Enhanced survival of lethally irradiated adenosine A₃ receptor knockout mice. A role for hematopoietic growth factors?

Michal Hofer · Milan Pospíšil · Ladislav Dušek ·
Zuzana Hoferová · Denisa Komůrková

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Abstract Adenosine A₃ receptor knockout (A₃AR KO) mice and their wild-type (WT) counterparts were compared from the point of view of their abilities to survive exposures to lethal doses of γ -radiation belonging to the range of radiation doses inducing the bone marrow acute radiation syndrome. Parameters of cumulative 30-day survival (experiment using a middlelethal radiation dose) or cumulative 11-day survival (experiment using an absolutely lethal radiation dose), and of mean survival time were evaluated. The values of A₃AR KO mice always reflected their higher survival in comparison with WT ones, the *P* values being above the limit for statistical significance after the middlelethal radiation dose and standing for statistical significance after the absolutely lethal radiation dose. This finding was considered surprising, taking into account the previously obtained findings on defects in numbers and functional properties of peripheral blood cells in A₃AR KO mice. Therefore, previous hematological analyses of A₃AR KO mice were supplemented in the present studies with determination of serum levels of the granulocyte colony-stimulating factor, erythropoietin, and thrombopoietin. Though distinct differences in these parameters were observed between A₃AR KO and WT mice, none of them could explain the relatively high postirradiation survival of A₃AR KO mice. Further studies on these mice comprising also those on other than hemopoietic tissues and organs can help to clarify their relative radioresistance.

Keywords Adenosine A₃ receptor · Postirradiation survival · Hematopoiesis · Granulocyte colony-stimulating factor · Erythropoietin · Thrombopoietin

Introduction

Adenosine, a naturally occurring nucleoside, was stated to possess receptor-mediated regulatory activities representing a universal intercellular communication system [1]. Signaling through adenosine membrane receptors was found to modulate cell proliferation, differentiation, and apoptosis [e.g., 2–4]. Adenosine receptors exist in four types, denominated as A₁, A_{2a}, A_{2b}, and A₃ [5].

In the recent years, an extensive amount of information was gathered on hematopoiesis-stimulating effects of agonists of adenosine A₃ receptors (A₃AR) [for review see 6]. These effects were observed in normal and irradiated mice in all hematopoietic compartments with the exception of those of hematopoietic stem and multipotent progenitor cells [7], and considered for therapeutical applications in man [8].

For a better understanding of the mechanisms of the abovementioned findings, the attention of our laboratory has recently been focused on the experimental object of adenosine A₃ receptor knockout (A₃AR KO) mice. These mice have been used previously in several studies aimed predominantly at studying the role of A₃AR in the nervous and cardiovascular systems [e.g., 9–13], but their use for the study of hematological regulation processes has been neglected.

The experiments performed until now have provided two main pieces of knowledge: First, A₃AR KO mice possess defects in the counts and/or functional parameters of mature peripheral blood cells (neutrophils, monocytes, erythrocytes, and platelets) [14–16]. Second, these mice show significantly higher values of their bone marrow parameters (femoral bone marrow cellularity, granulocyte/macrophage progenitor cells

M. Hofer (✉) · M. Pospíšil · Z. Hoferová · D. Komůrková
Department of Molecular Cytology and Cytometry, Institute of
Biophysics, v.v.i., Academy of Sciences of the Czech Republic,
Královopolská 135, 61265 Brno, Czech Republic
e-mail: hofer@ibp.cz

L. Dušek
Institute of Biostatistics and Analyses, Faculty of Medicine, Masaryk
University, Kamenice 126/3, 62500 Brno, Czech Republic

(GM-CFC), and erythrocyte progenitor cells (BFU-E)), explainable probably by the efforts of the hematopoietic system to overcome possible problems arising from peripheral blood cell defects [15, 16].

These results have still left unanswered numerous questions concerning the role of A₃AR in hematopoiesis. One of them is the question of whether the defects observed in the compartment of the peripheral blood cells of A₃AR KO mice represent a real threat to the organism as a whole. To uncover at least partially this issue, we have performed experiments in which A₃AR KO mice and their wild-type (WT) counterparts were exposed to lethal doses of ionizing radiation and their survival was monitored. Furthermore, we have supplemented our previously obtained hematological findings in A₃AR KO mice with data on serum levels of three important hematopoietic growth factors having generally a profound influence on the processes of production and maturation of blood cells, namely on those of the granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO), and thrombopoietin (TPO) in both nonirradiated and irradiated animals. The serum G-CSF, EPO, and TPO levels might, together with the previously observed findings on hematopoiesis in the A₃AR KO mice, help to evaluate their postirradiation survival following exposure to radiation doses inducing the bone marrow syndrome of the acute radiation disease.

