Physiol. Res. 48: 37-42, 1999

Granulocyte Colony-Stimulating Factor and Drugs Elevating Extracellular Adenosine Act Additively to Enhance the Hemopoietic Spleen Colony Formation in Irradiated Mice

M. HOFER, M. POSPÍŠIL, J. NETÍKOVÁ, V. ZNOJIL[1], J. VÁCHA[1]

Institute of Biophysics, Academy of Sciences of the Czech Republic and [1]Institute of Pathological Physiology, Medical Faculty, Masaryk University, Brno, Czech Republic

Received May 7, 1998
Accepted October 1, 1998

Summary

The effects of combined administration of two drugs elevating extracellular adenosine, namely dipyridamole (DP) and adenosine monophosphate (AMP), and granulocyte colony-stimulating factor (G-CSF) on hemopoietic stem cells *in vivo* were investigated. The experiments were performed on mice using the endogenous spleen colony formation in gamma-irradiated animals as an endpoint. The results have shown that DP and AMP act additively with G-CSF to enhance spleen colony formation and thus the erythroid repopulation of the spleen. These findings indicate that the signaling pathways of G-CSF and drugs elevating extracellular adenosine can interact at the level of primitive hemopoietic stem cells. The enhancement of hemopoiesis-stimulating effects of G-CSF by DP and AMP, which are low-priced and clinically available drugs, could improve the cost-effectiveness of the therapy with G-CSF.

Key words
Hemopoietic stem cells • Granulocyte colony-stimulating factor • Dipyridamole • Adenosine monophosphate

Introduction

Our earlier radiobiological studies have shown that elevation of extracellular adenosine induced in mice by the combined administration of dipyridamole (DP), a drug inhibiting the cellular uptake of adenosine, and adenosine monophosphate (AMP) serving as an adenosine prodrug, can exhibit radioprotective activity due to the enhancement of hemopoiesis (Pospíšil *et al.* 1993, 1995a, Hofer *et al.* 1995). These effects are probably mediated by the activation of cell membrane receptors by extracellular adenosine which seems to represent a universal regulatory system modulating several cellular functions including cell proliferation and differentiation (Abbracchio 1996, Neary and Burnstock 1996). Furthermore, we found that in normal non-irradiated mice the combination of DP+AMP can enhance the effect of the granulocyte colony-stimulating factor (G-CSF), a hemopoietic regulator that accounts for the production, differentiation, and activation of neutrophil granulocytes. Under the combined treatment

Fax +420 2 293 894
E-mail physres@biomed.cas.cz
http://www.lf2.cuni.cz/physiolres/physiolres.htm

with these drugs, the most expressively enhanced production of granulocytic progenitors, precursors, and mature cells has been demonstrated (Pospíšil *et al.* 1995b). Because both G-CSF (Patchen *et al.* 1990) and DP+AMP (Hofer *et al.* 1997) were shown to enhance endogenous spleen colony formation in irradiated mice, the question was raised of whether interactions between drugs elevating extracellular adenosine and G-CSF also occur at the level of the stem cells generating these colonies. For this reason, the present study examined the effects of the combined administration of DP+AMP with G-CSF on hemopoietic stem cells using endogenous spleen colony formation in gamma-irradiated mice as an endpoint.

Methods

Animals

Male B10CBAF$_1$ mice aged 3 months and weighing 25 g on the average were obtained from AnLab Ltd. (Prague, Czech Republic). The mice were kept under controlled conditions; a standardized pelleted diet and HCl-treated tap water (pH 2-3) were given *ad libitum*.

Irradiation

Mice were total-body irradiated with cobalt-60 radiation at a dose rate of 0.23 Gy/min. Single doses in the range of 6.5 to 8.5 Gy were applied.

Drugs and the experimental protocol

Dipyridamole (Sigma, USA) was dissolved in 0.4 % tartaric acid and injected s.c. (0.4 ml) in a daily dose of 2 mg per mouse. Adenosine 5'-monophosphate sodium salt from yeast (Sigma, USA) was dissolved in distilled water and injected i.p. (0.2 ml) at a dose of 5 mg free acid per mouse. Recombinant human G-CSF (Neupogen; purchased from F. Hoffman-LaRoche Ltd., Basel, Switzerland) was diluted with 5 % glucose and injected s.c. (0.1 ml) in a daily dose of 3 μg per mouse. The controls received injections of the same volumes of the pertinent vehicle. In the combined treatment, DP was administered 20 min before AMP, G-CSF was given 30 min after AMP. The drugs were given daily for 4 days between 09:00 and 10:00 h. Three hours after the last injection the mice were irradiated.

