Induction of Lung Tumors in C3H Strain Mice after Single or Fractionated Irradiation with X-Rays

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ABSTRACT. Murine model for lung tumor induction was studied in C3H/He male mice, a strain with low spontaneous incidence of lung tumors. Dose-response relationships in lung tumor induction were compared following irradiation with single doses and split doses of X-rays to the thorax either at night or in the daytime. The tumor incidence after a single 1.25 Gy dose at night during the period of nocturnal activity almost reached the maximum level after a 5 Gy dose in the daytime. Proliferative activity determined by observing the labeling index with tritiated thymidine in the normal lung was low as a whole, but tended to decrease in the daytime. When the proliferative response was induced by X-irradiation, significantly higher activity was observed at night. These circadian fluctuations were thought to affect radiosensitivity and lung tumor induction in mice. When split doses or fractionated doses of X-rays were applied to the thorax, lung tumor incidence definitely increased. The incidence after two 7.5 Gy doses with a 12 hr-interval was 41%, 3-fold higher than that after a single 15 Gy dose. Moreover, fractionated whole body irradiations (three times at 3 Gy with 3-month-interval) after a single 7.5 Gy thoracic irradiation was most effective in increasing not only the incidence (47%) but also the multiplicity of the lung tumor. More than 30% of tumor-bearing mice had two or more tumors following thoracic and whole body irradiations, while only 10% of tumor-bearers had multiple tumors after single or fractionated thoracic irradiation alone.—**KEY WORDS:** C3H/He mouse, fractionation, labeling index, lung tumor, X-ray.

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There are many factors affecting effectiveness in radiation carcinogenesis, including dose and dose-rate, radiation quality, single or fractionated irradiation, whole- or partial-body irradiation and tissue sensitivity [17]. Among these factors, differences in carcinogenic effects by either single, fractionated or continuous radiation exposure, however, remain to be elucidated.

The lung is one of the most important organs for radiation induced tumors in man as well as in mice. For a murine model of lung tumors, strain A mice are commonly used because of their high rate of tumor induction by carcinogens such as chemicals and/or ionizing radiation, despite their high spontaneous incidence of lung tumors. To study the mechanism of radiation carcinogenesis, tumor models are needed in which the tumor incidence is spontaneously low, while high induction occurs after irradiation. However, such models for radiation-induced lung tumors had not previously been reported in mice.

We have previously reported lung tumor induction in C3H/He strain mice which have low spontaneous incidence but have a four- to six-fold higher incidence after X-irradiation [9]. The highest incidence of lung tumors was, however, less than 40% following irradiation of either single or split doses. The present study was done to achieve more effective induction of lung tumors in C3H male mice by fractionated X-irradiation and combination with whole body irradiation. Moreover, the dose-response relationship of lung tumor induction after single dose of

X-rays was also determined in mice irradiated at night during the period of nocturnal activity.

MATERIALS AND METHODS

Animals: Male inbred C3H/HeSlc mice were used in all experiments. Mice aged 6 weeks under specific-pathogen-free conditions were purchased from Japan SLC, Inc. (Shizuoka, Japan) and kept under conventional conditions in an environment maintained at $22 \pm 2^\circ\text{C}$ with a 12-hr light/dark (6 a.m. to 6 p.m.) cycle. Four mice per cage were housed in plastic cages bedded with wood shavings irradiated with 10 kGy ^{60}Co γ -rays and covered with a filter top, and fed laboratory chow irradiated with 10 kGy γ -rays and tap water containing hydrochloric acid (pH 2.5–3.0) *ad libitum*.

Irradiation procedure: Partial irradiations to the thoracic cavities were performed with X-rays at 170 kVp and 25 mA with 0.5 mm Cu and 0.5 mm Al filters at a dose rate of 0.87 Gy/min. Briefly, the mice were anesthetized by intraperitoneal injection of sodium pentobarbital, and were shielded with 5 mm-thick lead, except for the thoracic area, so as to reduce the dose rate to 5% of the thorax. Whole body irradiations (WBI) were also performed with the same source at a dose rate of 0.95 Gy/min without anesthesia. Prior to both thoracic irradiation and WBI, thermoluminescent dosimetry (TLD) was carried out in paraffin-phantoms with probes calibrated with a model 550 Radocon III ionization chamber (Victoreen, Cleveland, Ohio).

