PROLIFERATIVE ACTIVITY OF CLONOGENIC BONE MARROW STROMAL PRECURSOR CELLS

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The proliferative activity of precursors of bone marrow stromal cells forming colonies (clones of fibroblasts) in monolayer cultures was studied by the thymidine-suicide method in vitro. Clonogenic cells of native bone marrow were shown not to be inhibited by thymidine-H^3 with high specific activity, i.e., they virtually do not proliferate in vivo. Having started to proliferate 24 h after explantation, they then give rise to colonies of fibroblasts. In 6-14-day bone marrow cultures 39 ± 4% of clonogenic cells die under the influence of thymidine-H^3. Hence it follows that, unlike precursor cells in native bone marrow, clonogenic cells in primary cultures proliferate actively.

KEY WORDS: bone marrow; monolayer culture; proliferation; stromal precursors.

Colonies, consisting of clones of fibroblasts, are formed in monolayer cultures of hematopoietic and lymphoid cells [1]. Autoradiographic studies have shown that precursors of fibroblasts found in monolayer cultures hardly proliferate at all in adult animals in vivo [2], and they start to divide only 24 h after explantation, when they give rise to colonies of fibroblasts [3]. In the process of formation of such colonies, some fibroblasts (about 20%) leave the proliferative pool [4]. The colonies of fibroblasts are composed of clonogenic cells capable of giving rise to new colonies of fibroblasts during recloning of primary cultures.

TABLE 1. Results of Action of Thymidine-H^3 on Fibroblast Colony Precursor Cells Contained in Bone Marrow Suspension

Experiment No.	Addition of thymidine: unlabeled	Addition of thymidine: labeled	Number of cells per flask	Mean number of colonies in flasks	Mean number of colony-forming units per 10^4 cells	Proportion of colony-forming units surviving after treatment with isotope (in %)
1	+	—	$5 \cdot 10^6$	14	0,03	
	—	+	$5 \cdot 10^6$	17	0,03	100*
2	+	—	$5 \cdot 10^6$	321	0,6	
	—	+	$5 \cdot 10^6$	330	0,6	100*
3	+	—	$5 \cdot 10^6$	550	1,1	
	—	+	$5 \cdot 10^6$	574	1,1	100
4	+	—	$2 \cdot 10^6$	374	1,9	
	—	+	$2 \cdot 10^6$	522	2,6	137
	+	+	$2{,}6 \cdot 10^6$	525	2,1	110
5	+	—	$4 \cdot 10^5$	221	5,5	
	—	+	$4 \cdot 10^5$	220	5,5	100
	+	+	$\cdot 4 10^5$	203	5,1	92

* Incubation in suspension for 2 h.

In this investigation the state of proliferative activity of cells giving rise to colonies of fibroblasts by primary explantation of bone marrow cells was compared with that of clonogenic cells forming colonies on recloning. The thymidine-suicide method was used for this purpose; it is based on the fact that proliferating cells synthesizing DNA and incorporating thymidine-H^3 of high specific activity lose their ability to proliferate and their colony-forming properties [5, 6].

EXPERIMENTAL METHOD

Adult guinea pigs weighing 180-300 g were used as experimental animals. A suspension of bone marrow cells was obtained by the method described previously [1]. The cells ($5 \cdot 10^6$-$10 \cdot 10^6$) were given in 100-ml flasks. The medium was 90% medium No. 199 or double-strength Eagle's medium + 10% embryonic calf serum. The cells were grown in an atmosphere of 5% CO_2 + 95% air. The medium was changed completely 24 h before addition of thymidine to the cultures, which then consisted of discrete foci of fibroblasts.

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TABLE 2. Results of Action of Thymidine-H^3 on Fibroblast Colony Precursor Cells Contained in Primary Cultures of Bone Marrow

Experiment No.	Age of culture (in days)	Addition of thymidine: unlabeled	Addition of thymidine: labeled	Number of recloned cells per flask	Mean number of colonies growing per flask	Mean number of colony-forming units per 10^3 cells		Proportion of colony-forming units surviving after treatment with isotope (in %)
1	6	+	—	$6{,}3 \cdot 10^3$	198	31		
		—	+	$12.5 \cdot 10^3$	269	22	24	79
		—	+	$6{,}3 \cdot 10^3$	172	27		
		—	+	$12{,}5 \cdot 10^3$	129	10		33*
2	6	+	—	$2 \cdot 10^3$	276	138		
		—	+	$2 \cdot 10^3$	126	63		46
		—	+	$2 \cdot 10^3$	160	80		58*
3	6	+	—	Flask 2	269	538	570†	
		+	—	Flask 4	145	580		
		+	—	Flask 8	74	592		
		—	+	Flask 2	140	280	262†	46*
		—	+	Flask 4	61	244		
4	7	+	—	$5 \cdot 10^2$	242	484		
		—	+	$5 \cdot 10^2$	120	240		50
5	14	+	—	$5 \cdot 10^3$	501	100		
		—	+	$5 \cdot 10^3$	259	52		52*
6	14	+	—	10^3	262	262	308	
		+	—	$5 \cdot 10^2$	177	354		
		—	+	10^3	165	165	198	64
		—	+	$5 \cdot 10^2$	116	232		
7	14	+	—	$2 \cdot 10^3$	251	125	114	
		+	—	10^3	103	103		
		—	+	$2 \cdot 10^3$	171	86	64	56
		—	+	10^3	43	43		
8	14	+	—	$2 \cdot 10^3$	307	154	158	
		+	—	$5 \cdot 10^2$	81	162		
		—	+	$2 \cdot 10^3$	214	107	108	68
		—	+	$5 \cdot 10^2$	54	108		
9	14	+	—	$5 \cdot 10^2$	130	260		
		—	+	$5 \cdot 10^2$	93	186		72
		+	+	$5 \cdot 10^2$	134	268		103
10	12	+	—	$5 \cdot 10^2$	110	220		
		—	+	$5 \cdot 10^2$	50	100		46
		+	+	$5 \cdot 10^2$	91	182		83
11	11	+	—	10^3	447	447		
		—	+	10^3	301	301		67

