Association between low doses of ionizing radiation, administered acutely or chronically, and time to onset of stroke in a rat model

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ABSTRACT

Exposure to high-doses of ionizing radiation has been reported to be associated with the risk of stroke. However, risks associated with lower dose exposures remain unclear, and there is little information available for the risk modification according to the dose-rate. There are few studies using animal models which might be able to provide complementary information on this association. In this study, the male stroke-prone spontaneously hypertensive rat (SHRSP) was used as a model animal. The rats were acutely irradiated with doses between 0 and 1.0 Gy or chronically irradiated with a cumulative dose of 0.5 or 1.0 Gy (at a dose rate of 0.05 or 0.1 Gy/day, respectively). The onset time of stroke related symptoms in SHRSP was used as an endpoint for evaluating the effects of low dose and the low dose-rate gamma-ray exposures. With respect to acute exposure, the time to the onset of stroke in the irradiated rats suggested the presence of a threshold around 0.1 Gy. For the low dose-rate chronically exposed, no significant increase in stroke symptom was observed. These findings are novel and demonstrate that the SHRSP system can be used to determine the association between the risk of stroke and radiation exposure with high sensitivity. Moreover, these studies provide important information regarding the association between the low dose and low dose-rate radiation exposure and circulatory diseases, especially stroke.

Keywords: Low dose radiation; Low dose-rate radiation; Stroke; Stroke-prone spontaneously hypertensive rat (SHRSP); Threshold

INTRODUCTION

Epidemiological studies of cancer patients treated with high dose radiotherapy have demonstrated an association between the risk of circulatory diseases (CD) and radiation exposure [1–6]. These studies revealed that vascular injury caused by high radiation doses was a major cause of late radiation morbidity. Moreover, an important cohort study, the Life Span Study (LSS) of Japanese atomic bomb survivors, who were exposed on average to radiation doses less than 0.5 Gy for the high dose-rate, demonstrated evidence of an increased risk of stroke and myocardial infarction [7,8]. An additional study using data from the Adult Health Study (AHS) also suggested that an increased risk of stroke increasing radiation dose. However, data from women suggested there was a threshold, while the dose response for men was well described by a linear model [9]. In contrast, other recent meta-analyses of low dose exposed population studies suggest that, if a linear dose incidence response is assumed, the risk of some types of CD at doses < 0.5 Gy may be positive and similar to that seen for radiation related cancer [10]. There is emerging but somewhat controversial evidence
that has indicated that exposure to low doses or low dose-rate radiation, in particular doses associated with occupational and diagnostic radiological exposures, may be associated with an excess risk of CD [11, 12]. Therefore, the effects of low dose-rate chronic exposure on the risk of CD are an important issue. However, there has been insufficient evidence collected for the non-malignant endpoints including stroke or CD, as there has been no compelling epidemiological data or animal data made available.

As mentioned above, collectively, there is persuasive evidence that has demonstrated that high dose radiation exposure is a risk factor for the development of stroke in humans [7, 8]. However, whether this effect occurs after exposure at low doses or with low dose-rates remains unclear. Therefore, experimental studies using various animal models could provide important complementary information [13].

In this study, we used the stroke-prone spontaneously hypertensive rat (SHRSP) as a model system. SHRSP is considered to be a good model of cerebrovascular diseases caused by severe hypertension and arteriosclerosis because the overall vascular changes in the brain and other organs of SHRSP are consistent with those observed during malignant hypertension. In this study, we demonstrated that the use of this SHRSP system made it possible to detect, with high sensitivity, the effects of the radiation on the stroke-related symptoms (stroke). We were also able to compare the risk of stroke following radiation exposure over a range of different doses that included both low dose and low dose-rate irradiations.

**Study design and methods**

All rats were kept at the Animal Center of the Research Institute for Radiation Biology and Medicine, Hiroshima University, in accordance with the Research Protocol (Approval number; A16-57) and the regulations of the Institutional Animal Care and Use Committee.

