CFU-S and Haematopoietic Microenvironment in Mice after Fractionated Irradiation. A Study on Spleen Colonies

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Abstract. The paper is aimed at evaluating the quantity and quality of the haematopoietic stem cells, CFU-S, in the bone marrow and the functional effectiveness of the haematopoietic microenvironment of the spleen in two time intervals after repeated exposure of mice to doses of 0.5 Gy gamma-rays once a week (total doses of 12 and 24 Gy). After irradiation, bone marrow was cross-transplanted between fractionatedly irradiated and control mice. The parameter evaluated were numbers of spleen colonies classified into size categories. The data obtained provide evidence for a significant damage to the CFU-S, concerning both their number and proliferation ability, after both total doses used. The functional effectiveness of the haematopoietic microenvironment of the spleen was impaired only in bone marrow recipients receiving a transplant after having been exposed to a total dose of 24 Gy; this dose combined with subsequent pre-transplantation irradiation resulted in a marked suppression of cell production within the spleen colonies formed from a normal bone marrow on the spleens of fractionatedly irradiated mice.

Key words: Haematopoietic stem cells — Haematopoietic microenvironment — Spleen colonies — Fractionated irradiation

Introduction

Essential components of the process of haematopoiesis are the stem cell compartment and the haematopoietic microenvironment (HM) (e.g. Tavassoli and Friedenstein 1983).

Transplantation of the bone marrow to lethally irradiated recipients results in the formation of spleen colonies (Till and McCulloch 1961) whose properties depend, among others, on the HM within the spleen (e.g. Trentin 1978).

Exposure of an experimental animal to ionizing radiation results in damage to both the stem cell compartment and the HM. The radiosensitivity of the CFU-

S stem cells has been determined relatively precisely (McCulloch and Till 1962; Boersma 1983). Moreover, the residual damage measurable by impaired ability to proliferate was described in the stem cells which survived irradiation (Hübner et al. 1985).

The postirradiation performance of the HM was studied by several research groups and by means of *in vitro* and *in vivo* methods (Tavassoli 1975; Zuckermann et al. 1986; Molineux et al. 1987) with conflicting conclusions.

Protracted exposure to doses in the order of tenths of Gy per week led to a serious damage of the haematopoietic system after several weeks of exposure (e.g. Lorenz et al. 1954; Seed et al. 1978; Praslička 1983; Hofer et al. 1990). Our experiment was designed to determine the effects on both the CFU-S compartment and the HM of an exposure to a total of 12 or 24 Gy in single weekly doses of 0.5 Gy. The degree of damage to both systems was evaluated by carrying out bone marrow cross-transplantations between fractionatedly irradiated mice and control mice of equal age. For these cross-transplantation experiments the bone marrow recipients were divided into two experimental groups. Each of the groups was compared with the control group where neither the donors nor the recipients were fractionatedly irradiated. The evaluation parameter for bone marrow recipients were the numbers of spleen colonies classified according to their sizes. A comparison of controls and the experimental group, with the control recipients receiving bone marrow of fractionatedly irradiated donors, served to evaluate the bone marrow stem cell compartment. Comparing data obtained from controls and the experimental group, with the fractionatedly irradiated recipients receiving bone marrow of control donors, enabled us to evaluate the HM of the spleen.

Materials and Methods

Experimental animals: The experimental animals were male (CBA x C57BL/10)F1 mice, aged 3 months at the start of the fractionated irradiation.

Fractionated irradiation: Mice were exposed once a week to single doses of 0.5 Gy gamma-rays of ^{60}Co (Chisostat, Chirana, Praha, Czechoslovakia), at an exposure rate of 0.65 Gy/min.

Cross-transplantation experiments were performed after the exposure to total doses of 12 or 24 Gy; the method used was based on that of Till and McCulloch (1961).

The control and experimental groups in cross-transplantation experiments were as follows:

Experimental group 1 comprised fractionatedly (0.5 Gy once a week) irradiated mice which received the bone marrow of control mice on day 9 after having received a total dose of 12 or 24 Gy.

