PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **179**, 283–287 (1985)

Correlation between Production of Colony-Stimulating Activity (CSA) and Adipose Conversion in a Murine Marrow-Derived Preadipocyte Line (H-1/A)[1] (42097)

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Abstract. The correlation between adipose conversion of cloned H-1 cells (H-1/A) and their production of colony-stimulating activity (CSA) was examined. The production of CSA from H-1/A cells declined after adipose conversion, although H-1/A cells are active producers of CSA during their fibrocytic stage. The addition of 2×10^{-5} *M* 5-bromo-2′-deoxyuridine to the cultures almost completely inhibited adipose conversion and there was no reduction of CSA levels after 9 days of culture. On the other hand, the addition of 10^{-6} *M* hydrocortisone sodium succinate to the culture markedly enhanced adipose conversion, and a greater reduction in the CSA level was observed in the supernatants than in the control cultures after 12 days of culture. Indomethacin had no effect on the production of CSA or on adipose conversion. Furthermore, there were no significant differences between the CSA levels of nondialyzed supernatants and dialyzed supernatants from the control cultures during the entire course of the experiment. Supernatants during the adipocyte stage of H-1/A cultures did not inhibit the CSA derived from the fibrocytic stage. There were no differences in colonies in agar cultures stimulated by supernatants derived from cultures that had undergone either of the above treatments. These results suggest that the reduction of CSA is not due to the production of inhibitors, but that the production of CSA declines after adipose conversion of H-1/A cells. Preadipocytes in bone marrow therefore appear to contribute to granulopoiesis during the fibrocytic stage and are hematopoietically inactive when they convert to adipocytes.

The maintenance and growth of murine hematopoietic stem cells in long-term liquid bone marrow culture systems (1) are dependent on a population of adherent cells. In this system, the adherent layer consists of a mixture of mononuclear phagocytic cells, epithelioid cells, fibroblasts, and fat-containing cells. Morphological investigation of *in vitro* hematopoiesis indicates that granulopoiesis is seen in the vicinity of the fat-containing cells (2).

A cell line (H-1) derived from the adherent cell layer of a 14-week-old Dexter bone marrow culture has been established (3). Functional and cytochemical studies suggest that this cell line is derived from the adventitial reticular cells of the bone marrow (4). This cell line develops many fat droplets as it ages and becomes confluent. Feeder layers or supernatants from this line markedly stimulate the formation of colonies derived from CFUc (3). The present study deals with the effects of adipose conversion of cloned H-1 cells (H-1/A) on the production of colony-stimulating activity (CSA).

Materials and Methods. H-1/A cells were cloned from original H-1 cells by dilution plating and then routinely cultured with Fisher's medium (GIBCO, Grand Island, N.Y.) supplemented with 10% horse serum (Irvine Scientific, Santa Ana, Calif.) and antibiotics (100 μg streptomycin and 100 units penicillin/ml, GIBCO) in an atmosphere of 5% CO_2/95% air at 33°C. They were subcultured weekly at a split ratio 1:8 using trypsin–EDTA dispersant (GIBCO).

For experimental purposes, 25-cm^2 plastic flasks (Corning Co., Corning, N.Y.) were inoculated with 1×10^5 cells in 5 ml of growth medium. In some cases, the growth medium was supplemented with the following test substances: 10^{-6} to 2×10^{-5} *M* concentrations of 5-bromo-2′-deoxyuridine (BrdU, Sigma Chemical Co., St. Louis, Mo.), 10^{-7} to 10^{-5} *M* concentrations of indomethacin (Sigma), or 10^{-6} to 10^{-10} *M* concentrations

[1] Research supported by Grant-in-Aids for Science Research from Ministry of Education, Science and Culture in Japan (57570154, 58480163, 58570178).

0037-9727/85 $1.50

of hydrocortisone (HDC, Upjohn Co., Kalamazoo, Mich.).

To develop the growth curve, single cell suspensions were prepared from two flasks on Days 1, 2, 3, 4, 5, 6, 9, 12, 15, 18, and 21, using trypsin–EDTA dispersant. The numbers of cells were counted by a hemocytometer.

To estimate adipose conversion of H-1/A cells, two flasks were fixed with 10% buffered neutral Formalin and stained with 0.5% oil red O solutions in propylene glycol on Days 3, 6, 9, 12, 15, and 21. Four photomicrographs were randomly taken at each time point using the Nikon TMD inverted microscope (Nihon Kogaku Co., Tokyo) under ×130 magnification. Three hundred cells were then counted in each photograph and the percentage of adipocytes was calculated.

