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Radioprotective Efficacy of Dipyridamole and AMP Combination in Fractionated Radiation Regimen, and Its Dependence on the Time of Administration of the Drugs Prior to Irradiation

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Summary

We have recently demonstrated that the combined administration of dipyridamole and adenosine monophospate to mice induces radioprotective effects in terms of postirradiation haemopoietic recovery in animals irradiated with a single dose. The aim of the present experiments was to investigate the radioprotective ability of the drug combination under conditions of fractionated radiation treatment. It has been shown that administration of drugs either 15 or 60 min before each of the five daily 3-Gy doses of gamma-radiation enhances haemopoietic recovery and survival of mice exposed to an additional "top-up" dose of 3.5 Gy. Furthermore, it has been ascertained that the regimen using administration of the drugs 60 min prior to irradiation is more effective than administration of the drugs 15 min prior to irradiation. Due to the evidence that administration of the drugs 15 min prior to irradiation protects the organism mainly *via* mechanisms of systemic hypoxia while the pretreatment 60 min before irradiation avoids the role of hypoxia and mainly induces cell proliferation effects, our results suggest a more effective protective role of mechanisms stimulating haemopoiesis under conditions of fractionated radiation. The data may provide a basis for more rational use of radioprotection in fractionated radiation regimens.

Key words

Radioprotection – Fractionated irradiation – Haemopoiesis – Adenosine monophosphate – Dipyridamole

Introduction

Our earlier experiments have shown that the elevation of extracellular adenosine by means of the combined administration of dipyridamole, a drug inhibiting the cellular uptake of adenosine, and adenosine monophosphate (AMP), a soluble adenosine prodrug, mediates at least two physiological effects in mice that can be evaluated as radioprotective (Pospíšil *et al.* 1989, 1992, 1993a,b, Hošek *et al.* 1992). Firstly, bradycardia and vasodilation induced by the elevation of extracellular adenosine can lead to short-lasting hypotension and subsequent hypoxia in radiosensitive tissues and exert radioprotective activity *via* the oxygen effect. As defined in radiobiology, the oxygen effect refers to the direct dependence of cell sensitivity to ionizing radiation on the concentration of oxygen (Tubiana *et al.* 1990). Secondly, receptor-mediated action of extracellular adenosine can increase the proliferation of haemopoietic cells and, consequently, enhance postirradiation regeneration of the perturbed haemopoietic tissue and thus exhibit the radioprotective effect.

The present study was conducted to understand the role of the two above-mentioned radioprotective mechanisms of the pharmacological elevation of extracellular adenosine under conditions of fractionated radiation treatment in mice. The fractionated radiation regimen consisted of five fractional irradiations with a dose of 3 Gy given in 24-h intervals. This 24-h fractionation has common features with the standard fractionated schedule used in clinical

radiotherapy. The combination of dipyridamole and AMP was administered before each of the five radiation fractions. Different timings of the preirradiation drug administration were chosen to separate the two proposed radioprotective mechanisms. Based on our earlier experiments (Pospíšil *et al.* 1993a), administration of the drugs 15 min before irradiation was assumed to protect the organism *via* mechanisms of hypoxia, administration of the drugs 60 min before irradiation was considered to avoid the role of hypoxia and mainly to induce cell proliferation. The efficacy of these two protective schemes was compared in terms of haemopoietic recovery and survival of mice.

Material and Methods

Experimental Animals

Three-month-old conventional male (CBAxC57BL/10)F_1 mice with average body weight of 25 g were used. Standardized pelleted diet and HCl-treated tap water (pH 2–3) were given *ad libitum*. The mice were kept under controlled light conditions (L:D 12:12) at a temperature of 22±1 °C.

Irradiation

Mice received total-body doses of gamma-radiation from a ^{60}Co source at a dose rate of 0.3 Gy/min. During the irradiation the mice were placed in ventilated Plexiglas containers. The fractionated regimen consisted of five radiation fractions of 3 Gy given at 24-h intervals. Such a fractionated schedule was found to be sublethal; no deaths of mice were recorded within 30 days after completion of the irradiation regimen. In order to investigate radiation damage in terms of lethality, 24 h after the last fractionated dose the mice were irradiated with an additional "top-up" dose of 3.5 Gy and deaths were recorded daily up to the 30th day after the additional exposure.

