Colony promoting activity and its target 'pre-CFUc' in genetically anemic mice of W/W^v genotype[1]

T. Miyanomae, M. Tsurusawa, H. Izumi, S. Aizawa, K.J. Mori and Y. Kitamura

Department of Microbiology, Faculty of Medicine, Kyoto University, Kyoto 606 (Japan), and Institute for Cancer Research, Medical School, Osaka University, Osaka 530 (Japan), October 11, 1982

Summary. Incubation of bone marrow cells with supernatant from long-term cultures of bone marrow cells increases the number of granulocyte-macrophage progenitor cells. This study reveals the presence of target cells of the colony promoting activity (CPA) in W/W^v mouse marrow. It is also shown that CPA does not stimulate erythroid colony formation in vitro.

Supernatants of long-term bone marrow cultures contain colony promoting activity (CPA)[2,3]. Incubation of murine bone marrow cells with CPA increases the number of granulocyte-macrophage progenitor cells (CFUc)[3], but further differentiation of CFUc to granulocytes and/or macrophages requires colony stimulating activity (CSA)[2,3] thus indicating that the target cell of CPA is a population younger than CFUc. Although supernatant with CPA activity is suppressive to proliferation of pluripotent stem cells (CFUs)[3], a subpopulation of CFUs may be one of the target cells of CPA. Increase in the number of CFUc in W/W^v marrow cells in response to CPA would indicate that cells other than CFUs are the targets of CPA, since W/W^v mice are depleted of genuine CFUs[4,5]. We found that such target cells did exist in these mice, and also showed an elevated activity of CPA in the supernatant of the long-term cultures of W/W^v marrow cells.

Materials and method. Mice of the strain WBB6F$_1$(WB-$W/+$ X C57BL/6-$W^v/+$) F$_1$-W/W^v were obtained from Jackson Laboratory, Bar Harbor, Maine. Bone marrow cells from W/W^v and from their littermate ($+/+$) mice were cultured as reported previously[6-8]. Cells from femur and tibia of a $+/+$ mouse were flushed directly into a culture flask (Falcon 3024, USA) in 10 ml Fischer's medium supplemented with 20% horse serum (Pel Freeze Biol., USA), antibiotics and 10^{-7} M hydrocortisone sodium phosphate, and cultures were maintained at 37 °C in 5% CO_2 in humidified air. One half of the growth medium was replaced with the same volume of fresh growth medium at weekly intervals. Three weeks later, freshly harvested bone marrow cells were added at a concentration of 5×10^5 cells/ml to the culture flask. Weekly feeding was continued as before, and supernatants of the culture media were collected at each refeeding and assayed for colony promoting activity. Supernatant thus collected was added to soft-agar cultures of bone marrow cells for CFUc assay in the presence of mouse lung and heart-conditioned medium as a source of CSA[7,8], and colony promoting activity was expressed as the ratio of the number of colonies formed in the presence of supernatant to that in the absence of the supernatant. Since most of the supernatants from $+/+$ mouse marrow cultures and from marrow cultures of other strains of mice showed a similar activity, they were pooled together and used for the present study as $+/+$ supernatant. CFUe, BFUe and CFUmix were assayed using techniques described by Hara and Ogawa[9]. Marrow cells from a BDF1 mouse were incubated in a mixture containing α-medium, 0.8% methylcellulose, 1% bovine serum albumin, 30% fetal calf serum, 1×10^{-4} M mercaptoethanol, 2.0 µg/ml of step III preparation of sheep plasma erythropoietin and 15% spleen conditioned medium. Small erythroid colonies on day 2[10] and erythropoietic bursts on day 9[11], mixed colonies containing erythroid subcolonies and more than 50 large cells were scored on day 14[9]. Spleen conditioned medium (SCM) was made from murine spleen cells according to the method described previously[9].

Results and discussion. Addition of the supernatant of long-term cultures of $+/+$ marrow cells increased the number of granulocyte-macrophage colonies in soft-agar culture of W/W^v marrow (table 1), indicating the existence of the target cells of CPA in hemopoietic tissues of W/W^v mice. The 2nd experiment was designed to elucidate whether or not supernatant from long-term cultures of W/W^v marrow cells contain CPA. Supernatant from W/W^v marrow cultures had sufficient activity to increase the number by a factor of 2.2, whereas supernatant from $+/+$ marrow cultures increased the number by a factor of 1.45 (table 2). This elevated level of CPA was confirmed by separate experiments, and also by soft-agar culture of $+/+$ marrow cells as target (table 2).

