



Effects of chronic low-dose-rate gamma irradiation on the antitumor activity and chemokine system in mice

Daisaku Takai^{1*}, Akiko Todate², Kazuaki Ichinohe¹, Jun-ichiro Komura¹

Abstract

Introduction: There has been a growing interest in the carcinogenic effects of low-dose-rate radiation following the exposure of many people after the nuclear power plant accidents in Chernobyl and Fukushima. However, estimating cancer risk is more difficult for low-dose radiation than for high-dose radiation. This study aimed to clarify the effects of chronic low-dose-rate gamma irradiation on antitumor activity in mice and the possible involvement of chemokine systems in this.

Methods: Mice were exposed to gamma rays for 400 days at low-dose rates of 0.05, 1.0, and 20 mGy/22 h/day, giving total accumulated doses of 20, 400, and 8000 mGy, respectively. The antitumor activity to a syngeneic tumor cell line and the expression levels of 84 genes involved in the chemokine system in recipient mice were analyzed.

Results: Reduced antitumor activity was reproducibly observed in the mice irradiated at a dose rate of 20 mGy/22 h/day but not in the mice irradiated at 0.05 or 1.0 mGy/22 h/day. Enhanced antitumor activity, previously reported for acute low-dose irradiation, was not detected under conditions of chronic irradiation. The expression levels of some genes involved in the chemokine systems were altered after low-dose-rate irradiation.

Conclusion: No apparent effects were revealed in mice with chronic irradiation at dose rates of 0.05 or 1.0 mGy/22 h/day. The suppression of antitumor activity observed after chronic irradiation at 20 mGy/22 h/day could be explained, at least in part, by alterations in chemokine/chemokine receptor systems.

Keywords: Low-dose-rate radiation, Tumor cell transplantability, Chemokines, PCR array

Introduction

Many studies have focused on the biological effects of ionizing radiation, particularly radiation carcinogenesis (1). Recently, there has been growing interest in the carcinogenic effects of low-dose exposure following the exposure of many people to low-dose-rate radiation as a result of the nuclear power plant accidents in Chernobyl and Fukushima (2). However, estimating the cancer risk is more difficult for low-dose radiation than for high-dose radiation because few cancers are induced and these cannot be easily detected (3).

Tanaka et al revealed the effect of chronic exposure to low-dose-rate gamma rays on lifespan and incidence of neoplasms using 4000 specific-pathogen-free (SPF) B6C3F₁ mice; statistically significant life shortening was observed in mice irradiated with 21 mGy/22 h/day but not in those irradiated with 0.05 mGy/22 h/day (4). Pathological analysis indicated that the life shortening was primarily the result of earlier death due to neoplasms, particularly malignant lymphomas (5). However, the mechanism responsible for early neoplastic death is not yet understood.

It has been thought that, during the process of radiation

carcinogenesis, ionizing radiation induces cellular neoplastic transformation (6). However, the results of tumor cell transplantation assay suggest that ionizing radiation not only induces neoplastic transformation but also promotes cancer progression (7). Tumor cell transplantation assay is an *in vivo* model for the study of cancer progression, in which tumor cells are transplanted into healthy mice. Almost all of these cells are eliminated by the antitumor response of the host mouse; however, if the antitumor response fails to eliminate tumor cells, they will proliferate, resulting in the formation of palpable tumors (8). Although the effects of radiation exposure on the antitumor activity are not well understood, some studies have reported a reduced tumor dose 50 (TD₅₀, the number of tumor cells needed to develop into a tumor in 50% of recipient animals) (9-12) and an enhanced transplantability of tumor cells (13,14). In these reports, mice were exposed to high-dose level acute irradiation (range 1.5–6.25 Gy). On the other hand, exposure to low-dose level acute irradiation (range 10–250 mGy) was reported to activate an antitumor response *in vivo* (15-17). Interestingly, we previously observed that 400 days of chronic exposure at

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¹Department of Radiobiology, Institute for Environmental Sciences, 2-121 Hacchazawa, Takahoko, Rokkasho, Aomori 039-3213, Japan. ²JAC Co. Ltd., 1-2-7 Higashiyama, Meguro, Tokyo 153-0043, Japan.

