Modulation of the growth of pulmonary tumour colonies in mice after single or fractionated low-level irradiations with X-rays

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Introduction

Exposures to low doses of ionizing radiation, i.e., to those which in case of an acute irradiation do not exceed 0.2 Gy [48], may stimulate detoxification and repair mechanisms in cells leading to the reduction of DNA damage even below the spontaneous level decreasing thereby the probability of neoplastic transformation [42]. On the other hand, such exposures may enhance immune reactions of the organism and attenuate harmful effects of higher doses of radiation. These mechanisms may at least partially explain a number of epidemiological observations indicating that cancer incidence and mortality are not elevated among inhabitants of the high- vs. low-background radiation areas [19, 20, 23, 33, 52] as well as among tenants of homes with elevated levels of radiation from $^{222}$Ra or $^{60}$Co [8–10, 49, 51]. Also, in many cohorts of nuclear workers and in the survivors of the Hiroshima and Nagasaki bombings who absorbed doses below 0.25 Gy incidence of leukaemia and some solid tumours has been reported to be lower than in the respective control groups [4, 7, 22, 26, 28, 33, 35, 41]. Moreover, accumulating evidence from animal studies demonstrates that exposures to low doses of X- or γ-rays are associated with the reduced
cancer rate and increased latency of spontaneous lymphomas and leukemias in the irradiated subjects [5, 6, 13, 14, 18, 19, 21, 30, 37, 38, 47, 50]. Importantly, most of the above anti-neoplastic effects were detectable after irradiations of whole bodies of the hosts indicating that stimulation of the tumour surveillance immune mechanisms comes into play [2, 5, 13, 14, 17, 21, 24, 45]. These mechanisms rely on the activities of natural killer (NK) cells, cytotoxic T lymphocytes (CTL) and activated macrophages (MΦ) whose functions are mediated by a variety of cytolytic factors and cytokines [3, 11, 15, 16, 25, 31, 32, 39, 40, 43, 44]. In view of this, the aim of the present investigation was to seek a possible correlation between the tumour-inhibitory effect of single or fractionated low doses of X-rays and the cytotoxic activities of NK cells and macrophages.

**Material and methods**

**Animals and irradiation**

Male BALB/c mice were obtained from the Nofer Institute of Occupational Medicine, Łódź, Poland, and at 6–8 weeks of age were used for the experiments. Whole bodies of the animals were exposed to: a) single irradiation from the HS320 Pantak X-ray generator (230 kV, 20 mA) supplied with the Al and Cu filters, at 2.2 Gy/h dose rate to obtain the absorbed doses of 0.1, 0.2 or 1.0 Gy per mouse, or b) fractionated (5 days/week for 2 weeks) irradiation from the ANDREX X-ray generator (150 kV, 3 mA) at 2.16 Gy/h dose rate to obtain the absorbed doses of 0.01, 0.02 or 0.1 Gy per mouse per fraction, so that total absorbed doses per mouse equalled to 0.1, 0.2 or 1.0 Gy, respectively. Control mice were sham-exposed (generator at the off-mode) in identical conditions. The absorbed doses were verified using thermoluminescent dosimeters implanted subcutaneously (s.c.) in the middle abdominal region. All mice were maintained under specific pathogen-free conditions. The investigations were carried out by permission of the Local Ethical Committee for Experimentation on Animals at the National Institute of Public Health in Warsaw.

**Tumour cells**

L1 sarcoma cells were used for the induction of pulmonary tumour nodules and as targets in the macrophage-mediated cytotoxicity assay. YAC-1 lymphoma cells were used as targets in the NK cell-mediated cytotoxicity assay.

The cells were maintained in the culture medium (CM): RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at 37°C, 5% CO₂.