Examination of lung tumors: Immediately after the mice were killed by an overdose of pentobarbital, the lungs

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were perfused with phosphate-buffered saline (PBS) and then with 5% phosphate-buffered formalin, dissociated and fixed for 3 days in 10% buffered formalin. 5 μ m-thick serial sections were obtained from whole paraffin-embedded lung tissues at 100- μ m intervals, and stained with hematoxylin and eosin. Tumor foci were detected under a light microscope and the diameters of the tumors in these sections were measured.

Microautoradiography: To identify diurnal variations in the population of lung cells in flux through the cell cycle, the DNA labeling indices were examined by microautoradiography. At 8 weeks of age, three mice in each group were given X-rays at a dose of 5 Gy to thoracic cavities or only anesthetic at 14:00. Twenty days after the treatments, microautoradiography experiments were conducted at 4-hr intervals for an entire day (0:00-24:00).

Three mice per group were intraperitoneally given 37 MBq/kg body weight of tritiated thymidine (pulse labeling). Sixty minutes later they were sacrificed by giving an overdose of pentobarbital, and the lungs were perfused with PBS, then with 5% buffered formalin. Representative 3 μ m-thick lung sections were coated with NR-M2 emulsion (Konica, Japan) diluted 2-fold with distilled water at 50°C, dried at room temperature and kept for 14 days in a light-tight box with silica gel at 4°C. The sections were developed and then stained with hematoxylin and eosin. To obtain a labeling index, more than 5000 nuclei of lung cells other than bronchial cells and visible vascular cells were counted, and the number of labeled nuclei over five grains was recorded.

Experimental protocol: Since the preliminary experiment demonstrated that the lung tumor incidence of mice irradiated at night reached the highest at doses below 5 Gy, the mice were exposed to single-doses ranged from 0 to 6.25 Gy with 1.25 Gy increments at times between 01:00 and 03:00 (night-irradiation) in order to know if higher incidences would be acquired than the incidences in mice irradiated in the daytime.

To expose the mice to high X-ray doses, two fractionated 7.5 Gy doses were given at 4, 8, 12, 18, and 24 hr intervals. Four fractionated doses were also applied to thorax at 1-week intervals to achieve total doses of 10, 20, and 30 Gy. To clarify the effect of the immune system on lung tumor induction [16], a single 7.5 Gy dose was first applied to the thorax and then WBI were performed by exposures to 3 Gy three times at 3 month intervals. All irradiations except the second protocol of irradiations by 2×7.5 Gy were carried out in the daytime between 13:00 and 15:00. All the experimental details are shown in Table 1.

Statistics: Statistical examination was carried out by means of the χ^2 -test in contingency tables for the tumor incidence, and Student's t-test for the labeling index and tumor diameter. Standard deviations (SD) in tumor incidences were calculated by means of the formula: $SD = \sqrt{p(1-p)/N}$.

Table 1. Experimental design and the number of mice in each group

Total dose of	Number of mice		Total No.
X-rays (Gy)	Initial	Surviving	of tumors
Experiment I: Single thoracic dose			
[Night-irradiation]			
0	72	72	7
1.25	32	32	9
2.5	36	35	9
3.75	32	31	9
5	48	47	13
6.25	32	32	10
[Day-irradiation]			
5	52	50	11
7.5	40	40	11
15	40	39	5
Experiment II: Two equal doses			
[thorax, 4~24 hr interval]			
15(4 hr)	38	38	11
15(8 hr)	28	28	5
15(12 hr)	32	32	14
15(18 hr)	32	32	5
15(24 hr)	32	32	7
Experiment III: Four equal doses			
[thorax, 1-week interval]			
0	28	28	1
10	28	27	6
20	28	28	10
30	28	26	7
Experiment IV:			
[7.5 Gy (thorax)+3×3 Gy (WBI, 3-months interval)]			
16.5	34	34	21

RESULTS

Tumor induction: Most of the lung tumors were alveologenic adenomas that appeared as local foci separated from their surrounding tissues. Some adenocarcinomas were also observed, while typical tubular- or papillary-form tumors were rarely observed.