Hematological methods

Endogenous hemopoietic spleen colonies were counted on day 10 after irradiation by standard techniques, either as macroscopically visible surface colonies on the spleens fixed in Bouin's solution (nodules with a diameter of 0.5 mm or larger), or as histologically evaluated colonies (more than 10 cells were considered as a colony) on the midline-longitudinal sections of the spleens embedded in paraffin. The histologically evaluated colonies were counted in four size categories: <0.25 mm, 0.25-0.5 mm, 0.5-1.0 mm, and >1.0 mm, in diameter. Taking into account the number of colonies in individual size categories and a roughly spherical shape of colony, mean colony volumes (mm^3) were estimated (a semiquantitative approach). The numbers of nucleated cells in the spleen cell suspension were determined on days 10 and 14 after irradiation using a Coulter counter, and differential counts were performed on smear preparations stained with the May-Grunwald-Giemsa method. The differential counts included morphologically recognizable erythroid (proerythroblasts through orthochromatic erythroblasts), granulocytic (myeloblasts through segmented neutrophils) and lymphoid cells.

Statistics

The results of experiments are presented as mean ± standard error (S.E.M.). Dose-survival curves for spleen hemopoietic colonies were fitted by the weighted least-squares method. The parameters of the curves, i.e. their slopes in semilogarithmic coordinates (D_0 values – doses that reduce the survival of colony-forming cells to 37 %) and intercepts with the straight segment of each curve on the zero-dose axis, or on the axis belonging to the 7.5 Gy dose, were computed. Analysis of variance and multiple-comparison techniques according to Holm (1979), Tukey or Mann-Whitney (CSS: Statistica, v 3.1, StatSoft, Inc., Tulsa, USA, 1992), as appropriate, were used to evaluate the significance of differences between the groups. The significance level was set at $P<0.05$.

Results

Drugs were administered in a 4-day regimen terminated three hours before irradiation. The effects of different treatments on the number of macroscopically visible surface spleen colonies on day 10 after irradiation with different radiation doses are shown in Figure 1. Dose-survival curves were defined by their slopes (D_0 values) and intercepts on the Y axis. The D_0 values for control, DP+AMP, G-CSF alone, and combination of all drugs were 0.64±0.08 Gy, 0.79±0.14 Gy, 0.70±0.12 Gy and 0.90±0.13 Gy, respectively. There were no significant differences between the D_0 values of the differently treated groups. The intercepts of the dose-

survival curves on the zero-dose axis suffer from inaccuracies resulting from the lengthy back extrapolation and cannot be tested statistically in this model. For this reason, the significance of these effects was evaluated at a reference radiation dose situated in the middle of the dose range used (7.5 Gy). At this dose, all the pairwise differences between the experimental groups were found to be significant at the $p<0.005$ level. It is evident that all the treatments enhanced colony formation and that the combination of DP+AMP with G-CSF was the most effective. In order to evaluate the contribution of the different treatments to the enhancing effects, the colony responses were converted into radiation doses and the effects of drugs were quantified as protection factors, i.e. the ratios of doses producing the isoeffects, i.e. six colonies. This reference value was estimated as the optimum with respect to the confidence limits of the regression straight lines. Thus, the computed protection factors related to the control, were 1.10±0.02 for DP+AMP, 1.21±0.02 for G-CSF alone and 1.30±0.02 for combination treatment, suggesting that the effect of G-CSF was greater compared to DP+AMP, with an approximately additive action of DP+AMP and G-CSF.