Materials and methods

Mice

A₃AR KO (Adora3^{tm1Jsb}/Adora3^{tm1Jsb}) mice, backcrossed onto a C57BL/6 background [17], were obtained from Merck Research Laboratories (West Point, PA, USA) and bred in the Laboratory Animal Breeding and Experimental Facility of the Faculty of Medicine, Masaryk University, Brno, Czech Republic. WT C57BL/6 mice were obtained from the same Laboratory Animal Breeding and Experimental Facility of the Faculty of Medicine, Masaryk University, Brno, Czech Republic. The mice were kept in controlled conditions; standardized pelleted diet and HCl-treated tap water were available *ad libitum*. For material sampling, 2.5-month-old mice were used. The use and treatment of the animals followed the European Community Guidelines as accepted principles for the use of experimental animals. The experiments were carried out with the approval of the Institute's Ethical Committee.

Irradiation

The mice were whole-body irradiated at a dose rate of 0.41 Gy/min using a γ -ray source (⁶⁰Co, Chisostat,

Chirana, Prague, Czech Republic). Single doses of 8.5 or 9.0 Gy were used for the postirradiation survival studies, and a single dose of 4.0 Gy was used in the experiments aimed at determination of serum hematopoietic growth factor levels.

Postirradiation survival

In the experiment using the 8.5 Gy dose, survival of the experimental male mice was recorded daily up to day 30 after irradiation. In the experiment using the 9.0 Gy dose, the survival was recorded daily up to day 18 after irradiation when all mice had died.

Determination of the granulocyte colony-stimulating factor, erythropoietin, and thrombopoietin (TPO) serum levels

The animals were anesthetized intraperitoneally with Narkamon (ketamine)/Rometa (xylazine) solution (5 % Narkamon and 2 % Rometa [both Spofa, Prague, Czech Republic]) in a ratio of 2.63:1, and the blood was sampled by cardiac puncture. Concentrations of granulocyte colony-stimulating factor (G-CSF), erythropoietin (EPO), and thrombopoietin (TPO) in mouse serum were determined using ELISA kits (R&D Systems, Minneapolis, MN, USA). For the determination of G-CSF serum levels, female mice were used, and for that of EPO and TPO, male mice were utilized. The assays had sensitivities (as stated by the manufacturer) of typically less than 5 pg/ml for G-CSF, ranging from 6.5 to 46.9 pg/ml for EPO, and typically less than 20 pg/ml for TPO.

Statistics

Comparisons of the numbers of surviving mice in individual experimental groups by day 30 (experiment using the 8.5 Gy radiation dose) and by day 11 (experiment using the 9.0 Gy radiation dose) were performed using a Fisher exact test. Day 11 was set as the evaluation time for the experiment using the 9.0 Gy radiation dose because it was the last day on which at least one mouse was alive in each experimental group.

The analysis of the mean survival time was carried out by Kaplan-Meier methodology. Estimates of the mean survival time were derived from the Kaplan-Meier curve. Differences in the mean survival time between individual experimental groups were tested by a log-rank test.

For statistical comparisons of the differences in serum G-CSF, EPO, and TPO levels between the groups, the Mann-Whitney *U* test was applied.

The significance level was set at $P < 0.05$.

The analyses were computed using SPSS 19.0.1 (IBM Corporation, 2010) and Statistica 9.1 (Statsoft, 2010).

Results

In the first survival experiment, A₃AR KO mice and their WT counterparts were exposed to a midlethal dose of 8.5 Gy of γ -rays. Their survival curves are shown in Fig. 1. By day 30 after irradiation, the cumulative percentage survival was higher in A₃AR KO mice (46.2 %) in comparison with the WT mice (23.1 %). This difference lay slightly below the level of statistical significance ($P=0.060$). The mean survival time was also higher in A₃AR KO mice (23.6 days) than in their WT counterparts (18.6 days), but this difference was not statistically significant ($P=0.411$).

In the second survival experiment, an absolute lethal dose of 9.0 Gy of γ -rays was applied to the mice. Their survival is illustrated in Fig. 2. The survival of A₃AR KO mice was again better when compared with the WT ones. Since all WT mice died by day 12 after irradiation and all A₃AR KO mice died by day 18, the time interval for evaluation of their cumulative percentage survival was arbitrarily set at day 11 (the last day on which at least one mouse from each group was still alive). On day 11, the cumulative percentage survival was 57.1 % in the group of A₃AR KO mice, that in WT mice amounted to 9.5 % ($P=0.001$). The mean survival time of A₃AR KO mice was 12.3 days, that of WT mice was 8.9 days and, thus, death in the A₃AR KO group was significantly ($P=0.003$) delayed.