Drugs

Protective combination of dipyridamole and adenosine monophosphate was used in doses shown to be effective in our earlier experiments with single radiation exposures (Pospíšil *et al.* 1993a). Dipyridamole (Sigma, USA) was dissolved in 0.4 % tartaric acid and injected subcutaneously at a dose of 2 mg per mouse in a volume of 0.4 ml. Adenosine 5'-monophosphate sodium salt from yeast (Sigma, USA) was dissolved in distilled water and injected intraperitoneally at a dose of 5 mg of free acid per mouse in a volume of 0.2 ml. AMP was injected to mice either 15 min or 60 min before each radiation fraction, dipyridamole was injected always 20 min before AMP. Control animals received injections of tartaric acid (0.4 %) and saline.

Haematological Methods

Endogenous spleen colony-forming units (CFU-S) were counted on day 10 after the completion of the fractionated irradiation. Animals were sacrificed by cervical dislocation, their spleens removed and fixed in Bouin's solution. The macroscopically visible nodules larger than 0.4 mm in diameter were counted (Till and McCulloch 1963). In some experiments the spleens were embedded in paraffin and used for histological analysis. A single midline longitudinal section was considered to be a representative sample of each spleen (Inoue *et al.* 1984) and was used for counting histologically evaluable colonies (more than ten cells were considered to be a colony). Haemopoietic progenitor cells committed to granulocyte and/or macrophage development (GM-CFC) were assayed in the femoral marrow by a semi-solid plasma-clot technique (Vacek *et al.* 1990). Bone marrow cell suspensions were plated in quadruplicates using 10 % murine lung conditioned medium as a source of the colony-stimulating factor. Colonies (>50 cells) were counted after 7 days of incubation in a 37 °C humidified environment containing 5 % CO_2. In addition, spleen weight, bone marrow cellularity and blood leukocytes and erythrocytes were determined. The counts of nucleated cells in the femur and blood cells were estimated with a Coulter counter. Blood was withdrawn from a fine incision in the tail vein.

Statistical evaluation

The concept of multiple testing (Miller 1966) as defined in more detail by Holm (1979) was used for treating the experimental data. Survival data were analyzed by the life-table method using the "long-rank" test (Peto *et al.* 1977). $P<0.05$ was considered as a basis of statistically significant differences.

Results

Table 1 gives the results of experiments evaluating the ability of dipyridamole and AMP combination administered before each of the five 3-Gy radiation fractions to modify the haemopoietic responses of the animals. In non-treated irradiated mice haemopoiesis was clearly suppressed on day 10 after the completion of fractionated radiation as compared to non-irradiated control animals. Administration of the protective drugs 15 min before each irradiation significantly elevated all examined parameters. The pretreatment 60 min before irradiation was significantly more effective in most of the evaluated indices than the administration of drugs 15 min prior to irradiation.

