region in front of the SOBP shows lower effects, due to this region receiving a lower dose than the SOBP region. In addition, the surface of the subject sees a much lower effect than the SOBP region and the region in front of SOBP. In this way, the effect of radiation quality could be digitized and compared with minimal animal sacrifice.

Late effects of radiation, which can occur several months after radiation therapy, are a frequent problem in clinical radiation protocols. No standard method to estimate late effects of radiation at clinical doses has been established. An estimation of radio-biological effects based on the 30-d survival rate of mice may be not suitable for estimating late effects of radiation. The estimation of radio-biological effects based on leg contraction proposed in this paper, which considers both a several-month time course and magnitude of the particle beam, the M level ·OH batch was probably generated in the SOBP region focused at the target tissue can give a larger effect than the same dose of X-rays. Therefore, SOBP carbon-ion beam. The method proposed in this paper can offer enough information to estimate radiation quality effects with minimal animal sacrifice.

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Conflict of Interest The authors declare no conflict of interest.

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