**Animals**

SHRSP/Izm (SHRSP) SPF (specific pathogen-free) male rats were purchased from the Disease Model Cooperative Research Association (DMCRA) (Kyoto, Japan) through Japan SLC, Inc. (Hamamatsu, Japan). DMCRA conducted the health inspections which included appearance, body weight, weeks of age, and sex prior to the shipment. In order to verify the condition of the animals after receipt, all animals were kept for one week before the irradiation. Rats were individually housed in plastic cages (41 cm [width(W)] × 26 cm [length(L)] × 22 cm [height(H)]) with a 12-hr day/12-hr night cycle in the room called “clean convention”. All animals were fed a normal diet (MF cubed food for mice and rats; Oriental Yeast, Co. Ltd. Tokyo, Japan) ad libitum without any additional salts. A series of SHR (spontaneously hypertensive rat) strains [14], which were originally derived from Wistar-Kyoto rats (WKY), are well-known rat models for examination of human essential hypertension and have been used in genomic studies performed to elucidate the mechanism of hypertension [15]. The SHRSP are established from the SHR and exhibit a nearly 100% incidence of stroke. This unique model makes it possible to experimentally study the effects of environmental factors on stroke.

To avoid possible bias among the groups, systolic blood pressure (SBP) was consistently measured using a non-invasive tail cuff system with a sphygmomanometer (BP-98A-L, Softron, Tokyo, Japan) according to the distributor’s manual. Details of the measuring methods used in this study are described elsewhere [16]. Briefly, blood pressure was measured once a week for 7 weeks post-acute irradiation and once a week for 7 weeks after the beginning of the low dose-rate exposure, respectively. The SBP measurement was stopped after 7 weeks post-irradiation based on our experience from preliminary experiments that demonstrated SHRSP started to show stroke symptoms at week 8 or later. In some exceptional cases, SBP measurement were stopped prior to 7 weeks due to the overexcitation in the rats, which made handling difficult after or just before showing stroke symptoms. In this study, the SBP data for the SHRSP were only used to identify the rats that exhibited significantly low values, which might correspond to reversion mutation to WKY. We worried that the rats having such mutation might become one of the principal causes of bias. We tried to detect rats showing significantly low values, which were defined as having an SBP level that showed less than 2 standard deviations from the mean values. Those tests were continued to 6 weeks post-irradiation, since SBP could be measured in most rats, except for three cases. No rat showed abnormal values in majority of 6 measurements which were conducting for each rat. (See Supplemental Data).

**Radiation exposure**

For acute exposures, the whole body of each rat was irradiated using a commercial cesium-137 irradiator (Gammacell 40 Exactor; Best Theratronics, Ottawa, Canada). Although rats were not anesthetized when they were irradiated, they were placed into small plastic boxes (13.5 cm (W) × 9.0 cm (L) × 5.0 cm (H)) in order to prevent their changing position. Rats at 5 weeks of age were irradiated using a dose rate of approximately 0.72 Gy/min to deliver a cumulative dose of 1.0 Gy and a dose rate of approximately 0.175 Gy/min to deliver cumulative doses varying between 0.05 and 0.75 Gy. In cases using the 0.175 Gy/min exposure, radiation shielding plates were used to attenuate the beam. In order to avoid bias caused from stress, all rats were kept in the irradiation chamber for the same period of time (5 minutes). For example, in the cases of a 1.0 Gy irradiation, rats were kept in the chamber of irradiator for 3 minutes and 35 seconds after 1 minute and 25 seconds of exposure at the dose rate of approximately 0.72 Gy/min. In the cases of 0.5 Gy irradiation, rats were kept in the chamber for 2 minutes and 10 seconds after 2 minutes and 50 seconds of exposure at the dose rate of approximately 0.175 Gy/min. Non-irradiated controls rats were also kept in the chamber for 5 minutes without any radiation exposure. When performing animal experiments, the difference among “lots or batches” (hereafter described as “lot”) always need to be taken into consideration. In order to avoid bias caused by a lot, we always included non-irradiated rats as “controls” in each experiment. For example, when rats were irradiated with 0.25, 0.5 or 0.75, positive controls who received 1.0 Gy were also examined in parallel. For the 1.0 Gy irradiation, we used 12 rats. For the SHRSP cases that were irradiated with 0.1, 0.075 or 0.05 Gy, we examined the irradiated rats of each dose group in parallel with the non-irradiated rats. The numbers of rats in each of the groups are described in each figure (Figures 1 and 2).