*Corresponding Author: Daisaku Takai, Department of Radiobiology, Institute for Environmental Sciences, 2-121 Hacchazawa, Takahoko, Rokkasho, Aomori 039-3213, Japan. Tel: +81-175-1753, Fax: +81-175-71-1982. Email: dtakai@ies.or.jp

■ Implication for health policy/practice/research/medical education

Estimating the cancer risk of low dose radiation is very difficult compared with that of high dose radiation, because there are few cancers induced by low level radiation and they cannot be easily detected. This study aims to clarify the effects of chronic low dose-rate gamma irradiation on anti-tumor activity in mice and the possible involvement of chemokine systems in this activity.

a dose rate of 20 mGy/22 h/day (a total dose of 8 Gy) suppressed the antitumor response (7).

In the present study, we investigated whether the reduced antitumor activity observed after chronic exposure to high doses could be extended to chronic exposure to lower total doses. The dose rates we examined were 0.05 and 1.0 mGy/22 h/day; the exposure was continued for 400 days, and the total doses were 20 and 400 mGy, respectively. The lower level is comparable to a dose limit for radiation workers (20 mSv/year) and also to the average dose level for airplane passengers (20 μ Sv/h or less) (18). The dose rate of 1.0 mGy/22 h/day is close to the level that astronauts are exposed to in a space station (approximately 1 mSv/day) (19). For comparison, we re-examined the effects of 20 mGy/22 h/day of irradiation for 400 days (a total dose of 8000 mGy), which had been shown to induce an increased failure in the elimination of transplanted tumor cells (7). We also measured the mRNA levels of 84 genes involved in chemokine systems to examine the possible involvement of these systems in the effects of low-dose-rate radiation on antitumor immunity as these proteins have been proven to play an important role in tumor growth (20,21).

This study aimed to clarify the effects of chronic low-dose-rate gamma irradiation on antitumor activity in mice and the possible involvement of chemokine systems in this activity.

Materials and Methods

Animals and the tumor cell line

One hundred SPF female B6C3F₁ mice were purchased from an animal breeding facility (Japan CLEA, Tokyo, Japan) at 6 weeks of age. The mice were divided into four groups: a 20 mGy/day irradiated group (n=20), a 1.0 mGy/day irradiated group (n=20), a 0.05 mGy/day irradiated group (n=20), and an age-matched non-irradiated control group (n=40). Continuous irradiation of the mice began at 8 weeks of age. After irradiation for 400 consecutive days with total doses of 8000, 400, and 20 mGy, respectively, the surviving mice (all 20 of the 20 mGy/day irradiated group and 19 from each of the other two irradiated groups) were transferred to animal rooms without irradiation. The surviving non-irradiated mice (n=39) were used as controls. All of the animals were housed in either irradiation rooms or in animal rooms maintained

at a temperature of 23 \pm 2°C with 50 \pm 10% humidity and a 12-hour light/dark cycle and were allowed *ad libitum* access to commercially prepared and gamma-sterilized feed pellets (FR-2; Funabashi Farm Co., Chiba, Japan) and chlorinated drinking water. All of the experiments were conducted according to the legal regulations in Japan and followed the Guidelines for Animal Experiments of the Institute for Environmental Sciences. The tumor cell line OV3121, which was derived from an ovarian granulosa cell tumor that arose in female B6C3F₁ mice after irradiation with high-dose-rate gamma rays (22), was obtained from Health Science Research Resources Bank, Osaka, Japan.

Irradiation

Irradiation equipment (Yoshizawa LA Co., Ltd, Chiba, Japan) using ¹³⁷Cs sources with activities of 74 GBq, 3.7 GBq, and 0.185 GBq were located in the three irradiation rooms, providing dose rates of 20, 1.0, and 0.05 mGy/day, respectively. Dosimetry was carried out by using two ionization chambers (1200 ml and 12 ml) and thermoluminescence dosimeters (AGC Techno Glass Co., Ltd, Shizuoka, Japan). The racks for the mouse cages were positioned using the ionization chambers to confirm the dose rates were as expected. Continuous radiation exposure lasted for 22 hours per day, with the remaining 2 hours used for clinical observation of the test animals, room cleaning and bedding replacement, and to provide a fresh supply of food and water.

Tumor cell transplantation

OV3121 cells cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal calf serum were trypsinized and suspended in normal saline to obtain 1 \times 10⁶ cells/ml cell suspensions. The cell suspensions (0.1 ml) were subcutaneously inoculated into the shaved back of the non-irradiated and irradiated mice. Tumor formation and development were examined twice a week until day 70 after inoculation. The tumor size was measured using a caliper, and the volume was calculated according to the formula (length \times width²)/2 (23). The number of tumor-bearing mice, in which a palpable tumor was detected during the observation period of 70 days after inoculation, was counted to assess tumor formation.