**Tumour colony assay**

To obtain the cells for the assay, 14 days after s.c. transplantation of 10⁶ L1 cells to a BALB/c mouse the developed tumour was removed, minced, and incubated for 30 min at room temperature with 0.25% trypsin-EDTA and a standard DNase I enzyme solution. After that, the cells were washed and resuspended in CM to give 10⁶ cells/ml. Two hours after the irradiation, mice were intravenously (i.v.) injected with 2.5 × 10⁶ L1 cells/mouse. Fourteen days later the animals were killed, their lungs injected with India ink, and visible colonies on the lung’s surface were counted.

**Preparation of the NK cell-enriched suspension**

On the selected days after the irradiation of mice spleens were removed, minced and the obtained single-cell suspensions were incubated on glass for 40 min at 37°C, 5% CO₂. Non-adherent cells were then collected, passed through a nylon wool column and the wool non-adherent cells were recovered to obtain the NK-enriched cell suspension.

**Harvest of peritoneal macrophages**

Two days before the macrophage collection, mice were intraperitoneally (i.p.) injected with 1 ml of 10% Sephadex G-25. Peritoneal macrophages were collected on the selected days after the irradiation by vigorous washing of the peritoneal cavity with cold CM. Following the incubation on glass for 2 h at 37°C, 5% CO₂, adherent cells were harvested and re-suspended in CM.

**NK cell-mediated cytotoxicity assay**

The NK cell-enriched splenocytes were mixed with the ⁵¹Cr-labelled YAC-1 cells at 100:1 effector-to-target (E:T) ratio and incubated for 4 h. Radioactivity released into the supernatant was measured in a γ-counter and the rate of cytotoxic activity was calculated using the formula: % cytotoxicity = [(experimental release – spontaneous release)/(maximum release – spontaneous release)] × 100.

**Macrophage-mediated cytotoxicity assay**

The collected peritoneal macrophages were mixed with the [³H]thymidine-labelled L1 cells at E:T = 20:1 and incubated in CM supplemented with 50 U/ml IFN-γ and 100 ng/ml LPS. Forty-eight hours later adherent cells were lysed with 0.1 ml of 0.1 N KOH. The lysates were harvested and the radioactivity was measured in a β-counter. The rate of cytotoxic activity was calculated using the formula: [(A – B)/A] × 100, where A is the number of disintegrations per minute (DPM) in the target cells cultured alone and B is the DPM in the assay culture.

**Suppression of the NK cell-mediated activity in vivo**

One day before the irradiation mice were i.p. treated with the anti-asialo GM₁ antibody (20 μl Ab in 0.5 ml
PBS per mouse). Two or 14 days later the animals were used for the assessment of the NK cell-mediated cytotoxicity or the numbers of the pulmonary tumour colonies, respectively.

**Suppression of the macrophage-mediated activity in vivo**

One day before the irradiation mice were i.p. treated with carrageenan (CGN, 4 mg in 0.4 ml PBS per mouse) and three or 14 days later assayed for the cytotoxic activity of peritoneal macrophages or the number of the pulmonary tumour colonies, respectively.

**Statistical analysis**

For the assessment of the differences, Mann-Whitney U test for non-parametric trials was used and \( p < 0.05 \) was regarded as significant.

**Results**

Figure 1 shows the rates of the pulmonary tumour colonies (expressed as percentages of the control values obtained in the sham-exposed animals) that grew in mice after the single WBI with various doses of X-rays. As indicated in all the four separate experiments irradiation with 0.1 or 0.2 Gy led to the significant inhibition of the development of the colonies. In contrast, in most of the trials, no statistically significant reduction in the number of pulmonary tumour nodules could be detected when mice were pre-exposed to 1.0 Gy X-rays.

As shown in Fig. 2, the two separate experiments indicated that the fractionated WBI of mice with both 0.1 and 0.2 Gy X-rays resulted in the insignificant retardation of the development of pulmonary tumour colonies (expressed as percentages of the control values obtained in the sham-exposed animals), whereas irradiation of mice with 1.0 Gy led to the slight increase in the number of the colonies.