*Incubation with unlabeled thymidine or with isotope for 2 h.

†Mean number of colony forming units per flask subjected to recloning.

Thymidine-H^3 with a specific activity of 19-25 Ci/mmole (UVVVR, Czechoslovakia) was used. The cells were incubated with the isotope for 30 min at 37°C.

Treatment of the Cell Suspensions and Cultures with Thymidine-H^3

1. A suspension of cells of freshly isolated bone marrow was made up in Hanks's solution at 37°C and the concentration adjusted to $5 \cdot 10^6$ cells/ml. Thymidine-H^3 was added to one portion of the suspension in a dose of 50 μCi to 1 ml of suspension, and another (control) portion was treated with unlabeled thymidine at the rate of 100 μg/ml suspension. The third portion, the isotope toxicity control, was treated with a mixture of unlabeled thymidine and thymidine-H^3 in the proportions specified above. At the end of incubation, unlabeled thymidine was added to the tube containing the isotope at the rate of 100 μg/ml and all three suspensions were placed on ice. The cells were then washed three times in cold medium No. 199 with the addition of 10% embryonic calf serum and unlabeled thymidine at the rate of 20 μg/ml medium. The cell suspension was explanted in flasks in doses of $2 \cdot 10^6$–$5 \cdot 10^6$ cells.

2. After the culture medium had been poured off, flasks containing 7-14-day primary monolayer bone marrow cultures were washed with Hanks's solution warmed to 37°C. Thymidine-H^3 was added to the flasks of group 1 containing a fresh portion of Hanks's solution in a dose of 50 μCi/ml solution, the flasks of group 2 (control) were treated with unlabeled thymidine at the rate of 100 mg/ml solution, and the flasks of group 3 were treated with a mixture of thymidine-H^3 and unlabeled thymidine.

After incubation and washing 3 times with cold medium No. 199 containing 20 μg/ml unlabeled thymidine and 10% embryonic calf serum, the cells were removed from the flasks with trypsin and recloned in doses of $0.5 \cdot 10^3$ to $12.5 \cdot 10^3$ cells per flask.

After treatment with thymidine-H^3 the bone marrow cells and the cloned fibroblasts were grown on ordinary medium, with the addition of unlabeled thymidine at the rate of 20 μg/ml medium daily in order to prevent reutilization of the isotope. Bone marrow cells irradiated in a dose of 4000 R ($2 \cdot 10^7$ cells per flask) were used as the feeder to create the necessary initial cell density. On the tenth day the cultures were fixed with ethanol, stained by the Romanovsky–Giemsa method, and the number of colonies of fibroblasts containing no fewer than 50 cells growing in the flasks was counted.

EXPERIMENTAL RESULTS

The results of these experiments are given in Tables 1 and 2. The cloning efficiency of cells treated with unlabeled thymidine only was taken as 100%. The number and size of the colonies growing on suspensions of bone marrow cells treated either with isotope or with unlabeled thymidine were identical (Table 1). Hence it follows that cells from which colonies of fibroblasts were formed after primary explantation of bone marrow did not incorporate thymidine-H^3, i.e., they belonged to the category of cells not proliferating in vivo.

After incubation of 7-14-day bone marrow cultures for 30 min with the isotope the number of colonies formed on recloning was reduced by 39 ± 4% (by 53 ± 5% after incubation for 2 h) compared with the control (Table 2). Hence it follows that about 40% of clonogenic cells included in the colonies were at any given moment in the S phase of the cell cycle. Since the cultures were not synchronized, these results confirm that virtually all the clonogenic cells in the colonies belonged to the proliferative pool No significant difference could be found in the reduction of the number of colony-forming cells after the addition of thymidine-H^3 to cell cultures aged 6-7 or 11-14 days.

It thus follows from these results that precursor cells for fibroblast colonies contained in primary bone marrow cultures proliferate actively, unlike precursors in native bone marrow.

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