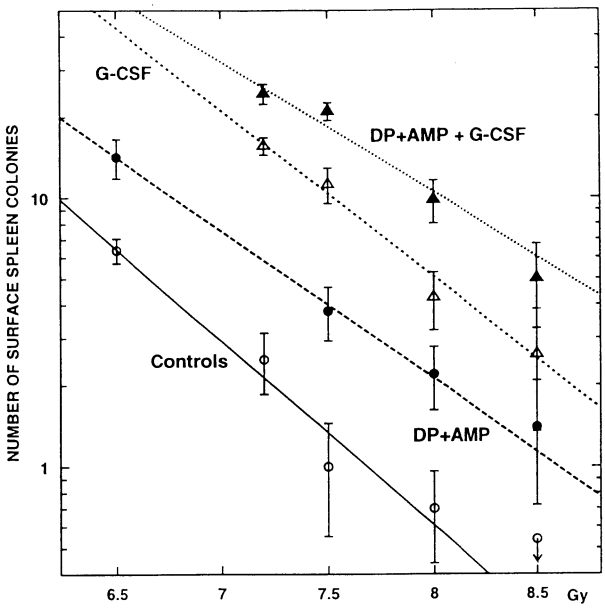

Fig. 1. *Dose-survival curves for the macroscopically visible surface spleen colonies in mice subjected to various treatment regimens. Each point on the curves represents mean value from 5 mice. Statistical analysis of the effects is given in the Results.*

Table 1. Numbers and average volumes (mm^3) of histologically evaluated colonies on day 10 after 7.5 and 8.5 Gy of radiation

	Control	DP+AMP	G-CSF	DP+AMP+G-CSF
7.5 Gy - number	1.4± 0.5	3.0 ±0.7	7.4± 1.0[a]	12.4± 0.9[a,b]
- volume	0.006± 0.005	0.486± 0.165[a]	0.376 ±0.148[a]	0.485± 0.080[a]
8.5 Gy - number	1.0± 0.8	1.4± 0.7	2.0 ±0.3	5.0± 1.4[a,b]
- volume	0.039 ±0.032	0.032± 0.019	0.258± 0.185	0.415± 0.105[a]

Five mice per group were studied. Statistical significance (for the sake of simplicity only the level of $P<0.05$ was used for all comparisons): a - compared to control, b - compared to G-CSF alone.

Histologically evaluated colonies were counted in differently treated groups of mice irradiated with doses of 7.5 and 8.5 Gy. The data given in Table 1 show that the values obtained from these colonies correspond approximately to the findings obtained when investigating macroscopically visible surface colonies. Again, the highest stimulatory effects were observed after the treatment with a combination of all drugs. After 7.5 Gy, average volumes of the histologically evaluated colonies were significantly higher in all the treated groups compared to the controls. Following the dose of 8.5 Gy, only the group treated with the combination of all drugs exhibited a significant elevation. About 90 % of colonies were of the erythropoietic type. There was no evidence that the treatments modified the differentiation pattern of colonies.

Table 2. Erythroid, granulocytic and lymphoid cells in the spleen (x 10^7) on days 10 and 14 after 6.5 Gy dose

	Control	DP+AMP	G-CSF	DP+AMP+G-CSF
Erythroid cells				
- day 10	0.41± 0.07	1.61± 0.12[a]	2.95± 0.08[a]	4.95± 0.45[a,b]
- day 14	4.63± 0.40	4.76± 0.31	8.63± 0.58[a]	9.74± 0.91[a,b]
Granulocytic cells				
- day 10	0.11± 0.02	0.19± 0.07	0.27± 0.11	0.18± 0.03
- day 14	0.52± 0.09	0.49± 0.11	0.35± 0.07	0.44± 0.08
Lymphoid cells				
- day 10	1.49± 0.14	0.85± 0.13	1.31± 0.32	1.32± 0.13
- day 14	1.94± 0.18	2.06± 0.16	1.46± 0.15	1.55± 0.20

5 - 10 mice per group were studied. Statistical significance (for the sake of simplicity only the level of $P<0.05$ was used for all comparisons): a - compared to control, b - compared to G-CSF alone. The mean values obtained in non-irradiated controls were 4.71±0.43 x 10^7 for erythroid cells, 0.74±0.14 x 10^7 for granulocytic cells, and 5.71±0.84 x 10^7 for lymphoid cells.

