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MODULATION OF NORMAL MYELOPOIESIS IN VITRO BY RETINOIC ACID

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SUMMARY: The effects of retinoic acid (RA) on the proliferation and differentiation of normal myeloid progenitor cells (CFU-C) were studied. In general, RA at 10^{-10} to 10^{-6} M enhanced primary myeloid colony formation in the presence of colony-stimulating factor(s). However, macrophage colony formation was strongly inhibited by RA. This may be related to the finding that RA is able to differentiate bipotential HL-60 cells into granulocytes but not into macrophages. Moreover, secondary colony formation was always suppressed by the addition of RA to the primary cultures. It means that self-renewal capacity of CFU-C was suppressed by RA. This finding suggests that normal myelopoiesis will be suppressed eventually by RA.

Leukemia cells are characterized by their impaired maturation and differentiation. These cells can be induced to differentiate by several chemical agents, including dimethyl sulfoxide (1), phorbol esters (2), retinoic acid (RA) (3), and 1, 25-dihydroxyvitamin D_3 (4). Since RA has been reported to enhance normal hemopoiesis in vitro (5, 6) and inhibit leukemic cell proliferation (7), therapeutic usefulness of RA in leukemia is currently discussed (8). However, sufficient information has not been obtained about the effects of RA on normal myelopoiesis in vitro. In the present study, we have examined the effects of RA on the proliferation and differentiation of normal myeloid progenitor cells (CFU-C) using several different sources of colony-stimulating factor(s) (CSF).

MATERIALS AND METHODS

Normal Myeloid Colony Formation: Bone marrow specimens were obtained from normal human volunteers after written informed consent. Single-layer soft agar cultures were performed according to the method of Robinson et al. (9) with minor modifications. Mononuclear cells were isolated by Ficoll-Metrizoate (Lymphoprep; Nyegaad, Oslo, Norway) density centrifugation at 400 x g. Sheep erythrocyte (E)-rosette-forming cells were subsequently removed. Cells from the E-rosette-negative fraction were cultured at a concentration of 3×10^4 cells/ml in 0.3% agar medium containing 20% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.) and 10% CSF.

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Four different sources of CSF were used in this study. Phytohemagglutinin-stimulated lymphocyte-conditioned medium (PHA-LCM) was obtained from the supernatants of cultured lymphocytes (1 x 10^6 cells/ml) incubated for 7 days in alpha-medium with 10% fetal calf serum and 1% PHA (Wellcome HA-15). Human placental conditioned medium (HPCM) was prepared according to the method of Burgess et al. (10). GCT-CM (Grand Island Biological Co., Grand Island, N.Y.) was derived from a giant-cell tumor cell line (11). Cystic fluid containing CSF (Cystic F.) was obtained from a transplanted CSF-producing tumor in nude mice (12). Culture plates were incubated for 7 or 14 days at 37°C in a fully humidified atmosphere containing 5% CO_2 in air. Colonies containing 40 or more cells were counted under an inverted microscope. Permanent preparations were made (13) and colony types were determined by dual esterase staining (14) and Biebrich-Scarlet staining (15).

Effects of Retinoids on Colony Formation: Stock solutions of RA and retinol (Sigma Chemical Co., St. Louis, Mo.) were prepared in 100% ethanol at 10^{-3} M and stored at -20°C. These were diluted to the required concentration in alpha-medium. The effects of retinoids on primary colony formation were tested by the direct addition to the culture mixture at concentrations ranging from 10^{-14} to 10^{-6} M. Ethanol equivalent to that present in the final concentration of RA had no effect on colony growth. To investigate the efect of RA on self-renewal capacity of CFU-C, secondary colonies were formed. Briefly, 5 x 10^4 cells were cultured in the presence or absence of RA (10^{-7} M) in 1 ml of 0.8% methylcellulose (Dow Chemical Co., Midland, Mich.) medium instead of agar-medium under the otherwise same conditions. After 7 days of incubation, colonies were pooled, washed, resuspended, and then used for secondary colony formation. These cells were cultured for further 7 days without the addition of RA in 0.3% agar-medium containing 10% GCT-CM. Secondary colonies formed from 5 x 10^4 cells initially plated in primary cultures were counted.

RESULTS

Effect of RA on Primary Colony Formation

Addition of RA to normal bone marrow cultures enhanced the formation of myeloid colonies stimulated by PHA-LCM, GCT-CM, or Cystic F. (Table 1). Apparent enhancement by RA was observed at concentrations ranging from 10^{-10} to 10^{-6} M on Day 14 of culture (Fig. 1). In the HPCM-stimulated cultures,such enhancement was not observed. RA alone did not stimulate colony growth (data not shown). Addition of retinol instead of RA as a control had no effect on colony growth. Analysis of colony types revealed that macrophage colony formation was strongly inhibited by RA. In the PHA-LCMstimulated cultures, neutrophilic and eosinophilic differentiation besides macrophage differentiation was inhibited by RA and the proportion of esterase-negative colonies markedly increased. Retinol had no effect on colony morphology (data not shown).