![Fig. 1. Relative numbers (percentages of the control values indicated as solid line at 100%) of pulmonary L1 sarcoma cell colonies in mice single exposed to 0.1, 0.2 or 1.0 Gy X-rays and two hours later i.v. injected with L1 sarcoma cells. Data are mean values ± SD. Results of four independent experiments are shown: each experimental group consisted of 12 mice. * – indicates statistically significant \((p < 0.05)\) difference from the control (100%) value.](image1)

![Fig. 2. Relative numbers (percentages of the control values indicated as solid line at 100%) of pulmonary L1 sarcoma cell colonies in mice exposed to fractionated 0.1, 0.2 or 1.0 Gy X-rays and two hours later i.v. injected with L1 sarcoma cells. Data are mean values ± SD. Results of two independent experiments are shown: each experimental group consisted of 12 mice.](image2)

![Fig. 3. Cytotoxic activity of NK cells tested on various days after the single (A) and fractionated (B) WBI. C – sham-exposed mice; 0.1 Gy – mice exposed to WBI with 0.1 Gy X-rays; 0.2 Gy – mice exposed to WBI with 0.2 Gy X-rays; 1.0 Gy – mice exposed to WBI with 1.0 Gy X-rays. Mean values ± SD obtained from three independent experiments are presented; each experimental group consisted of five mice.](image3)
exposures to either 0.1 or 0.2 Gy X-rays. Fractionated WBI of mice with either of the three applied doses of X-rays led to the significant enhancement of the cytotoxic function of NK cell-enriched spleen cells to the comparable level in all the three groups (Fig. 3B).

As shown in Fig. 4A a single WBI of mice with either 0.1 or 0.2 Gy X-rays led to the significant elevation of the cytotoxic activity of the IFN-γ- and LPS-stimulated peritoneal macrophages against the L1 tumour targets on the third day post-exposure to X-rays compared to the activity of these cells obtained from both the sham-irradiated and 1.0 Gy exposed mice. Macrophages collected from mice pre-injected with CGN were significantly less cytotoxic against the L1 cells in vitro than macrophages obtained from the CGN-untreated animals in all the examined groups (Table 1).

A single WBI of mice with either 0.1 or 0.2 Gy X-rays resulted in the significant boosting of the cytotoxic function of the NK cell-enriched lymphocytes obtained from the spleens of mice two days after the exposure to X-rays compared to the activity of NK splenocytes obtained from the control, sham-irradiated mice. When mice were injected with anti-asialo GM₁ antibody, the activity of these cells tested two days later was totally abrogated and this inhibition could not be reversed by WBI with 0.1, 0.2 or 1.0 Gy X-rays (Table 1).

Irradiation with each of the applied doses of X-rays resulted in the significant boosting of the cytotoxic function of the NK cell-enriched lymphocytes obtained from the spleens of mice two days after the exposure to X-rays compared to the activity of NK splenocytes obtained from the control, sham-irradiated mice. When mice were injected with anti-asialo GM₁ antibody, the activity of these cells tested two days later was totally abrogated and this inhibition could not be reversed by WBI with 0.1, 0.2 or 1.0 Gy X-rays (Table 1).

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Table 1. Cytotoxic activity of the NK cell-enriched splenocytes and peritoneal macrophages on the second and third day, respectively, after irradiation of mice with 0.1, 0.2 or 1.0 Gy X-rays

<table>
<thead>
<tr>
<th>Group</th>
<th>NK-enriched splenocytes</th>
<th>Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>Ab</td>
</tr>
<tr>
<td>Control</td>
<td>7.2 ± 1.1</td>
<td>0.5 ± 0.1*</td>
</tr>
<tr>
<td>0.1 Gy</td>
<td>11.1 ± 1.5*</td>
<td>0.3 ± 0.1*</td>
</tr>
<tr>
<td>0.2 Gy</td>
<td>11.4 ± 1.2*</td>
<td>0.3 ± 0.1*</td>
</tr>
<tr>
<td>1.0 Gy</td>
<td>14.2 ± 1.5*</td>
<td>0.5 ± 0.1*</td>
</tr>
</tbody>
</table>

Control – sham-exposed mice; 0.1 Gy – mice exposed to a single WBI with 0.1 Gy X-rays; 0.2 Gy – mice exposed to a single WBI with 0.2 Gy X-rays; 1.0 Gy – mice exposed to a single WBI with 1.0 Gy X-rays; PBS – mice i.p. injected with phosphate buffered saline; Ab – mice i.p. injected with anti-asialo GM₁ antibody; CGN – mice i.p. injected with CGN. Presented are means ± SD from three independent experiments; each experimental group consisted of at least three mice. * – indicates statistically significant (p < 0.05) difference from the control/PBS value.