In mice irradiated with the dose of 6.5 Gy and pretreated with G-CSF alone, or with the combination of all drugs, surface spleen colonies began to be confluent and could not accurately be counted. In order to determine the interactions of adenosine signaling and G-CSF under these conditions, the repopulation potential of splenic hemopoiesis was evaluated in terms of the counts of morphologically recognizable cells of the erythroid, granulocytic and lymphoid lineages. As is shown in Table 2, only the erythroid cells were influenced. There were no significant differences in the counts of granulocytic and lymphoid cells in the spleens of the compared groups. Relative to normal counts observed in non-irradiated mice, the counts of erythroid cells in the spleen on day 10 after irradiation were reduced in control mice to about 9 %, in DP+AMP-treated mice to about 34 %, in mice treated with G-CSF alone to about 63 %, whilst in mice treated with the combination of all drugs erythroid cells attained normal values. Thus, the stimulatory interactions of DP+AMP with G-CSF have been confirmed for the multiplicative and maturation pools which are fed from the stem cell pool. On day 14 after irradiation, the erythroid cells in the spleens of control mice recovered to normal values, and the effects of drugs were less pronounced probably due to the action of feedback control damping the erythroid production.

Discussion

The counting of endogenous hemopoietic spleen colonies in irradiated mice provides information on the size of the hemopoietic stem cell pool repopulating the spleen. It is inferred that spleen colonies, which can be counted on day 8 to 11 days after irradiation indicate the number of pluripotent colony-forming cells that have survived radiation exposure and have the capacity to proliferate and differentiate (Till and McCulloch 1963). Because the spleen of rodents presents a very suitable environment for erythroid differentiation, spleen colonies are mainly of the erythropoietic type (Curry and Trentin 1967).

Our results show that the combination of drugs elevating extracellular adenosine, i.e. dipyridamole and adenosine monophosphate, act additively with G-CSF to enhance the spleen colony formation and thus the erythroid repopulation of spleen. Several mechanisms can participate in this effect. The agents administered before irradiation can increase the radioresistance of stem cells. This would be characterized by a lower slope of the dose-survival curves (increase of the D_0 value). As has been shown, this effect does not seem to be involved because of the lack of significant differences in D_0 values between the compared groups. Other mechanisms may be of importance. According to Hanks and Ainsworth (1967), they can include increased migration

of colony-forming cells into the spleen from other sites, the rate of *in situ* proliferation of these cells derived from either active or inactive hemopoietic pools, preferential differentiation in cell lines which have shorter generation or maturation time, or earlier initiation of the postirradiation proliferation. The relative contribution of any of the above factors is unknown. However, it should be mentioned that migration of stem cells from the marrow to the spleen under G-CSF treatment has been repeatedly described (Bungart *et al.* 1990, Molineux *et al.* 1990, Drize *et al.* 1993). As has been demonstrated by our results, colony sizes increased significantly after both radiation doses of 7.5 and 8.5 Gy in the groups treated with the combination of DP+AMP with G-CSF. This suggests a higher proliferation and/or differentiation potential of colony-forming cells after the combined treatment with all three drugs.

Besides the traditional concept considering the cycling activity of hemopoietic cells as the main mechanism of cell amplification, programmed cell death (apoptosis) has also been recognized to play a role (Nečas *et al.* 1995). Various cytokines, including G-CSF, were found to promote the viability of hemopoietic cells and to suppress apoptosis (Williams *et al.* 1990). Adenosine signaling can also be involved in mechanisms of apoptosis of blood cells. Gasmi *et al.* (1996) reported that diadenosine polyphosphates and adenosine triphosphate, acting on cells *via* purinoceptors, interact with granulocyte-macrophage colony-stimulating factor to delay human neutrophil apoptosis.

Although the mechanisms underlying the additive effects of G-CSF and drugs elevating extracellular adenosine need to be further analyzed, our results clearly indicate that both these regulatory pathways can interact at the level of primitive hemopoietic stem cells. This might be of importance in some therapeutic indications for G-CSF, such as mobilization of stem cells into blood and amelioration of the effectiveness of marrow transplantation (Welte *et al.* 1996). It seems of interest to point out that enhancement of G-CSF effects by low-priced and clinically available drugs could reduce the high costs of G-CSF therapy.

Acknowledgments

This work was supported by the Grant Agency of the Czech Republic (Grant No. 306/96/1721).

References

ABBRACCHIO MP: P_1 and P_2 receptors in cell growth and differentiation. *Drug Dev Res* **39:** 393-406, 1996.