Our results demonstrate the presence of CPA in long-term cultures of W/W^v marrow cells and its target cells in hemopoietic tissues of W/W^v mice. CPA induces the differentiation or maturation of CSF-nonresponsive cells (pre-CFUc) to CSF-responsive CFUc with or without cell growth, but further differentiation of the CFUc requires CSA[2,3]. Early studies have suggested the presence of CPA and immature granulocyte-macrophage progenitor cells or 'pre-CFUc' being its target[12,13]. Moreover, the state of

Table 1. Effects of CPA on granulocyte/macrophage colony formation by bone marrow cells from $+/+$ and W/W^v mice

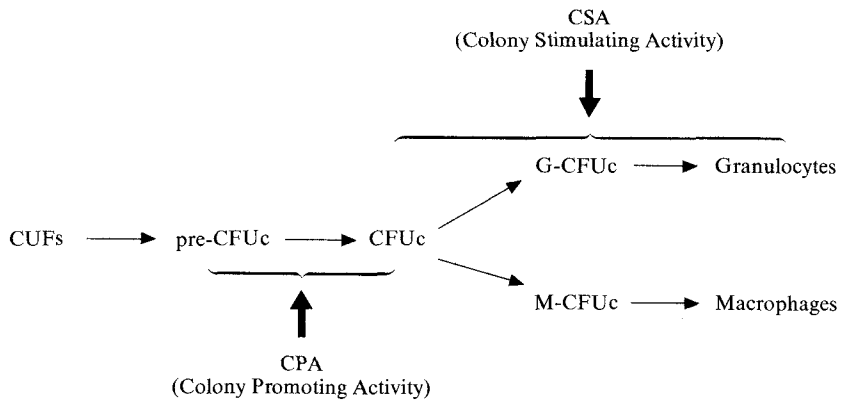
	No. of colonies/10^5 cells	
	$+/+$ marrow	W/W^v marrow
CSA alone*	128.1 ± 10.3	133.3 ± 6.3
CSA + CPA**	201.0 ± 5.8	219.5 ± 3.3
Ratio (CPA$^+$/CPA$^-$)	1.57	1.65
CPA alone (50%)	0	0

Bone marrow cells were incubated in semi-solid agar cultures with or without CPA. Gross average of triplicate experiments. *Mouse lung and heart-conditioned medium was used as CSA at the final concentration of 10%. **Supernatant from long-term culture of $+/+$ mouse marrow cells was added at the final concentration of 20%.

Table 2. Presence of CPA and its target in $+/+$ and W/W^v mice

Supernatant from*	No. of colonies/10^5 $+/+$ marrow cells			No. of colonies/10^5 W/W^v marrow cells		
	CSA alone	CSA + 20% sup.	Ratio**	CSA alone	CSA + 20% sup.	Ratio**
$+/+$ marrow culture	148.2 ± 16.5	209.4 ± 8.8	1.41	152.5 ± 11.2	221.8 ± 8.6	1.45
W/W^v marrow culture		342.7 ± 21.1	2.31		338.5 ± 14.9	2.22

Bone marrow cells were incubated in semi-solid agar culture with or without supernatant from long-term bone marrow culture. Gross average of 7 separate experiments. *Supernatant from 4-14-week cultures. **Ratio = (sup. +/sup. −).



Maturation sequence from CFUs to granulocytes and macrophages

maturation of CFUc subpopulations has been determined[14]. However, these observations do not rule out the possibility that a subpopulation of CFUs may be one of the targets of CPA, since some of the CFUs can also give rise to granulocyte-macrophage colonies in the semi-solid culture system[15,16].

Mice of W/W^v genotype are known to be depleted of genuine CFUs. Transplantation of bone marrow cells from W/W^v mice does not give rise to macroscopical colonies in the spleens of lethally irradiated recipient mice[4,17]. Accordingly, the presence of comparable numbers of 'pre-CFUc' in W/W^v marrow cells and in +/+ marrow cells might exclude the possibility that the target of CPA is CFUs. Indeed, there were as many target cells of CPA in W/W^v marrow ($86/10^5$ cells) as in +/+ marrow ($73/10^5$ cells) (calculated from the data in table 1). It is also worth remarking that W/W^v marrow cells are capable of producing CPA to the same extent as +/+ marrow cells (table 2).