Experimental group 2 comprised control mice which received bone marrow from fractionatedly irradiated mice on day 9 after having been exposed to a total dose of 12 or 24 Gy. The control group comprised control mice which received bone marrow of other control mice.

The experimental and control mice used in both cross-transplantation experiments were always of equal age.

Bone marrow transplantation: The bone marrow recipients included in the cross-transplantation experiment after having been exposed to a total dose of 12 Gy were additionally exposed to a dose of 8.5 Gy gamma-rays 2–3 hours before the transplantation. The pre-transplantation dose applied in analogy to bone marrow recipients having been exposed to a total dose of 24 Gy was only 8 Gy, because of a very high death rate in this group by day 10 after a pre-transplantation dose of 8.5 Gy. Each recipient received 10^6 nucleated bone marrow cells. The number of cells in the bone marrow transplants was higher than usually used owing to markedly reduced numbers of spleen colonies expected in both experimental groups, especially in experimental group 2. The suspension of bone marrow cells for the transplantation was always a mixture of femoral cells obtained from 5 fractionatedly irradiated or 5 control mouse donors. The numbers of bone marrow recipients in the individual groups and the numbers of animals surviving and sampled on day 10 after the irradiation are given in Tab. 1.

Table. 1. Numbers of bone marrow recipients and of living recipients on day 10 after bone marrow transplantation

	Week 24 Bone marrow recipients/ living recipients	Week 48 Bone marrow recipients/ living recipients
Experimental group 1 (fractionatedly irradiated recipients of bone marrow from control donors)	6/2	7/3
Experimental group 2 (control recipients of bone marrow from fractionatedly irradiated donors)	7/6	7/7
Control group (control recipients of bone marrow from control donors)	6/6	7/6

The parameter studied in the cross-transplantation experiments was the number of macroscopic spleen colonies (on the spleen surface) evaluated on day 10 after bone marrow transplantation. The colonies were counted in four size categories: (I) 0.5–1.0 mm, (II) 1.0–2.0 mm, (III) > 2.0 mm, (IV) all colonies > 0.5 mm.

Estimation of the relative extent of total cell production: Spleen colonies have been supposed to be approximately spheric in shape. In the colony size categories I, II, and III, the approximate average diameters were 0.75 mm, 1.5 mm, and 2.5 mm, respectively. The volume of each colony was supposed to be directly proportional to the cell content. The volume of a sphere, $V = 4/3\pi r^3$, where r is the radius of the sphere. If the ratio of the

Table. 2. Numbers of macroscopic spleen colonies in recipients of bone marrow on day 10 after marrow transplantation; cross-transplantation experiment performed with animals exposed to a total dose of 12 Gy

	Macroscopic spleen colonies							
	Size 0.5 – 1.0 mm		Size 1.0 – 2.0 mm		Size > 2.0 mm		All sizes > 0.05 mm	
	Absolute	mean ± SD	Absolute	mean ± SD	Absolute	mean ± SD	Absolute	mean ± SD
Control group	4		12		8		24	
(control recipients	4		10		6		20	
of bone marrow	9		10		3		22	
from control	16		9		3		28	
donors)	19		4		4		27	
	16		6		3		25	
		11.3 ± 2.7		8.5 ± 1.2		4.5 ± 1.2		24.3 ± 1.2
Experimental								
group 1 (frac-	0		5		18		23	
tionatedly irra-	0		13		7		20	
diated recipients		0.0 ± 0.0		9.0 ± 4.0		12.5 ± 5.5		21.5 ± 1.7
of bone marrow								
from control								
donors)								
Experimental	8		3		2		13	
group 2 (control	4		2		0		6	
recipients of bone	7		4		1		12	
marrow from frac-	6		4		0		10	
tionatedly irra-	4		7		1		12	
diated donors)	4		10		3		17	
		5.5 ± 0.7		5.0 ± 1.2		1.2 ± 0.6[a]		11.7 ± 1.5[a]

Legend: [a] – significantly ($P < 0.01$) lower in comparison with control group

average radii of colonies of the size categories I, II, and III is 1 : 2 : 3.33, then the ratio of their volumes is 1 : 8 : 36.9. To express the extent of cell production in spleen colonies of the individual spleens in mutually comparable terms, the following formula was used: *total relative cell production in colonies per spleen = numbers of size I colonies* + (8× numbers of size II colonies) + (36.9× numbers of size III colonies). The resulting value is an estimate of total cell production in the colonies per spleen expressed as the number of average sized (r = 0.75 mm) colonies of size category I, which would produce such amount of cells contained in all colonies of the respective spleen.