Low dose-rate irradiation was conducted using the cesium-137 gamma-ray irradiator (PSCT-3003Hs Type, Pony Industry Co. Ltd.,
Osaka Japan) for approximately 23 hours every day. The last hour of the 24-hour cycle was used for measuring blood pressure of the rats and husbandry procedures. Dose rates were controlled by the distance from the source to the cages. Rats were housed in plastic cages (23 cm (W) × 13 cm (L) × 16 cm (H)) and irradiated without anesthesia. A cumulative dose of 0.5 Gy was delivered at a rate of approximately 0.1 Gy/day over 5 successive days or at the rate of approximately 0.05 Gy/day over 10 successive days. To deliver a cumulative dose of 1.0 Gy, rats were irradiated at rate of 0.1 Gy/day over 10 successive days or at the rate of 0.05 Gy/day over 20 successive days. After irradiation was completed, rats were housed in the plastic cages (41 cm (W) × 26 cm (L) × 22 cm (H)) that were placed in a chamber with controlled temperature and humidity and that were set in a non-irradiated area. Control (0 Gy) rats were handled, housed and transported in the same manner as exposed rats and were placed in non-irradiated chambers during the exposure of the test group. Other conditions, such as the type of food, ad libitum water and the light/dark cycle, were the same as those used in the acute radiation exposure experiments. The number of weeks after exposure was counted from the day when the irradiation started. Figure 3 presents information on the number of rats examined in this study.

All rats used for both the acute exposure experiments and low dose-rate exposure experiments were randomly selected for each dose group, with all of the experiments conducted under blind conditions. Radiation doses delivered to the rats irradiated acutely and chronically were verified using glass photoluminescence dosimeters (GD-302 M, AGC Techno Glass Co., Ltd, Shizuoka, Japan). For the acute irradiation, the radiation dose for the rats was measured using dosimeters taped on the surface of walls of the boxes. In the case of the chronic irradiation, the dosimeters were also taped on the surface of the cages and maintained in the same place during the period of irradiation, with the dose/day then calculated by the total doses measured divided by total days kept in the radiation room.

Measurement of the onset time of stroke

The onset of stroke was used as an end-point that was determined by the fainting of rats and/or rapid body weight loss (20 g or more per one week). The association between fainting and/or weight loss and hemorrhage was validated by preliminary pathological examinations using rats that were sacrificed at 8 weeks post-irradiation. Rats showing fainting and/or excessive weight loss were shown to have cerebral hemorrhage. To investigate the radiation effect on the time of stroke onset, we constructed Kaplan-Meier (KM) curves for each experimental group over a 30-week post-irradiation time period, with the difference between the symptom-free probability curves of each irradiated group and the non-exposed group then tested by Cox regression analysis and the proportional hazards assumption evaluation described in the following "Statistical Methods".

Statistical Methods

To investigate the association between radiation dose and stroke, we compared the time to stroke onset among different dose groups using log-rank tests and performed graphical evaluations using KM curves for each dose category (0.05 to 1.0 Gy, and a non-irradiated control). The difference between the symptom-free probability curves for each dose group and the control group was tested using the log-rank test and a Cox proportional hazards model. The hazard ratios of each irradiated group as compared to the controls were estimated using survival package of R statistical software (The R Foundation, Vienna, Austria). The radiation dose response was evaluated based on the results of the Cox proportional hazards model and the possible lot effect among the control groups used in the experiments. The different durations of experiments were taken into account by including a categorical variable for the experimental group and a mixed effects Cox regression or frailty model was also applied. The unobserved heterogeneity of the background in each experiment conducted in the different duration was considered to be a random effect in the frailty model in order to be able to take the possible lot effects into account. The mathematical expression of the model is described in the "Appendix". The proportional hazards assumption in the Cox regression was checked using the Grambsch-Therneau test [17].