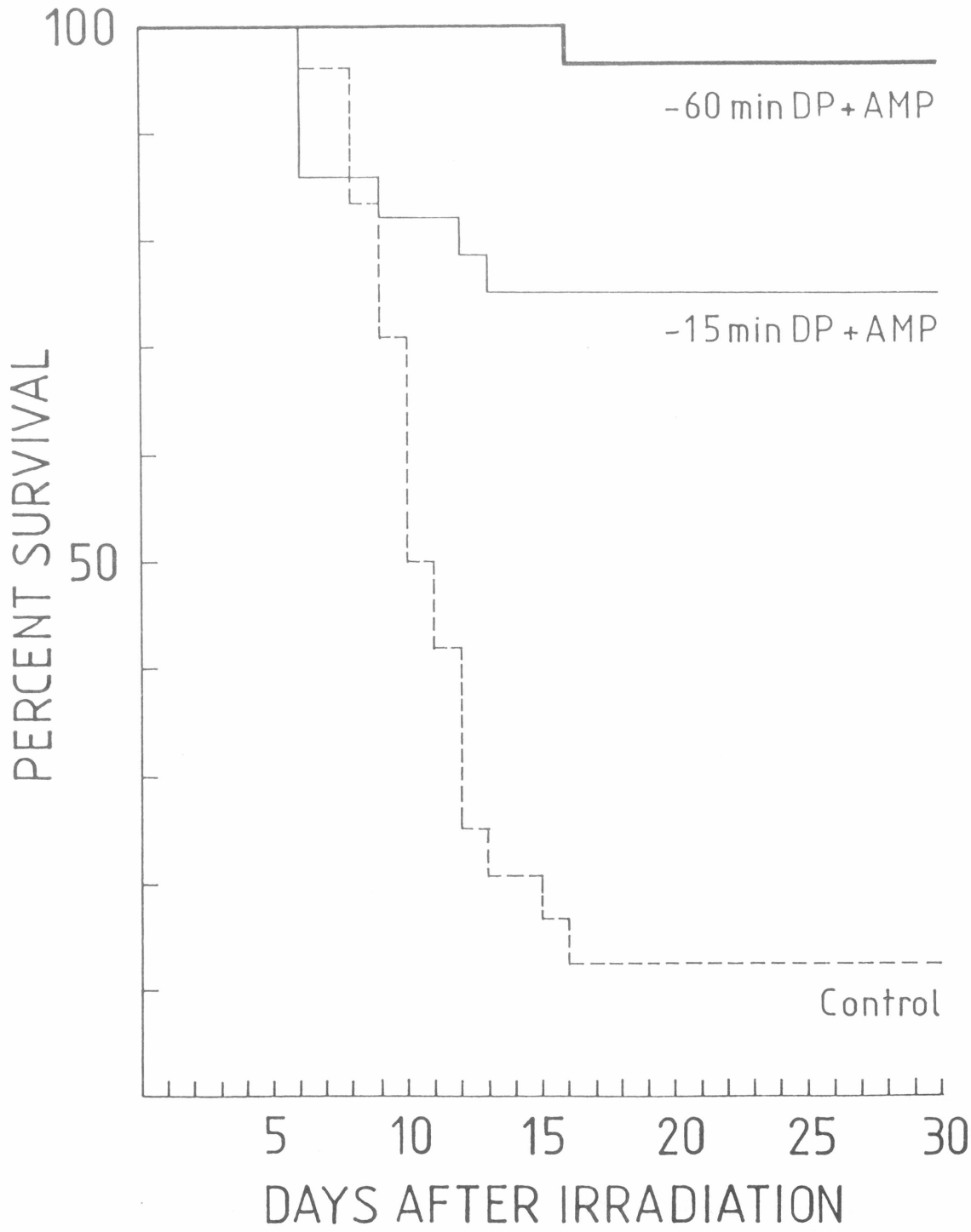

Fig. 1
Survival of mice gamma-irradiated with five daily fractions of 3 Gy and an additive "top-up" dose of 3.5 Gy, treated with solvents (control) or dipyridamole (DP) + AMP in two schemes (15 or 60 min before each of the five radiation fractions). The respective groups comprised 24–28 animals, data were obtained from four replicate experiments. Differences in death rate between the control group and groups treated with the drugs either 15 or 60 min before irradiation are significant at $P<0.001$, the difference between the two protected groups is significant at $P<0.05$.

Table 1
Haemopoietic indices in non-irradiated animals , and in control and dipyridamole (DP) + AMP protected groups of mice on day 10 after the completion of fractionated irradiation (5 x 3 Gy)

	Non-irradiated control	Irradiated control	DP+AMP 15 min before irradiation	DP+AMP 60 min before irradiation
Spleen weight (mg)	72.0±3.2 (15)	21.1±0.6 (34)	24.5±1.7* (26)	30.1±1.2# (40)
Macroscopically visible spleen colonies	–	0.5±0.2 (23)	4.2±0.9** (14)	5.9±0.7 (26)
Histologically evaluated spleen colonies	–	3.2±0.9 (6)	15.1±1.6** (7)	24.2±2.8 # (7)
Nucleated cells per femur (x10^7)	2.55±0.14 (15)	0.73±0.03 (34)	1.14±0.07** (26)	1.33±0.05# (40)
GM-CFC per femur	20 393±1 152 (15)	285±29 (17)	5 269±264** (13)	8 603±523## (14)
Leukocytes per 1 μl of blood	6 440±480 (15)	537±33 (34)	1 224±54** (26)	1 389±90# (40)
Erythrocytes per 1 μl of blood (x 10^6)	10.20±0.20 (15)	5.48±0.13 (34)	6.25±0.17** (26)	6.59±0.10 (40)

*Data represent means±S.E.M., the number of animals is given in parentheses. Significant differences between irradiated groups: *,** – compared to irradiated control P<0.05, P<0.01, respectively, #,## – compared to the group treated with DP+AMP 15 min before radiation fractions, P<0.05 P<0.01, respectively.*

The data on the survival of animals irradiated with five 3-Gy doses and subjected to an additional "top-up" dose of 3.5 Gy (without protection) are shown in Fig. 1. The survival of both treated groups of mice was increased significantly compared with that of the controls. However, the protective effect of the drugs administered 60 min before each irradiation was significantly higher than the effect induced by the pretreatment 15 min prior to irradiation.