Discussion

The results of the present study indicate that development of the pulmonary tumour colonies in mice i.v. injected with L1 sarcoma cells is significantly inhibited after a single whole-body irradiation with 0.1 or 0.2 Gy of X-rays as compared to the sham-exposed as well as 1.0 Gy-irradiated mice. Similarly, mice exposed to each
of the fractionated low doses of X-rays tended to have less induced tumour metastases than the sham-exposed as well as 1.0 Gy-irradiated mice. These observations corroborate the findings of Hosoi and Sakamoto [14] who detected a marked inhibition of both artificial and spontaneous pulmonary metastases in mice inoculated with tumour cells a few hours before or after exposure to 0.15, 0.2 and 0.5 Gy X-rays. Likewise, significant reduction in the number of pulmonary tumour nodules was reported by Ju et al. [21] and Cai [5] who irradiated mice with single doses of X-rays ranging from 0.05 to 0.15 Gy 24 h before the i.v. injection of B16 melanoma or Lewis lung cancer cells. Decreased incidence of lung and lymph node metastases was also reported by Hashimoto et al. [13] who exposed rats to 0.2 Gy of γ-rays 14 days after s.c. implantation of hepatoma cells; the same dose, however, neither reduced the number of metastases after local irradiation of the primary tumour nor affected the in vitro growth of the tumour cells irradiated in the culture medium. Recently, Sakai et al. [47] reported that protracted irradiation of mice with γ-rays for over 250 days attenuated the growth of the 20-methylocholantrene-induced tumours. These results collectively suggest that the inhibitory effect of low doses of low-LET radiation on the development of metastases may result from the stimulation of anti-cancer immune mechanisms of the host rather than from the direct reduction of proliferation and/or viability of cancer cells.

Natural killer (NK) cells and activated macrophages are primary effectors of the non-specific anti-tumour surveillance system [1, 3, 11, 31, 39, 40]. Hence, it was interesting to note in the present study that the activity of NK cell-enriched splenocytes obtained from mice exposed to each of the three doses of X-rays applied both as single and fractionated treatment was significantly elevated compared to the counterpart cells collected from the control, sham-irradiated animals. Similar stimulation was also described by Liu et al. [31] 24 h after a single exposure of mice to 0.075 or 0.5 Gy X-rays and by Kojima et al. [24, 25] who detected similar effect between fourth and sixth hour after a single irradiation of mice with 0.5 Gy γ-rays. Other authors [21], however, showed the enhanced cytoidal function of murine lymphocytes 2–6 days after the single exposures of the animals to 0.075 Gy X-rays. Moreover, our present results indicate that both single and fractionated exposures of mice to 0.1 or 0.2 Gy X-rays led to the statistically significant stimulation of cytolytic activity of macrophages. The fractionated exposures of the animals to 1.0 Gy also stimulated the cytotoxic activity of macrophages, whereas this function was not affected by a single irradiation of mice with 1.0 Gy X-rays. The present results support and supplement earlier studies of Ibuki and Goto [15, 16] who reported similar stimulation of the cytotoxicity of the IFN-γ- and LPS-treated peritoneal macrophages which were assayed already on the day of exposure. These authors, however, employed a markedly smaller dose of radiation (0.04 Gy γ-rays) than the ones applied in the present investigation and used different tumour cells (P815 mastocytoma cells) as targets for cytotoxic macrophages.