The statistical significance of the differences in arithmetic means between recorded and calculated parameters was determined using the distribution-independent Wilcoxon's sequential test, whenever data for sufficiently numerous groups of animals were available.

Results

Tabs. 2 and 3 show the counts of macroscopic colonies on the surface of the spleens of bone marrow recipients on day 10 after bone marrow transplantations performed in weeks 24 and 48 of the long-term radiation exposure.

In animals exposed to a total dose of 12 Gy (Tab. 2), reduction in total numbers of colonies larger than 0.5 mm was observed in experimental group 2 as compared with the control group; in particular, less colonies of larger sizes were counted. The differences in the average number of colonies between experimental group 2 and the control group were statistically significant for colonies larger than 2.0 mm and for those larger than 0.5 mm.

The low numbers of animals surviving in experimental group 1 did not enable an exact statistical evaluation. The data presented show but a slight difference in total spleen colony counts in comparison with the control group (Tab. 2). Nevertheless, although there were no colonies below 1 mm in diameter found in experimental group 1, relatively numerous in this group were colonies with a diameter exceeding 2 mm.

The other cross-transplantation experiment performed with animals exposed to a total dose of 24 Gy (Tab. 3) yielded similar results when comparing total counts of colonies larger than 0.5 mm between the control group and both experimental groups. However, there was a difference between control group and experimental group 2: in the latter group all colonies detected were of the smallest size category (0.5–1.0 mm). In experimental group 1, the total colony counts remained at a level comparable with those observed in the previous cross-transplantation experiment, but the majority of the colonies were of the smallest size category (0.5–1.0 mm); there were no colonies larger than 2.0 mm in the second cross-transplantation experiment mice of group 1.

Estimation of the relative extent of total cell production in all spleen colonies in the individual animal groups: The results are summarized in Tab. 4. After the exposure to a total dose of 12 Gy, the estimated cell production in the spleen colonies

Table. 3. Numbers of macroscopic spleen colonies in recipients of bone marrow on day 10 after marrow transplantation; cross-transplantation experiment performed with animals exposed to a total dose of 24 Gy

	Macroscopic spleen colonies							
	Size 0.5 – 1.0 mm		Size 1.0 – 2.0 mm		Size > 2.0 mm		All sizes > 0.05 mm	
	Absolute	mean ± SD	Absolute	mean ± SD	Absolute	mean ± SD	Absolute	mean ± SD
Control group	16		12		2		30	
(control recipients	17		10		0		27	
of bone marrow	13		7		1		21	
from control	15		14		3		35	
donors)	18		9		2		29	
	9		12		2		23	
		14.6 ± 1.3		11.2 ± 1.4		1.7 ± 0.4		27.5 ± 2.0
Experimental	17							
group 1 (frac-	25		7		0		24	
tionatedly irra-	17		2		0		27	
diated recipients		19.7 ± 2.7	6	5.0 ± 1.5		0.0 ± 0.0	23	24.7 ± 1.2
of bone marrow								
from control								
donors)								
Experimental	0		0		0		0	
group 2 (control	1		0		0		1	
recipients of bone	6		0		0		6	
marrow from frac-	0		0		0		0	
tionatedly irra-	12		0		0		12	
diated donors)	22		0		0		22	
	0	5.9 ± 3.2		0.0 ± 0.0[b]		0.0 ± 0.0[a]	0	5.9 ± 3.2[b]

Legend: [a] – significantly ($P < 0.05$) lower in comparison with control group
[b] – significantly ($P < 0.01$) lower in comparison with control group

Table. 4. Estimated extent of total cell production in all spleen colonies in the individual groups of animals