RESULTS

Examination of low-dose exposure

As shown in Figure 1, the onset time of stroke in the SHRSP irradiated with doses varying between 0.25 and 1.0 Gy were significantly earlier than the onset time for the non-irradiated rats. In the SHRSP rats irradiated with 0.1 Gy, the onset time (median time to onset = 21 weeks, 95% confidence intervals (CI); [13, 25]) was slightly earlier than the onset time for the non-irradiated rats (25 weeks, 95% CI; [19, not available (N.A.)]) (p = 0.059) (Figure 2A). In contrast, SHRSP irradiated with 0.05 or 0.075 Gy did not show any acceleration for the time of stroke onset (Figures 2B and 2C). Table 1 shows the increase in the
Association between radiation and stroke

Fig. 2. KM curves for onset time of stroke for the SHRSP acutely irradiated with 0.1, 0.075 or 0.05 Gy. (A) Rats irradiated with 0.1 or 0 Gy. The number of rats at the beginning of study was 40 for rats receiving 0.1 Gy and 35 for the non-irradiated rats. (B) Rats irradiated with 0.05 or 0 Gy. The number of rats at the beginning study was 35 rats in both the 0.05 Gy and non-irradiated groups. (C) Rats irradiated with 0.075 or 0 Gy. The number of rats at the beginning of the study was 28 rats in both the 0.075 Gy and non-irradiated groups. Since some rats accidentally died during the blood pressure measurement, the actual number of rats examined in each dose group is shown in the parentheses. The p-values estimated by Cox regression are indicated.

hazard ratio with increasing radiation dose. As shown in Table A1, there were no statistical differences in the hazard ratio among the non-irradiated groups of the different experiments, but the indicators of the experiments were retained in the Cox model. Furthermore, the hazard ratios estimated for the non-irradiated groups were very similar to those obtained by the frailty model. The hazard ratio estimated for stroke in the 0.1 Gy group when compared to the non-irradiated group with the maximum number of rats was 1.56 (95% CI: [0.93, 2.64]) (Table 1). These data suggest that there might be a threshold around 0.1 Gy for the dose response for the stroke onset after acute irradiation.

Examination of low dose-rate exposure

Next, we investigated the effect of dose-rate on stroke. As shown in Figure 3, time to stroke onset in each of the four low dose-rate groups was not different from that found for the non-irradiated group. Moreover, as shown in Table 2, the hazard ratios for each radiation dose group were less than one, even though the cumulative radiation doses of the rats were as high as 0.5 or 1.0 Gy. In contrast, as mentioned in Table 1, when the doses were acutely delivered, hazard ratios were found to be one or more.

DISCUSSION

There were two primary results found for this SHRSP animal model study. First was the presence of a possible threshold for the dose response for stroke onset following an acute radiation exposure. The second was a lack of detectable effects following the low dose-rate exposure.

With respect to the first result, as seen in Figures 1 and 2, and in Table 1, the results obtained from acutely irradiated SHRSP indicated that the threshold dose appeared to be around 0.1 Gy. As far as we know, this is the first animal study that has experimentally demonstrated the presence of a threshold dose for the association between stroke and low dose-acute exposure. With regard to the second result, as seen in Figure 3 and in Table 2, our findings indicated that chronically irradiated SHRSP did not show any acceleration in the onset time of stroke in conjunction with the dose, while the acutely irradiated rats did show a dose effect for the same endpoint (Figure 1 and Table 1).

The first results (Figures 1, 2 and Table 1) support the proposal of the International Commission on Radiological Protection (ICRP), which classified CD, including stroke, as a tissue reaction with an approximate threshold dose. ICRP Publication 118 [18] stated that the threshold dose for CD is about 0.5 Gy, although the estimated dose is still uncertain. In contrast, our current result appeared to be around 0.1 Gy. The estimate used in the ICRP report was derived from epidemiologic data and then selecting the dose below which there was less than a 1% chance of an effect. As such, this value does not represent a true no-effect dose threshold. In contrast, we were able to experimentally estimate the no-effect dose, that is a "threshold value", albeit our data pertained only to stroke as an end point in this SHRSP model.