PCR array analysis of the mRNA levels

Small samples of blood were obtained from the retro-orbital venous plexus of mice using heparinized 75- μ l micro-hematocrit capillary tubes (Hirschmann Laborgerate, Germany) just prior to the tumor cell inoculation. RNAs were extracted from the blood samples using an RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) and were analyzed for the mRNA levels of the different genes involved in chemokine systems using the RT² Profiler™ PCR Array (PAMM-022Z; QIAGEN). The levels of glyceraldehyde-3-phosphate dehydrogenase gene were used as ref-

erences. The fold changes in the irradiated mice and the 95% confidence intervals for each mRNA were calculated using the RT² Profiler™ PCR Array Data Analysis version 3.5 (QIAGEN).

Statistical analysis

Differences in the tumor cell transplantability between the non-irradiated and irradiated mice were examined using the log-rank test. The tumor growth rates were examined using the *t* test. *P* < 0.05 was considered statistically significant. When estimating the gene expression levels, the *P* values were calculated using the RT² Profiler™ PCR Array Data Analysis version 3.5.

Results

Comparison of tumor cell transplantability

Female B6C3F₁ mice were irradiated continuously with gamma rays at three different dose rates: the lowest low-dose-rate (0.05 mGy/22 h/day; LL), the middle low-dose-rate (1.0 mGy/22 h/day; ML) and the highest low-dose-rate (20 mGy/22 h/day; HL). Tumor cells of the OV3121 cell line, derived from an ovarian granulosa cell tumor,

were inoculated into the irradiated mice and age-matched, non-irradiated control mice. The tumor formations were examined twice a week for 70 days after inoculation. The results are shown in Figure 1A. When compared with non-irradiated control mice, the antitumor activity was significantly reduced in the mice irradiated with HL (*P* < 0.05), but not in the mice irradiated with ML (*P* = 0.11) and LL (*P* = 0.35). The tumor formation in the ML-irradiated mice had a different appearance from that in non-irradiated mice until 40 days after inoculation, but this difference disappeared thereafter. The statistical analysis showed that the difference was not significant. There were no significant differences between the growth rates of the tumor in each group of mice (Figure 1B).

Gene expression pattern of the chemokine system

Small samples of blood were obtained from the retro-orbital venous plexus of mice just before tumor cell inoculation. RNAs from the blood cells were analyzed by the

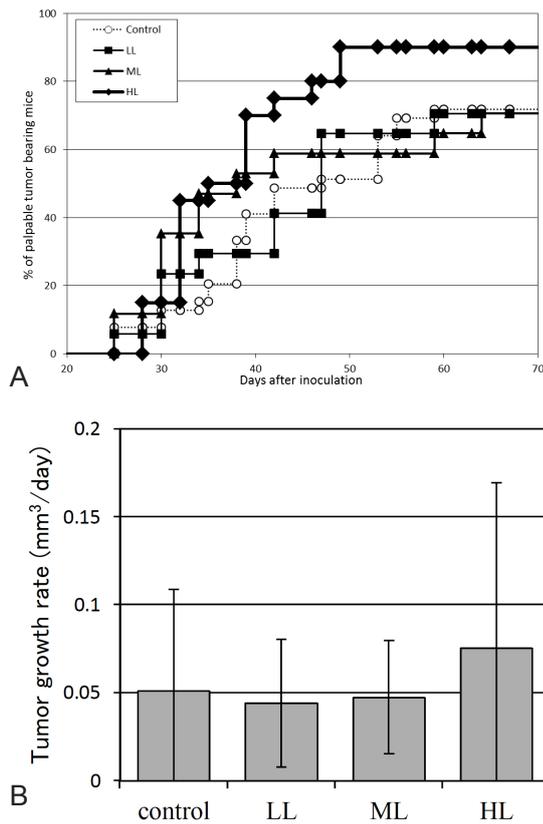


Figure 1. Tumor development in irradiated mice and non-irradiated control mice. A) A total of 1.0×10^5 OV3121 cells were inoculated in LL (square), ML (triangle), or HL (rhombus) irradiated mice and non-irradiated control (circle) mice. The number of tumor-bearing mice, where a palpable tumor was detected, was counted to assess tumor formation. B) The growth rate of the tumor was calculated by dividing the increase in the tumor volume by the days spent for the increase. The bars show standard deviations.