Interestingly, in our study the activity of NK cell-enriched splenocytes was also markedly stimulated by a single irradiation with 1.0 Gy X-rays, the dose that did not lead to inhibition of the growth of the pulmonary tumour nodules. However, stimulatory effect of this dose of X-rays on the activity of NK cell-enriched spleen cells could be partially explained by the possible elimination of radiosensitive T and B cells from the spleen leading to the relative increase in the percentage of the NK effectors in the cytotoxic assay (data not shown). In fact, as indicated by Lin et al. [27] and Harrington et al. [12] NK cells appear to exhibit the greatest radioresistance among the splenic lymphoid cells. On the other hand, a single irradiation of mice with 1.0 Gy X-rays did not affect the cytoidal function of macrophages collected from the animals. These results suggest that the enhanced activity of macrophages may, to a large extent, account for the tumour-inhibitory effect of single exposures to low doses of X-rays.

In the present study i.p. injection of both the anti-asialo GM₁ antibody and CGN suppressed the cytolytic activity of NK cell-enriched splenocytes and macrophages and abrogated the differences between the numbers of the pulmonary tumour colonies developed in mice exposed to single irradiations with 0.1, 0.2 and 1.0 Gy X-rays. These results suggest that stimulation of the NK cell- and macrophage-mediated activities was responsible for the retardation of the development of tumour metastases by the low doses of X-rays. Notably, injection of CGN appeared to be a more potent suppressor of the anti-neoplastic effect of the low-level exposures to X-rays than the anti-asialo GM₁ antibody. This observation may be explained by the possible suppression by CGN of the cytotoxic functions of both macrophages and NK cells. Indeed, Minaarovits et al. [36] demonstrated that concurrent application of the inhibitors of NK cells and macrophages promoted tumour growth in mice transplanted with the SP94
adenocarcinoma and BaF1 fibrosarcoma cells to the same extent as did the sole injection of CGN. Moreover, several cytokines produced by macrophages (e.g., IL-12 and IL-18) are potent modulators of the activity of NK lymphocytes [53] and suppression of the activity of the former may compromise the function of the latter cells.

Interestingly, in the present investigation fractionated exposures of mice to all three doses of X-rays resulted in a similar stimulation of the cytotoxic activities of the NK-enriched splenocytes and peritoneal macrophages. The lack of the difference in the effect of low (0.1 and 0.2 Gy) vs. higher (1.0 Gy) total doses of radiation applied in ten fractions suggests that absorption of the consecutive doses of 0.1 Gy (total dose of 1.0 Gy) separated by the 24-h intervals leads to the similar up-regulation of the cytolytic function of the two cell populations as absorption of the smaller fractions of each of the total low dose of X-rays. Although no directly comparable data are available, Safwat showed [45, 46] that splitting a higher dose into several fractions results in the enhanced mitogen-induced proliferative response of T lymphocytes derived from peripheral blood of lymphoma patients which is comparable to that caused in murine T lymphocytes by a single exposure of mice to a low dose of X-rays [29, 31]. Since, in our hands, a significant stimulation of the macrophage- and NK cell-mediated cytotoxicities by a single irradiation of mice with 0.1 or 0.2 Gy X-rays was accompanied by a markedly lower number of the artificial tumour colonies in the lungs the inability of the fractionated exposures to the two low doses to significantly compromise the development of these colonies is difficult to explain. Apparently, more studies using larger numbers of animals are needed to clarify the relationship between the cytocidal functions of NK cells and macrophages activated by fractionated low doses of X-rays and the growth of tumours in mice exposed to such irradiation protocols.

In conclusion, our present results suggest that suppression of the development of pulmonary tumour colonies by single or fractionated irradiations of mice with low doses of X-rays may result from stimulation of the natural anti-tumour defence reactions mediated by NK cells and/or cytotoxic macrophages. It remains to be explored in future studies the possible mechanistic differences underlying the end-results of the single vs. fractionated exposures to X-rays and/or whether other immune cells and reactions are also involved in the tumour-suppressive effect of the low-dose irradiations with low-LET radiation.

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