There have been various reports on the association between radiation and CD, including information on mortality from the LSS, on the incidence from the AHS and meta-analyses that examined therapeutically, diagnostically, occupationally and environmentally exposed...
Table 1. Hazard ratio of each dose group for 0 Gy based on a frailty model

| Radiation dose | Hazard ratio | 95% confidence intervals |
|----------------|--------------|-------------------------|
| 0 Gy           | —            |                         |
| 0.05 Gy        | 1.05         | [0.61, 1.80]            |
| 0.075 Gy       | 0.98         | [0.53, 1.81]            |
| 0.1 Gy         | 1.56         | [0.93, 2.64]            |
| 0.25 Gy        | 3.03         | [1.52, 6.04]            |
| 0.5 Gy         | 5.30         | [2.68, 10.50]           |
| 0.75 Gy        | 4.92         | [2.48, 9.76]            |
| 1 Gy           | 4.92         | [2.25, 10.75]           |

Table 2. Hazard ratio of each group for 0 Gy estimated by a Cox regression

| Radiation dose                  | Hazard ratio | 95% confidence intervals |
|---------------------------------|--------------|-------------------------|
| 0 Gy                            | —            |                         |
| 0.1 Gy/day × 5 days             | 0.77         | [0.37, 1.58]            |
| 0.1 Gy/day × 10 days            | 0.73         | [0.36, 1.51]            |
| 0.05 Gy/day × 10 days           | 0.89         | [0.45, 1.78]            |
| 0.05 Gy/day × 20 days           | 0.99         | [0.50, 1.95]            |

Fig. 3. KM curves for the onset time of stroke in the chronically irradiated SHRSP receiving the low dose-rate. The p-values estimated by Cox regression are indicated. There were 20 rats in each of the four groups for the SHRSP and 25 rats for the non-irradiated controls. Since some rats accidentally died during the blood pressure measurement, the number of rats examined in each dose is shown in the parentheses.

groups, as well as a part of the atomic bomb survivors [9,10,20,21]. They collectively demonstrated that radiation is a risk factor for the development of human CD. However, the shape of the dose-response relationship, particularly at low doses and low dose-rate, remains uncertain. This is likely due to the limitation of epidemiological studies, for which there can be difficulties in detecting an increased risk at low doses due to large sample size requirements. This issue is a problem even for common outcomes such as CD and for multiple potential confounding due to contributory risk factors. Thus, our current data could provide a kind of important information on the shape of the dose-response when using only a relatively small number of animals, as our system makes it possible to examine the association between stroke and radiation with high sensitivity. Furthermore, this is a good example that reveals that reliable and pertinent data can be obtained from an experimental animal model. Another benefit is that since these animals can be kept in a controlled environment, this helps to minimize some of the confounding and modifying factors.

In addition to an association between CD and low-dose acute exposure [10, 21], the effects demonstrated for the low dose-rate chronic exposure on CD risk also have important implications. Exposures from nuclear accidents, such as that occurred in Fukushima along with other radioactive contaminations, occupational radiation exposure (e.g. [22]), frequent-flyer exposures [23], and manned deep-space explorations [24] have shown that low dose-rate research is more important and urgent than ever before.

The findings found for the chronically irradiated SHRSP supported the hypothesis that generally there would be a reduction of the effects after chronic exposures but not after acute exposures, even when the cumulative doses are large (Tables 1, 2). In contrast, this result suggests a different conclusion from that suggested in previous findings [18,19], which suggested that similar threshold doses would be expected if the risk for doses up to the threshold dose are governed by a single-hit irreparable injury, and if total number of irreparable injuries was not large enough to alter tissue homeostasis. For reactions manifesting very late after the low total doses, particularly for cataracts and CD, it is especially stated in ICRP publication 118 [18] that the rate of dose delivery does not modify the incidence. On the other hand, that publication also emphasized that the currently available evidence does not conclude whether or not the threshold is the same for acute, fractionated, and chronic exposures and the publication only pointed out that the concept shown for ‘the rate of dose delivery does not modify the incidence’ is hypothetical for the purpose of radiological
protection. In other words, our current data appear to provide a piece of scientific evidence that indicated the dose-rate affected incidence for the radiation associated stroke. Moreover, our system might also be able to play an important role in the examination of the association between the risk of CD, especially stroke, and chronically low dose-rate radiation exposure, as there has currently only been a little number of publications on these issues.