Determinations of the serum levels of G-CSF, EPO, and TPO were performed in nonirradiated as well as in sublethally (4.0 Gy of γ -rays) irradiated A₃AR KO and WT mice. As regards G-CSF (Fig. 3), its mean serum concentration in nonirradiated WT mice was higher (183 ± 39 pg/ml) than in nonirradiated A₃AR KO mice (107 ± 7 pg/ml), with the P value approaching the limit for statistical significance ($P=$

Fig. 1 Postirradiation survival of adenosine A₃ receptor knockout (A₃AR KO) male mice and their wild-type (WT) counterparts following a midlethal γ -ray dose of 8.5 Gy. Number of animals per group: 13

Fig. 2 Postirradiation survival of adenosine A₃ receptor knockout (A₃AR KO) male mice and their wild-type (WT) counterparts following an absolutely lethal γ -ray dose of 9.0 Gy. Number of animals per group: 21

0.053). On day 3 after irradiation, the G-CSF serum levels were found to be statistically significantly higher in the WT mice (399 ± 21 pg/ml) in comparison with the A₃AR KO ones (316 ± 18 pg/ml) ($P=0.011$). By day 6 after irradiation, the mean value of serum G-CSF was only slightly and non-significantly different when comparing A₃AR KO and WT mice (257 ± 57 vs. 213 ± 19 pg/ml, $P=0.429$).

When evaluating the EPO serum concentrations, we found that the relationship between A₃AR KO mice and their WT counterparts was different if nonirradiated or irradiated mice were taken into account. In nonirradiated mice, the EPO serum levels were observed to be significantly higher in A₃AR KO animals (860 ± 230 pg/ml) than in WT ones (183 ± 11 pg/ml) ($P<0.001$). In both the postirradiation time intervals of days 3 and 6, the situation was opposite, with significantly higher EPO serum concentrations in WT mice when compared with A₃AR KO animals (1415 ± 54 vs. 1255 ± 38 pg/ml, respectively, $P=0.025$, for day 3; 2448 ± 119 pg/ml vs. 1311 ± 187 pg/ml, respectively, $P=0.002$, for day 6) (Fig. 4).

The recorded serum levels of TPO in both A₃AR KO mice and their WT counterparts were low (between 7 and 20 pg/ml) and fluctuated in the vicinity of the limit for the assay sensitivity (see “Materials and methods”). The differences between the A₃AR KO and WT mice were not significant in any comparison.

Discussion

Taking into account the survival curves for A₃AR KO mice and for the corresponding WT mice obtained in our

Fig. 3 Serum concentration of the granulocyte colony-stimulating factor (G-CSF) in nonirradiated and 4 Gy of γ -rays-irradiated adenosine A_3 receptor knockout (A_3 AR KO) female mice and their wild-type (WT) counterparts; the values are given as means $\pm$ SE; Days—days after

irradiation, KO— A_3 AR KO mice, WT—WT mice; asterisk indicate the pertinent value in WT mice is statistically significantly higher ($P<0.05$) than that in A_3 AR KO mice. Number of animals per group: 10–12

experiments, and the P values computed for comparisons of the cumulative percentage survival and the mean survival times in these groups, it can be stated that the postirradiation survival of A_3 AR KO mice following lethal doses of γ -rays is higher than in their WT counterparts. This finding is rather surprising if previously obtained results on positive effects of

A_3 AR agonists on hematopoiesis [for review see 6], as well as those on the defects in the numbers and/or functional properties of peripheral blood cells in the A_3 AR KO mice [14–16] are considered.

In this connection, it should be mentioned that the Kaplan-Meier survival curves from our experiments match the figure

Fig. 4 Serum concentration of erythropoietin (EPO) in nonirradiated and 4 Gy of γ -rays-irradiated adenosine A_3 receptor knockout (A_3 AR KO) female mice and their wild-type (WT) counterparts; the values are given as means $\pm$ SE; Days—days after irradiation, KO— A_3 AR KO mice, WT—WT mice; asterisks indicate the pertinent values in WT mice are

statistically significantly higher ($P<0.05$, $P<0.01$, respectively) than those in A_3 AR KO mice; number symbol indicates the pertinent value in A_3 AR KO mice is statistically significantly higher ($P<0.001$) than that in WT mice. Number of animals per group: 8–12

of the bone marrow acute radiation syndrome induced to the mice in our survival studies [18]. Thus, it is reasonable to evaluate the results of the survival studies on A_3AR KO mice in the context with the hematological findings in these mice. On the other hand, it cannot be excluded that other, still undescribed parameters of A_3AR KO mice, including the non-hematological ones, positively influence their postirradiation survival.