Discussion

From the general point of view, chemical or biological reduction of radiation damage to the haemopoietic tissue of the mammalian organism can be achieved in two different ways: (1) by a reduction of the radiosensitivity of cells by modifying the initial radiation damage (induction of hypoxia, sulfhydryl compounds) (Tubiana *et al.* 1990); (2) by enhancing cellular repair and/or regeneration of the haemopoietic cell populations by activating various biochemical and physiological regulatory mechanisms (growth factors, biological response modifiers) (Chirigos and Patchen 1988). As was shown in our earlier experiments (Pospíšil *et al.* 1993a), the combined administration of dipyridamole and AMP, which increases the extracellular level of adenosine, can mediate both these mechanisms. Radiation damage to animals irradiated shortly, i.e. 15 min after the administration of the drugs can be decreased *via* cardiovascular hypoxia induced by the drug action. Bradycardia and vasodilatory effects of extracellular adenosine can induce transient hypotension and, consequently, hypoxia in radiosensitive tissues. Vasodilation is probably linked to A2 receptors and thus to adenylate cyclase activation (Collis 1989), and bradycardia is affected by A1 receptors inhibiting adenylate cyclase (Belardinelli *et al.* 1989). Irradiation of the animals at a longer interval (60 min) after the administration of the drugs, when the hypoxia effects already fade away, excludes this radioprotective mechanism so that mechanisms based on the haemostimulative action of extracellular adenosine become mainly effective. Even though the signalling pathways of this action remain to be analyzed further, the elevation of extracellular adenosine seems to act as a physiological stimulant of haemopoietic cell

proliferation (Pospíšil *et al.* 1992). It has been suggested that purine nucleotides are probably a universal system of intercellular signals which are capable of modulating several cellular functions, including cell proliferation (Rathbone *et al.* 1992a). In some cell lines, the activation of A2 receptors has been proposed to play a role in the effects elicited by adenosine (Rozengurt 1982, Meininger and Granger 1990, Rathbone *et al.* 1992b). In the murine multipotential haemopoietic stem cells and progenitor cells, both cAMP and cGMP linked to adenosine receptors have been implicated as second messengers in the regulation of cell proliferation and differentiation (Byron 1973, Kurland *et al.* 1977).

As compared with our earlier findings, which showed that both protective schemes are equally effective under conditions of single sublethal and lethal radiation exposures (Pospíšil *et al.* 1993a), the data presented here clearly demonstrate that after fractionated irradiation with lower doses the proposed haemostimulative mechanism is more effective than that acting *via* the effect of hypoxia. This is evident not only from the evaluation of indices of haemopoietic recovery at the level of pluripotent stem cells (endogenous spleen colonies), committed cells (GM-CFC) and mature white blood cells, but also from the survival of mice irradiated with an additional "top-up" dose. The enhanced survival observed in the drug-treated mice correlates with indices of haemopoietic recovery and can be assessed as a result of the severity of the bone marrow radiation syndrome. When interpreting these findings, the peculiarities of the haemopoietic response to split-radiation should be taken into consideration.

Splitting of the radiation treatment into lower doses separated by a time interval produces a smaller degree of total cell-killing effect than when the same total dose is delivered in a single fraction. This is due to the operation of recovery processes taking place between the fractional irradiations. The enhanced survival of haemopoietic stem cells during a 4–6 h rest period between radiation treatments has been interpreted to be consistent with an early intracellular repair process (Till and McCulloch 1963). With a rest period of 24 h and more between the fractional irradiations, repopulation on account of proliferating haemopoietic cells has been implicated in the recovery process during radiation treatment (Chaffey and Hellman 1969). However, this sparing effect of fractionated radiation on haemopoiesis can be decreased *via* mechanisms damping down cell repair and/or proliferation. Phillips and Hanks (1968) demonstrated in split-radiation experiments the absence of cellular repair and delayed proliferation in the mouse haemopoietic colony-forming cells when radiation injury occurred under conditions of hypoxia. From the general point of view, the possible inhibitory effect of hypoxia on cell proliferation may be considered to be a defense mechanism against the damaging effects of hypoxia which would occur if the cells were able to initiate DNA synthesis (Amellem and Pettersen 1991). Even though hypoxia is in principle a protective mechanism, its presence under conditions of fractionated irradiation may be unfavorable due to its inhibitory effect on recovery processes. In view of these suggestions, our results demonstrating the lower protective efficacy of the drugs when administered 15 min before irradiation might be ascribed to an unfavorable effect of concomitant hypoxia. Avoiding this contribution of hypoxia by increasing the preirradiation time interval of drug administration enhances the radioprotective haemostimulative action of the drugs.

When considering the clinical aspects of our findings, the need of radioprotection to decrease radiation damage to normal tissues, including the haemopoietic ones, under multifractionated radiation regimens as used in radiotherapy of tumors, has to be taken into account (Milas *et al.* 1988). Our results are, therefore, aimed at contributing to a rational use of radioprotective agents in radiotherapy.

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