Our findings are also consistent with previous radiobiological data (e.g., [25, 26]), which demonstrate a dose-rate-dependent reduction in malignant diseases. In contrast, this trend was reversed for non-malignant diseases, as in general, there is an increased risk per unit dose for fractionated exposures [25, 26]. For example, a previous study demonstrated that cardiovascular disease mortality in JANUS mice exposed to 60Co-gamma radiation appeared to be enhanced when the dose was protracted [25]. This apparent discrepancy and contradiction with our results appears to be due to the different dose delivery procedures, as our study used chronic low dose rate exposure versus the fractionated exposure that was used in the previous study. However, further studies will need to be undertaken in order to obtain securely establish a definitive conclusion.

Mechanistic derangements, such as fibrinoid necrosis of the small arteries and periarteritis, were observed in our preliminary SHRSP studies [21], in which we only examined a limited number of SHRSP. Fibrinoid necrosis in vessels was also preceded by proinflammatory cytokines and observed in late cerebral radio necrosis when using radiotherapy doses [27]. More recently, our SHR study revealed that the SBP increased with increasing doses [16]. Furthermore, the elevated blood pressure [28] and inflammation (C-reactive protein and interleukin-6) that is found among the atomic bomb survivors [29, 30] might further promote a fibrinoid necrosis link with hemorrhagic stroke. It has also been reported that hypertension has a greater impact on hemorrhagic stroke incidence as compared to cerebral infarction [31]. In the absence of any clear explanation for these previous findings, it is possible that a further study of subclinical arteriosclerosis or biological evidence from SHRSP may be able to provide additional insight into the role of radiation in promoting stroke and its subtypes.

Finally, it should be noted that only male rats were used in our current study, as it is well known that female sex hormones frequently cause bias in studies that examine circulatory systems. However, since data obtained from female atomic bomb survivors were different from the male survivors [9], it seems reasonable to include female rats in future studies, as this addition could potentially provide complementary results.

CONCLUSION

A novel animal system using SHRSP was established in order to evaluate the association between stroke and low dose and low dose-rate radiation exposure. The results from SHRSP demonstrated the presence of a threshold dose for the association between the acceleration of time to onset of stroke symptoms, and no increased risk of radiation-related stroke observed for low dose-rate chronic exposure.

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Table A1. Results of Cox regression after adjusting for the experimental indicators

| Radiation dose     | Hazard ratio | 95% confidence intervals |
|--------------------|--------------|--------------------------|
| 0 Gy (Experiment 1) | -            |                          |
| 0 Gy (Experiment 2) | 0.85         | [0.47, 1.52]             |
| 0 Gy (Experiment 3) | 0.69         | [0.40, 1.18]             |
| 0 Gy (Experiment 4) | 0.70         | [0.36, 1.35]             |
| 0.05 Gy            | 1.03         | [0.60, 1.78]             |
| 0.075 Gy           | 0.97         | [0.52, 1.82]             |
| 0.1 Gy             | 1.58         | [0.93, 2.69]             |
| 0.25 Gy            | 3.08         | [1.51, 6.27]             |
| 0.5 Gy             | 5.39         | [2.66, 10.90]            |
| 0.75 Gy            | 5.01         | [2.47, 10.13]            |
| 1 Gy               | 5.00         | [2.25, 11.13]            |

Appendix

The frailty model that fits the hazard model is as follows:

$$\lambda(t) = Z_i \lambda_0(t) \exp(X_\beta),$$

where $\lambda_0(t)$ is an unspecified baseline hazard function, $X$ is the design matrix that consists of the indicator variables of the dose category, a random effects $Z_i$ is distributed as an independently identically distributed gamma random variable for the $i$th experiment, and $\beta$ is the vector of fixed-effects coefficients interpreted as estimates of the logarithm of the hazard ratio, as they are in the usual Cox regression. We distinguished the usual Cox regression, which estimated only the fixed effects of the hazard ratio from 0 Gy and which is described in the same mathematical form when we excluded the frailty term $Z_i$ from the above frailty model. The unobserved heterogeneity among the different experiments was considered to be a random effect in this model in order to be able to take the possible lot effects into account.