Table 1. Effects of Retinoic Acid (RA) on Colony Morphology

Source of CSF	RA (M)	No. of colonies (% of control)	Colony morphology[a] (%)						
			N		NM	M		Est(-)	Eo
			+	±		+	±		
PHA-LCM	0	100	4	8	23	40	5	21	20
	10^{-7}	183	1	3	2	0	13	81	6
HPCM	0	100	82	0	0	2	0	16	17
	10^{-7}	98	80	2	0	0	0	18	15
GCT-CM	0	100	71	0	0	13	0	16	14
	10^{-7}	165	81	0	0	0	3	16	11
Cystic F.	0	100	72	1	0	20	0	7	10
	10^{-7}	131	83	1	0	0	1	15	7

[a] Colony morphology was evaluated by dual esterase and Biebrich-Scarlet stainings. N, neutrophilic colonies; NM, neutrophil-macrophage mixed colonies; M, monocyte-macrophage colonies; Est(-), esterase-negative colonies; Eo, eosionophilic colonies. Most of esterase-negative colonies were eosinophilic ones. Esterase activity: +, strongly positive; ±, weakly positive.

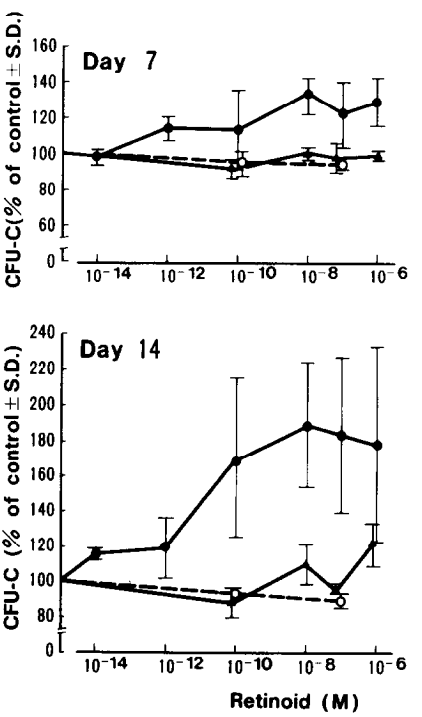

Fig. 1. Effects of retinoids on CFU-C growth. Bone marrow cells were cultured in the presence of 10% PHA-LCM and retinoic acid (●), 10% PHA-LCM and retinol (○), or 10% HPCM and retinoic acid (▲). Colonies were counted on Day 7 or on Day 14 of culture.

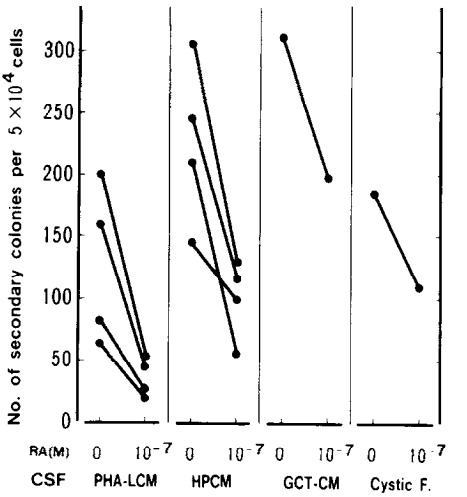

Fig. 2. Effects of retinoic acid (RA) on self-renewality of CFU-C. Primary cultures were done in the presence or absence of 10^{-7} M RA in methylcellulose-medium containing different kind of CSF. Secondary cultures were done without the addition of RA in agar-medium containing 10% GCT-CM. Further details are described in "MATERIALS AND METHODS"

Effect of RA on Secondary Colony Formation

To investigate the effect of RA on self-renewal capacity of CFU-C, secondary colonies were formed. As shown in Fig. 2, the number of secondary colonies decreased when 10^{-7} M RA was added to the primary cultures. The results were almost similar among the four different sources of CSF.

DISCUSSION

The accumulation of data on the effects of RA on normal hemopoiesis may be important, because a therapeutic trial of RA in patients with leukemia has started. As reported by Douer et al. (5), RA generally enhanced normal myeloid colony formation. However, the effects of RA were variable, depending on the sources of CSF used. Particularly, in the PHA-LCM-stimulated cultures, marked changes in colony morphology were observed. Since PHA-LCM contains many kinds of lymphokines, some factors other than CSF may be involved in RA-mediated modulation of colony formation. The analysis of colony types revealed that macrophage colony formation was preferentially suppressed by RA. This seems to be related to the finding that RA has the ability to differentiate bipotential HL-60 cells into granulocytes but not into macrophages (3).

To understand eventual effect of RA on normal myelopoiesis, it is important to examine the effects of RA on self-renewality of CFU-C. In the present study, secondary colony formation was always suppressed by the addition of RA to the primary cultures. It is possible that RA-induced CFU-C growth was accompanied by their enhanced maturation and that, as a result, self-renewal capacity of CFU-C was suppressed. This finding suggests that normal myelopoiesis will be suppressed eventually by RA. This point may become an issue when RA is actually used as a therapeutic agent in patients with leukemia. To compare the in vitro effect of RA on leukemic growth with that on normal myelopoiesis will be important to predict the clinical response to RA in individual cases.

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