	Total dose 12 Gy Estimated cell production[a]		Total dose 24 Gy Estimated cell production	
	Absolute	mean ± SD	Absolute	mean ± SD
Control group	395.2		185.8	
(control recipients	305.4		97.0	
of bone marrow from	199.7		105.9	
control donors)	198.7		261.7	
	278.6		163.8	
	230.7		178.8	
		268.0 ± 30.8		165.5 ± 22.4
Experimental group 1	704.2		73.0	
(fractionatedly irradiated	362.3		41.0	
recipients of bone mar-			65.0	
row from control donors)		533.2 ± 170.9		59.7 ± 9.8
Experimental group 2	102.8		0.0	
(control recipients of	20.0		1.0	
bone marrow from frac-	75.9		6.0	
tionatedly irradiated	38.0		0.0	
donors)	96.2		12.0	
	194.7		22.0	
			0.0	
		87.9 ± 25.1[b]		5.9 ± 3.2 [b]

Legend: [a] - estimated cell production was expressed as the number of average sized ($r = 0.75$) colonies of size category I (0.5 – 1.0 mm) which would be necessary to produce the amount of cells contained in spleen colonies of the respective mice (see Materials and Methods)
[b] - significantly ($P < 0.01$) lower in comparison with control group

in experimental group 1 exceeded that of the control group (statistical significance was not determined due to the low number of animals in this experimental group). The estimated cell production in experimental group 2 was significantly lower as compared with the control group. After the exposure to a total dose of 24 Gy, the estimated cell production was markedly reduced in both experimental groups. In experimental group 2, this decrease was found to be significant ($P < 0.01$). In this group, the estimated cell production in the spleen colonies represented only about 4 % of the control group production.

Discussion

Postirradiation regeneration of haematopoiesis depends on the amount of stem cells surviving the irradiation and, secondly, on their ability to produce sufficient amounts of functional offspring. The latter condition comprises the internal state of the stem cells (the absence or presence of residual damage (Hübner et al. 1985)) and, secondly, the functional effectiveness of the HM (Tavassoli 1975; Zuckermann et al. 1986; Molineux et al. 1987). All the factors mentioned which determine successful regeneration can be studied by the technique of spleen colonies (Till and McCulloch 1961) provided the colonies are also classified according to their size.

Information concerning the post-irradiation performance of the HM is relatively inaccessible. Numbers of stromal stem cells, CFU-F, can be determined using *in vitro* techniques (Friedenstein et al. 1976; Piersma et al. 1985; Nikkels et al. 1987). This method, however, does not reflect the post-irradiation functional effectiveness of the HM, which is formed by a variety of cell types and their products as a whole. A better approach is to study the postirradiation kinetics *in vitro* in long-term cultures of haematopoietic tissues (Tavassoli 1975; Zuckermann et al. 1986).

However, *in vivo* methods can be considered as the most valuable approach to assessing the post-irradiational functional effectiveness of the HM; this approach also includes regulatory mechanisms which link other systems of the organism with haematopoiesis. Molineux et al. (1987) investigated the radiosensitivity of the HM by studying the production of colony-forming cells in ectopic ossicles formed after ectopic transplantation of the bone marrow of donors irradiated with single doses up to 10 Gy at 0.016 Gy/min or 4 Gy/min. The numbers of CFU-S were determined by the spleen colony technique. After the exposure to 10 Gy at the lower rate, ossicles were produced containing one tenth of the number of CFU-S in control ossicles. After 10 Gy given at the higher dose rate, a lack of ossicle formation (and, consequently, of CFU-S) was noted.

In our experiments the post-irradiation functional effectiveness of the HM was evaluated using the only technique of spleen colonies (Till and McCulloch 1961). In addition to spleen colony counts the colonies were also classified according to their size as defined in our previous studies (Hofer et al. 1987) and those of other authors (Nečas and Znojil 1989). This classification served not only to determine the CFU-S numbers, but also to estimate the extent of cell production within the colonies.