Concerning the serum G-CSF levels, their significantly higher values in WT mice, either nonirradiated or on day 3 after irradiation with the dose of 4 Gy, can be perceived as being in agreement with the reported findings on the ability of agonists of A_3AR to induce production of this hematopoietic growth factor [19–21, for review see [8, 22] (there exists, however, also our report on unchanged serum G-CSF levels in mice after the administration of IB-MECA, an agonist of A_3AR [23]). IB-MECA was also reported by us not to produce higher GM-CFC numbers in vitro when added to the cultures jointly with G-CSF in comparison with those produced by G-CSF itself [24]. The numbers of neutrophils in the peripheral blood, one of the chief cell types, whose production is stimulated by G-CSF, were reported in one study to be significantly decreased in otherwise uninfluenced A_3AR KO mice [15], whereas in another study, the decrease of their values in these mice was non-significant [14]. Taken together, the research performed to date on the relationships of G-CSF and A_3AR does not provide any clear explanation for the better survival of A_3AR KO mice observed following radiation doses inducing the bone marrow acute radiation syndrome.

To address the issue of the relationships of A_3AR vs. EPO and erythropoiesis, a few remarks should be made. First, we

have found that the gene for A_3AR is expressed on the erythroid bone marrow cells [25], which are thus responsible to the stimulation by its respective agonists. Second, we have also observed that IB-MECA stimulates the proliferation of erythroid progenitor cells (BFU-E) in vitro [26]. On the other hand, there exists a communication by Bhanu et al. [27], who reported inhibitory effects of chloro-IB-MECA (CI-IB-MECA), another A_3AR agonist, on erythropoiesis in an ex vivo culture system of primary human CD34+ cells. Previous studies on A_3AR KO mice have repeatedly shown defects in their peripheral blood erythrocytes comprising decreased values of the mean erythrocyte volume and the mean erythrocyte hemoglobin [14–16]. On the contrary, increased numbers of erythroid progenitor cells (BFU) were found in the femoral bone marrow of these mice [15, 16], which suggested the existence of compensatory efforts of the hematopoietic system of A_3AR KO mice trying to overcome possible functional insufficiencies connected with the defects in mature erythrocytes. When looking at the EPO serum levels in non-irradiated A_3AR KO and WT mice (Fig. 4), it seems that the significantly increased EPO serum concentration in A_3AR KO mice supports the assumption about the ongoing compensatory processes in their erythropoietic system. The reversed situation demonstrating significantly lower EPO serum levels in the irradiated A_3AR KO mice (Figs. 4 and 5) may reflect partial exhaustion of the ability of A_3AR KO mice to further intensify the production of EPO under the postirradiation pressures on hematopoiesis. Nevertheless, the previously determined values of total hemoglobin levels in A_3AR KO mice, which are either approximately equal compared with those in their WT counterparts, or, in some cases, even significantly

Fig. 5 Serum concentration of thrombopoietin (TPO) in nonirradiated and 4 Gy of γ -rays-irradiated adenosine A_3 receptor knockout (A_3AR KO) female mice and their wild-type (WT) counterparts; the values are

given as means $\pm$ SE; *Days*—days after irradiation, *KO*— A_3AR KO mice, *WT*—WT mice. Number of animals per group: 5–6

exceed the values in the WT mice [14–16], bear evidence on a generally good functional state of the erythroid component of hematopoiesis in A₃AR KO mice. In connection with the evaluation of the survival experiments, it should be also noted that, concerning the relatively long life span of mouse erythrocytes of 50 to 55 days [28], the peripheral blood erythrocyte number status does not need to play an essential role during the 30-day duration of the survival experiments.

Our experiments, whose results are presented here, do not suggest any significant participation of TPO in the previously reported defects in the compartment of peripheral blood platelets of the A₃AR KO mice [14–16]. No significant differences between the serum concentrations of TPO between A₃AR KO mice and WT mice were observed in any of the three comparisons made.

It follows from our results and their abovementioned considerations that also other mechanisms than those discussed here up to now can participate in the compensatory responses of the A₃AR KO mice to ionizing radiation. For instance, the expression of other adenosine receptors might be affected in the A₃AR KO mice. However, Salvatore et al. [17] stated that in the A₃AR KO mice, the density of A₁ and A_{2a} adenosine receptor subtypes was the same as in their WT counterparts, and that the A_{2b} receptor transcript expression was not affected by ablation of A₃AR gene. Thus, significant participation of other adenosine receptors in the above-described processes in the A₃AR KO mice is not probable. It should be also mentioned that, to the knowledge of the authors, there exist no other published survival studies on any adenosine receptor knockout mice which could also help to shed light on our findings.

To summarize, no finding from any of the three components of hematopoiesis investigated in previous studies and present experiments (granulocytopoiesis, erythropoiesis, thrombopoiesis) can clearly explain the better postirradiation survival of A₃AR KO mice as reported here in comparison with their WT counterparts. Further studies on A₃AR-lacking mice, including those on their lymphocytic system and other tissues and organs are needed to explain the surprising finding of their relative radioresistance.

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Conflicts of interests The authors declare that there are no conflicts of interests.

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