However, previous studies have shown the presence of microscopic colonies in the spleens of irradiated recipients receiving W/W^v marrow cells[17,18]. It has also been shown that W/W^v marrow contains many pluripotent hemopoietic precursors in vitro (CFU-mix) which are closely related to CFUs[19]. Thus, the results do not rule out the possibility of the CPA-responsive cells being one of these precursor populations. The effect of CPA on CFUmix and erythroid colony formation was studied to clarify the possibility. As summarized in table 3, CPA did not stimulate the proliferation and maturation of CFUmix, BFUe or CFUe. On the contrary, CPA was rather suppressive to the proliferation of these progenitor cells. It has also been reported that CPA does not stimulate the proliferation of CFUs[3]. Accordingly it may be concluded that CPA acts on myeloid committed progenitors.

Based on the present findings and previous observations[2,3,12,14], it can be hypothesized that CPA induces maturation of 'pre-CFUc' to CSA-responsive CFUc but not any further, while CSF plays an essential role in the terminal differentiation to granulocytes and/or macrophages (fig.). We have recently shown the presence of CSA in long-term marrow cultures[20]. Shadduck et al.[21] also found CSA in their cultures. Thus, it is most likely that granulopoiesis in long-term bone marrow cultures is controlled by 2 different humoral regulators, namely CPA and CSA, and that both regulators act in sequential co-operation.

Table 3. Effect of CPA on erythroid colony formation in vitro

	No. of colonies/10^5 bone marrow cells		
	CFU-mix	BFUe	CFUe
SCM (5%)	6.8 ± 1.0	15.7 ± 3.6	48.1 ± 9.0
Sup. (CPA) (5%)	0	0	0
SCM + Sup.	0	5.7 ± 2.3	8.5 ± 3.4

Bone marrow cells were incubated in 0.8% methylcellulose in alpha medium in the presence of erythropoietin with further addition of spleen conditioned medium (SCM) and/or supernatant from long-term bone marrow culture containing CPA. Average of duplicate experiments.

1 This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

2 Tsurusawa, M., Miyanomae, T., Izumi, H., and Mori, K.J., Leuk. Res. (1983) in press.
3 Izumi, H., Tsurusawa, M., Miyanomae, T., Kumagai, K., and Mori, K.J., Leuk. Res. (1983) in press.
4 McCulloch, E.A., Siminovitch, L., and Till, J.E., Science *144* (1964) 144.
5 Mori, K.J., Kitamura, Y., and Seto, A., Biomedicine *35* (1981) 125.
6 Dexter, T.M., Allen, T.D., and Lajtha, L.G., J. Cell Physiol. *91* (1977) 335.
7 Mori, K.J., Fujitake, H., Okubo, H., Ito, Y., and Dexter, T.M., Gann *69* (1978) 689.
8 Bradley, T.R., and Metcalf, D., Aust. J. exp. Biol. med. Sci. *44* (1966) 287.
9 Hara, H., and Ogawa, M., Am. J. Hemat. *4* (1978) 23.
10 Stephenson, J.R., Axelrad, A.A., McLead, D.C., and Shreeve, M.M., Proc. natl Acad. Sci. *16* (1971) 1542.
11 Iscove, N.N., and Sieber, F., Exp. Hemat. *3* (1975) 32.
12 Van den Engh, G.J., and Bol, S., Cell Tissue Kinet. *8* (1975) 579.
13 Wagemaker, G., and Peters, M.F., Cell Tissue Kinet. *11* (1978) 45.
14 Bol, S., and Williams, N., J. Cell Physiol. *102* (1980) 233.
15 Dicke, K.A. Platenburg, M.G.C., and Van Bekkum, D.W., Cell Tissue Kinet. *4* (1971) 463.
16 Miyanomae, T., Tsurusawa, M., Mori, K.J., Mikawa, H., and Ito, Y., Gann *73* (1982) 408.
17 Lewis, J.P., O'Grady, L.F., Bernstein, S.E., Russell, E.S., and Trobaugh, F.E., Blood *30* (1967) 601.
18 Kitamura, Y., Kawata, T., Okano, K., Asayama, S., and Seki, M., Cell Tissue Kinet. *8* (1975) 145.
19 Hara, H., Ohe, Y., Noguchi, K., Nagai, K., Tsuyama, K., and Kitamura, Y., Cell Tissue Kinet. *15* (1982) 25.
20 Izumi, H., Miyanomae, T., Tsurusawa, M., Fujita, J., and Mori, K.J., Leuk. Res., (1983) in press.
21 Shadduck, R.K., Pigoli, G., Waheed, A., and Boegel, F., J. supramolec. Struct. *14* (1980) 423.

0014-4754/83/070776-02$1.50 + 0.20/0