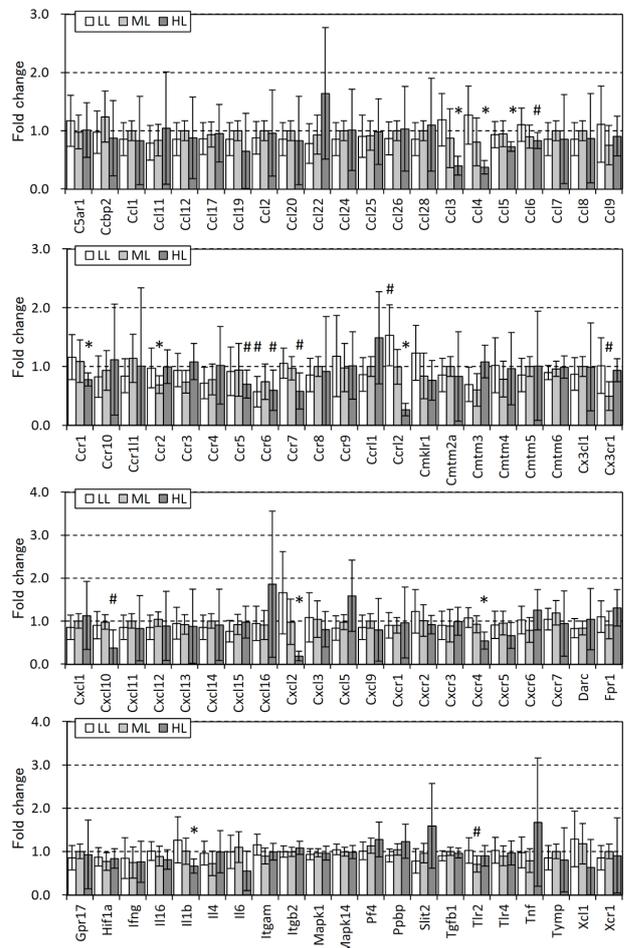


Figure 2. The gene expression profiles of LL (n = 12), ML (n = 12), and HL (n = 12) irradiated mice. RNAs from the blood cells of mice with or without low-dose-rate irradiation were analyzed using the RT² Profiler™ PCR Array for the chemokine pathway-focused gene expression analysis profiling of 84 related genes. The fold changes against non-irradiated control mice were calculated using the RT² Profiler™ PCR Array Data Analysis. The bars show 95% confidence intervals. #, *P* < 0.05; *, *P* < 0.01.

RT² Profiler™ PCR Array for chemokine pathway-focused gene expression analysis profiling 84 related genes. The expression levels of some genes were found to be significantly altered after low-dose-rate irradiation (Figure 2). Mice irradiated with HL gamma rays showed a wider effect on the expression of genes than did mice irradiated with ML or LL. Of the 84 genes, the expression of 13 (15.5%) were altered in the HL irradiated mice, whereas the expression of only three (3.6%) and two (2.4%) were altered in the LL and ML irradiated mice, respectively.

After LL irradiation, the expression of *Ccr6* (chemokine (C-C motif) receptor 6) was reduced and the expression of *Ccr12* (chemokine (C-C motif) receptor-like 2) was increased. After ML irradiation, the expressions of *Ccr2* (chemokine (C-C motif) receptor 2), *Cx3cr1* (chemokine (C-X3-C) receptor 1), and *Tlr2* (toll-like receptor 2) were reduced. After HL irradiation, reductions in the expression level were observed in the following 13 genes: *Ccl3* (chemokine (C-C motif) ligand 3 or MIP-1-alpha (macrophage-inflammatory protein-1-alpha)), *Ccl4* (chemokine (C-C motif) ligand 4 or MIP-1-beta (macrophage-inflammatory protein-1-beta)), *Ccl5* (chemokine (C-C motif) ligand 5 or RANTES (regulated upon activation normal T cell expressed and secreted)), *Ccl6* (chemokine (C-C motif) ligand 6), *Ccr1* (chemokine (C-C motif) receptor 1), *Ccr5* (chemokine (C-C motif) receptor 5), *Ccr6*, *Ccr7* (chemokine (C-C motif) receptor 7), *Ccr12*, *Cxcl10* (chemokine (C-X-C motif) ligand 10), *Cxcl2* (chemokine (C-X-C motif) ligand 2 or MIP2 (macrophage inflammatory protein 2)), *Cxcr4* (chemokine (C-X-C motif) receptor 4), and *Il1b* (interleukin-1-beta).