To confirm again the previous observation [9], single 5, 7.5 or 15 Gy doses were given to mice in the daytime between 13:00 and 15:00 (day-irradiation). Figure 1 shows the dose response relationships of lung tumor induction in C3H/He male mice 12 months after day-irradiation. The lung tumor incidence after day-irradiation in the present series of experiments (closed symbols) approximately reproduced that in the previous observation [9] (open symbols), and reached the maximum at doses over 5 Gy but decreased at 15 Gy. The dose-response relationships of lung tumor induction after night-irradiation are shown in Fig. 2. The lung tumor incidence after night-irradiation was significantly ($p < 0.05$) higher even at a 1.25 Gy dose than the spontaneous incidence, but were similar to the highest incidence after day-irradiation as shown in Fig. 1. Figure 3 shows the dose-response curves for tumor diameter after day-irradiation (open symbols) and night-irradiation (closed symbols). The diameter after day-irradiation increased at 5 Gy and then decreased at doses of over 7.5 Gy. At a 15 Gy dose the diameter decreased to

Fig. 1. Lung-tumor incidence after thoracic single-dose X-irradiation in the daytime. The points indicate the mean $\pm$ SD of data from the previous report [13] (open circle), and the present study (closed circle).

Fig. 2. Lung-tumor incidence after thoracic single-dose X-irradiation at night. Data are the mean $\pm$ SD for irradiated mice (closed circle) and unirradiated controls (shaded areas).

the control level. Reductions of both incidence and diameter at 15 Gy demonstrated that a 15 Gy thoracic dose in the daytime was an overdose in lung tumor induction. On the other hand, the diameters after night-irradiation at doses below 6.25 Gy were larger than the control. These results in both incidence and diameter demonstrated that night-irradiations at doses below 6.25 Gy did not reach the overdose in lung tumor induction.

Figure 4 shows the lung tumor incidence after two fractionated 7.5 Gy doses (total 15 Gy) at different time intervals. The lung tumor incidence with irradiation at 12 hr intervals was significantly ($p < 0.01$) higher (40.6%) than that with a single 15 Gy irradiation (*i.e.* 0-hr interval).

The dose response relationships of lung tumor induction after four fractionated doses are next compared. As shown in Fig. 5, the lung tumor incidence after 4 equal doses with a week-interval linearly increased and reached the maximum (36%) at a total dose of 20 Gy. As

Fig. 3. Diameters of lung-tumors induced by thoracic single-dose X-irradiation. Data are the mean $\pm$ SE for mice irradiated in the daytime (open circle), mice irradiated at night (closed circle) and unirradiated controls (shaded areas).

Fig. 4. Lung-tumor incidence after two fractionated thoracic 7.5 Gy X-irradiations at various time intervals. Data are the mean $\pm$ SD for irradiated mice (closed circle) and unirradiated controls (shaded areas).

compared to the incidence (47%) with a single (7.5 Gy) thoracic and subsequent three (3×3 Gy) WBI, four fractionated thoracic irradiations resulted in lower lung tumor incidence. In contrast, the mean tumor diameter at 10–20 Gy after four fractionated thoracic irradiations was greater than that of combinations of thoracic and whole-body irradiations (Fig. 6). For the multiplicity of tumors, more than 30 percent of tumor-bearing mice had 2 or 3 tumors in the group given WBI, while in other groups including single or fractionated irradiation, most of the tumor-bearing mice ($>90\%$) had a single tumor.

Cell kinetics: Changes in the labeling index of the lung cells are shown in Fig. 7 as a function of time of pulse-labeling. As few cells are in flux through the cell cycle in the normal lung, the indices were very low and

Fig. 5. Lung-tumor incidence after four equal fractionated thoracic X-irradiations with a 1 week interval (closed circle), or a single thoracic 7.5 Gy irradiation and subsequent 3 Gy WBI three times at 3 month intervals (open circle). Data are the mean $\pm$ SD for irradiated mice and unirradiated controls (shaded areas).

Fig. 6. Diameters of lung tumors induced in the same experimental groups as in Fig. 5. Data are the mean $\pm$ SE for irradiated mice and unirradiated controls obtained in experiment I (shaded areas). Note: The data with no error for unirradiated mice originated in a single tumor.

little change was observed (closed symbols). The labeling index was, however, lower in the daytime than at night, and statistically significant differences ($p < 0.05$) were observed between 12:00 or 16:00 and 24:00 (data not shown). To clarify the diurnal variations in the labeling index, the mice were irradiated with a 5 Gy X-ray dose and significant changes were expected (Fig. 7). The indices 20 days after a single dose of 5 Gy (open symbols) at 4:00, 8:00, 20:00 and 24:00 were significantly higher than that at 12:00 ($p < 0.05$).