The post-irradiation effectiveness of the HM was assessed by comparing the findings from experimental group 1 with those from the control group (see Tabs. 2 and 3). In the control group the pre-transplantation lethal irradiation, routinely used in the spleen colony technique, fell upon previously non-irradiated ground, while in experimental group 1 it hit an organism (including the spleen) already

exposed to 12 or 24 Gy at doses of 0.5 Gy given once weekly. The resulting damage was then due to the summed effects of the repeated exposures to smaller doses and the pre-transplantation dose of 8.5 or 8.0 Gy.

When comparing experimental group 1 with the controls, the results of the first cross-transplantation experiment indicate that the pre-transplantation dose of 8.5 Gy combined with the previous exposures to individual doses of 0.5 Gy (a total dose of 12 Gy) did not result in a damage to the HM. The difference in total colony numbers was not marked. Moreover, in contrast to the control group, the maximum occurrence of colonies in experimental group 1 was shifted towards the highest size category. Thus, experimental group 1 can be regarded, in the first cross-transplantation experiment, as having a more powerful cell production in the spleen colonies than the control group. This may have been the result of stronger regulatory pressures on cell proliferation in the organism exposed to long-term irradiation.

In the second cross-transplantation experiment performed with animals exposed to a total dose of 24 Gy, the difference in total colony numbers between experimental group 1 and the control group was not pronounced, either. However, large colonies over 2 mm were completely absent from the spleens of the group 1 animals and maximum numbers of colonies in this group were in the smallest size category of up to 1 mm. Cell production in the spleen colonies was therefore, in this group and at this exposure, substantially smaller than in the respective control group. Apparently, the combined effects of the exposure to doses of 0.5 Gy adding up to a total of 24 Gy and the pre-transplantation dose of 8.0 Gy already inflicts a significant damage to the HM which no longer supports full implementation of the proliferative potential of the homed CFU-S.

The degree of the damage to the colony-forming cell compartment due to repeated exposures to doses of 0.5 Gy once a week was evaluated by comparing experimental group 2 with the control group (see Tabs. 2 and 3). The total numbers of colonies in experimental group 2 in both cross-transplantation experiments were significantly smaller as compared with the control group. Moreover, compared with the control group, in both cross-transplantation experiments experimental group 2 showed a marked shift towards higher proportions of the smaller category colonies; in the second cross-transplantation experiment, all the colonies found in experimental group 2 were classified in the smallest size category. The level of cell production in the colonies of experimental group 2 was even considerably lower than would seem from estimations based solely on total colony counts. These results suggest the existence of a residual damage (Hübner et al. 1985) to the colony-forming cells of long-term fractionatedly irradiated mice.

All the above conclusions are further supported by the results of the estimation of the total extent of cell production in all spleen colonies of the individual groups of animals studied. In experimental group 2, the resulting low volume of

cell production in comparison with the control group is supposed to be due to low amounts of colony forming cells combined with their residual damage. The functionally still fully effective HM studied in the first cross-transplantation experiment (total dose 12 Gy) enabled the experimental group 1 with the haematopoiesis probaly stimulated by the decreasing numbers of peripheral blood cells (unpublished results), to slightly enhance total cell production in spleen colonies at nearly the same numbers of CFU-S. The observation of about a threefold reduction of the estimated cell production in experimental group 1 (total dose 24 Gy) suggests a serious damage to the HM. Nevertheless, further experiments with higher numbers of animals surviving the radiation treatments are to be performed to reach higher precision.

A pronounced decrease of the estimated cell production was found in the spleen colonies of the control group between the first and the second cross-transplantation experiment (see Tab. 4). At the same time, a slight increase in the total number of spleen colonies (Tabs. 2 and 3) and a decrease in peripheral erythrocyte counts (unpublished results) were recorded in this group. The latter finding agrees with the age-related decrease in erythrocyte counts reported by Wolford et al. (1986). As the spleen colonies are predominantly erythropoietic (Wolf and Trentin 1967), the above finding may be the result of age-related decreasing demands for erythrocytes. It can be concluded based on reports in the literature that probably neither seasonal variations in the production of blood cells (Berger 1979) nor contingent aging of colony-forming cells (Boggs et al. 1984) are the reason for the described findings.

Acknowledgements. The authors wish to thank Mrs. V. Reichmannová for skillful technical assistance.

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Final version accepted February 4, 1992