Thus, all of the alterations observed were reductions in mRNA levels after irradiation, with the single exception of *Ccr12* after LL irradiation, which showed an increase of approximately 50%. When the dose dependency of the alterations was examined, the expression levels of *Ccr6* were reduced after LL and HL and showed a similar tendency after ML. In contrast, *Ccr12* showed an elevation after LL, with no effect after ML and suppression after HL.

Discussion

Significant suppression of antitumor activity was observed only in HL-irradiated mice. LL and ML irradiation did not have such an influence. It is known that acute low doses of radiation (range 10–250 mGy) activate the antitumor response, as shown by the reduction of tumor cell transplantability (15), increase of TD₅₀ (16), and prolongation of tumor latency (17). In our chronic low-dose-rate experiments, however, no such response was detected. The reason for this may be that the antitumor activity induced by acute low-dose radiation may diminish when the low dose is administered as chronic low-dose-rate irradiation. A direct comparison of the effects of acute and chronic irradiation using the same strain of mice and under the same laboratory conditions would be needed to confirm this explanation.

The growth rates of tumors were not different from each other (Figure 1B), indicating that tumor growth after palpable tumors had arisen was not affected by irradiation. This result also suggests that the influence of low-dose-rate irradiation was limited to the earlier phase of antitumor activity, such as immune cell recruitment or construction of the tumor microenvironment. One of the factors involved in the recruitment of immune cells to tumor sites and the construction of tumor microenvironment is the activity of chemokines (20,21). As chemokine systems are involved in antitumor immunity, it is likely that the imbalance in the expression pattern of chemokine/receptor systems disturbs antitumor immunity. The expression patterns of chemokine/receptor systems after low-dose-rate (LL, ML, and HL) irradiation were compared with those in the non-irradiated control. Some of the genes showed altered expression, mostly reduction, after chronic low-dose-rate irradiation. HL irradiation reduced the mRNA levels of some receptors and ligands, whereas LL and ML irradiation induced alterations only in a small number of receptors. Chemokine ligands and receptors are known to be connected in a complex way (20,21). For example, ligands *Ccl3*, *Ccl4*, and *Ccl5* associate with the chemokine receptors *Ccr1* and *Ccr5*. The expression of these ligands and receptors was found to be reduced after HL irradiation. Thus, the reduced level of the axis of these chemokine ligands and chemokine receptors may contribute to the reduced antitumor activity after HL irradiation. How does chronic low-dose-rate irradiation induce alterations in the expression of specific chemokine ligands or receptors? One hypothesis for *Ccr5* could be that this is related to obesity. Nakamura et al reported that chronic low-dose-rate gamma irradiation induces obesity in B6C3F₁ female mice (24). The obese mice have more adipose tissue than do normal non-irradiated control mice. Furthermore, Kitade et al reported that macrophages expressing *Ccr5* accumulate in the adipose tissues of obese mice (25). Thus, it is likely that the adipose tissues of obese mice can hold more *Ccr5*-positive macrophages than those in non-obese mice. Taken together, it is hypothesized that low-dose-rate irradiation-induced localization of immune cells expressing *Ccr5* in adipose tissue results in an imbalance of the chemokine system in the blood of irradiated mice. The decrease in *Ccr5*-positive cells in the blood will show a reduced level of *Ccr5* mRNA in blood samples.

Conclusion

Although all three levels of gamma rays (LL, ML, and HL) used in the present study were categorized as low-dose-rate radiation (<100 mGy/day), significant effects on the antitumor activity were observed only in the HL-irradiated mice, but not in mice after ML and LL irradiation. The reduced activity of the antitumor response after HL irradiation may be induced through alterations of the gene expression of chemokine systems. A possible correlation between the modulated antitumor activity after irradiation

and alterations of the expression of some genes involved in chemokine systems warrants further study.

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Authors' contribution

DT contributed to the design of the study and acquisition, analysis, and interpretation of data for the study. AT contributed acquisition and analysis of data for the study. KI contributed animal experiments for the study. J-iK participated in its design and coordination and helped write the manuscript.

Conflict of interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

Ethical considerations

Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors. Also, this article does not contain any studies with human subjects.

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