Fig. 7. Labeling index for lung cells as a function of time of day. Data are the mean $\pm$ SD for untreated mice at 11 weeks of age (closed circle) and irradiated mice 20 days after a single thoracic 5 Gy irradiation. The dark periods are shown as shadow bars on the x-axis.

DISCUSSION

There are many differences between mouse strains in the spontaneous lung tumor incidence. The spontaneous incidence is relatively higher in SAS/4 [5], RFM/Un [18], and A/He [13] male mice and low in C3H/He and C57BL/6J male mice [13]. As for radiation induction of lung tumors, SAS/4 male mice are more sensitive than female mice [5]. A/He male mice are more sensitive in radiation-induced lung tumors than C57BL/6J male mice. The sensitivity of C3H/He male mice is halfway between those of A/He and C57BL/6J male mice [13]. Thus, C3H/He male mice have a low spontaneous incidence and are susceptible to the induction of lung tumors by radiation. C3H/He male mice were therefore chosen for this study.

Lung tumor incidence after a single thoracic X-irradiation in the daytime increased in a dose-dependent manner and reached its maximum at doses between 5 and 10 Gy, but, together with the mean diameter, it decreased at doses greater than 10 Gy, suggesting an overdose in our model of lung tumor induction.

In the cell renewal organs such as the skin, intestines, tongue and esophagus, diurnal variations in proliferative activities have been reported [4, 6, 8, 12, 14, 15]. Although the lungs have not generally been recognized as cell renewal organs, the higher proliferative activity at night suggested that there could also be a diurnal variation in the proliferative activity of the lungs during the period of nocturnal activity in mice. Some reports described a cell-cycle dependent tumorigenicity in inducing skin tumor with alkylating agents [3, 11], which showed that high tumor induction was observed when carcinogenic treatment was carried out at a time when many cells were in the late G_1 phase or in the early S phase. Since the majority of lung cells are in the static stage and a few cells

are switched in the flux of cells through the S phase, the change in the labeling index of lung cells after pulse-labeling could reflect the number of static cells switched in the S phase, rather than a synchronized cell population in flux throughout the cell cycle. The high labeling index at night would therefore possibly mean that many lung cells pass through the G₁-S phase, in which they are radiosensitive in both cell-killing and tumor induction. In fact, lung tumors were more effectively induced by night-irradiation at a lower dose (1.25 Gy) than by day-irradiation. Maximum tumor incidence following night-irradiation was, however, almost comparable with that following day-irradiation (Figs. 1 & 2) and was less than 30% with both irradiation regimens.

The reasons for bell-shaped dose-response curves obtained by single irradiations would be a certain competition between the inductive and suppressive effects of radiation that have been reported [5, 7, 18]. The cell-killing was thought to cause the suppressive effects at higher doses [1, 10]. Since such suppressive effects due to higher doses of radiation can be reduced by split doses [9], the total 15 Gy dose was divided into two equal doses. The lung tumor incidence after fractionated irradiation twice at 7.5 Gy with a 12 hr interval was significantly higher than that after a single 7.5 Gy or 15 Gy irradiation. The incidences after fractionated irradiations with any other time-intervals were not different from that after a single irradiation (Fig. 4). Although the role of suppressive effects in radiation-induced tumors might be complicated, higher incidences could be achieved by split doses. As additional evidence of suppressive effects, exposure to four fractionated equal doses at 1-week intervals was more effective in inducing lung tumors at an optimal total dose of 20 Gy. Four equal doses were also relatively effective in inducing larger lung tumors in the dose range lower than a total of 20 Gy (Fig. 6), but not in inducing a higher incidence than single irradiation followed by WBI (Fig. 5).

The highest incidence was obtained with combinations of a single thoracic irradiation and subsequent fractionated WBI three-times (Fig. 5). Although no evidence has been reported showing a relationship between lung tumor induction and immunosuppression, WBI should suppress an immune system other than that in lung tissue [16]. The so called host immunosurveillance mechanisms in tumor induction proposed by Burnet (1970) may partly explain the increased incidence caused by WBI [2]. In fact, the multiplicity of tumors also increased in the group given WBI.

In conclusion, despite a much lower spontaneous lung tumor incidence in C3H/He strain mice, fractionated thoracic irradiation with variations in time-intervals more effectively induced lung tumors (3- to 4-fold of spontaneous incidence). Furthermore, the combination of thoracic and fractionated whole-body irradiations significantly increased the multiplicity as well as lung tumor incidence. Our lung tumor model would be useful in investigating the mechanism of radiation carcinogenesis.

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