BUNGART B, LOEFFLER M, GORIS H, DONTJE B, DIEHL V, NIJHOF W: Differential effects of recombinant human colony stimulating factor (rh G-CSF) on stem cells in marrow, spleen and peripheral blood in mice. *Br J Haematol* **76:** 174-179, 1990.

CURRY JL, TRENTIN JJ: Hemopoietic spleen colony studies. I. Growth and differentiation. *Dev Biol* **15:** 395-413, 1967.

DRIZE N, GAN O, ZANDER A: Effect of recombinant human granulocyte colony-stimulating factor treatment of mice on spleen colony-forming unit number and self-renewal capacity. *Exp Hematol* **21**: 1289-1293, 1993.

GASMI L, McLENNAN AG, EDWARDS SW.: The diadenosine polyphosphates Ap_3A and Ap_4A and adenosine triphosphate interact with granulocyte-macrophage colony-stimulating factor to delay neutrophil apoptosis: Implications for neutrophil:platelet interactions during inflammation. *Blood* **87:** 3442-3449, 1996.

HANKS GE, AINSWORTH EJ: Endotoxin protection and colony-forming units. *Radiat Res* **32:** 367-382, 1967.

HOFER M, POSPÍŠIL M, NETÍKOVÁ J, ZNOJIL V, VÁCHA J, HOLÁ J: 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. *Physiol Res* **44:** 93-98, 1995.

HOFER M, POSPÍŠIL M, NETÍKOVÁ J, ZNOJIL V, VÁCHA J: Enhancement of haemopoietic spleen colony formation by drugs elevating extracellular adenosine: effect of repeated in vivo treatment. *Physiol Res* **46:** 285-290, 1997.

HOLM S: A simple sequentially rejective multiple test procedure. *Scand J Stat* **6:** 65-70, 1979.

MOLINEUX G, POJDA Z, DEXTER TM: A comparison of hematopoiesis in normal and splenectomized mice treated with granulocyte colony-stimulating factor. *Blood* **75:** 563-569, 1990.

NEARY JT, BURNSTOCK G: Purinoceptors in the regulation of cell growth and differentiation. *Drug Dev Res* **39:** 407-412, 1996.

NEČAS E, ŠEFC L, BRECHER G, BOOKSTEIN N: Hematopoietic reserve provided by spleen colony-forming units (CFU-S). *Exp Hematol* **23:** 1242-1246, 1995.

PATCHEN ML, MacVITTIE TJ, SOLBERG BD, SOUZA LM: Therapeutic administration of recombinant human granulocyte colony-stimulating factor accelerates hemopoietic regeneration and enhances survival in a murine model of radiation induced myelosuppression. *Int J Cell Cloning* **8:** 107-122, 1990.

POSPÍŠIL M, HOFER M, NETÍKOVÁ J, PIPALOVÁ I, VACEK A, BARTONÍČKOVÁ A, VOLENEC K: Elevation of extracellular adenosine induces radioprotective effects in mice. *Radiat Res* **134:** 323-330, 1993.

POSPÍŠIL M, HOFER M, ZNOJIL V, VÁCHA J, NETÍKOVÁ J, HOLÁ J: Radioprotection of mouse hemopoiesis by dipyridamole and adenosine monophosphate in fractionated treatment. *Radiat Res* **142:** 16-22, 1995a.

POSPÍŠIL M, HOFER M, ZNOJIL V, VÁCHA J, NETÍKOVÁ J, HOLÁ J: Synergistic effect of granulocyte colony-stimulating factor and drugs elevating extracellular adenosine on neutrophil production in mice. *Blood* **86:** 3692-3697, 1995b.

TILL JE, McCULLOCH EA: Early repair processes in marrow cells irradiated and proliferating in vivo. *Radiat Res* **18:** 96-105, 1963.

WELTE K, GABRILOVE J, BRONCHUD MH, PLATZER E, MORSTYN G: Filgrastim (r-metHuG-CSF): the first 10 years. *Blood* **88:** 1907-1929, 1996.

WILLIAMS GT, SMITH CA, SPOONCER E, DEXTER TM, TAYLOR DR: Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. *Nature* **343**: 76-79, 1990.

Reprint requests

M. Hofer, Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, 612 65 Brno, Czech Republic.