The growth medium was replaced with fresh medium and the supernatant medium was collected on Days 3, 6, 9, 12, 15, 18, and 21. The media were centrifuged at 800*g* for 10 min: parts of these samples were dialyzed for 24 hr against 200 vol of Dulbecco's phosphate-buffered saline (GIBCO) through tubing with a molecular weight cutoff of 6000 to 8000 (Spectrum Medical Industries, Inc., Terminal Annex, Los Angeles, Calif.). All samples were stored at −20°C until G/M–CFUc assay after filtration through a 0.2-μm membrane filter (Gelman Science Inc., Ann Arbor, Mich.).

CFUc were allowed to proliferate in semisolid soft agar cultures as described (5). Fresh murine bone marrow cells were obtained from 8- to 12-week-old female C57BL/6 mice by flushing the tibias and femurs. One hundred thousand fresh marrow cells were inoculated in 1 ml of the complete McCoy's medium containing 0.3% agar (Difco Laboratories, Detroit, Mich.) and 10% appropriate conditioned media in 35-mm plastic petri dishes (Lux, Lab. Tek Division, Miles Laboratories, Inc., Napenville, Ill.). Pokeweed-mitogen-stimulated spleen cell conditioned medium (PWM-SCM) was used as a positive control (6). In some experiments various conditioned media prepared from adipocytes stage of H-1/A cultures were added to the CFUc cultures stimulated by either PWM-SCM or 9-day H-1/A CM. After incubation for 7 days at 37°C in an atmosphere of 7.5% CO_2 in humidified air, the specimens were fixed with absolute methanol, air-dried, and stained with Harris' hematoxylin. Colonies of greater than 50 cells were scored with the aid of a Nikon optical microscope (Nihon Kogaku Co., Tokyo).

Half of the agar cultures were processed for enzyme–histochemical analysis by the whole plate technique (7) with some modification. After fixation the specimens were stained by means of a double-staining technique using naphthol–ADS–chloroacetate and α-naphthyl butyrate as substrate (8). Colonies mainly composed of naphthol–ASD–chloroacetate esterase (Cl-E) positive cells were designated as granulocyte colonies (G-CFUc), and colonies containing both Cl-E positive and α-naphthyl-butyrate esterase (N-E) positive cells were designated as granulocyte–macrophage colonies (G/M-CFUc). Colonies composed of N-E positive cells were designated as macrophage colonies (M-CFUc).

Results. *Growth of H-1/A cells.* The H-1/A cell line showed logarithmic growth from Day 3 to 6 of culture with an initial lag phase of about 48 hr. The number of cells reached a plateau level on Day 9. The mean doubling time of this cell line during the logarithmic growth period was about 24 hr.

The addition of BrdU did not have a significant effect on the growth of H-1/A cells in any of the concentrations used ($P < 0.05$). HDC (10^{-10} to 10^{-6} M) had an inhibitory effect on the growth of H-1/A cells and the cultures did not reach a plateau level as the control group did. The inhibitory effect was dose dependent. HDC was added to the cultures on the sixth day after inoculation when the cells reached subconfluence. This made for similar numbers of cells per flask in various HDC-treated cultures. Irrespective of concentration, indomethacin did not influence the growth of H-1/A cells throughout the 3 weeks of the culture period.

Adipose conversion of H-1/A cells. The routinely maintained H-1/A cells had a fibroblastoid morphology and were split when the cultures reached subconfluence. In control cultures, the cells had a fibroblastoid morphology until they reached confluence after 6 days of culture. Then, they accumulated fat droplets in their cytoplasm. A few adipocytes were found before Day 7 of culture;

the percentage of adipocytes rose rapidly after Day 9, reaching about 60 and 70 on Day 15 and 21, respectively (Fig. 1). One hundred percent adipose conversion was not attained at any time during the observation period of 2 months. The addition of a 2×10^{-5} *M* concentration of BrdU completely inhibited the adipose conversion of H-1/A cells (Fig. 1). Inhibition of adipose conversion in the cultures with BrdU was dose dependent within the concentration range of 10^{-6} to 2×10^{-5} *M*. HDC enhanced the adipose conversion of H-1/A cells (Fig. 1). The frequency of adipocytes in the H-1/A cell cultures with 10^{-6} *M* HDC was over 90% on Day 18 of culture. This enhancement was dose dependent within the concentration range of 10^{-10} to 10^{-6} *M* of HDC. Indomethacin had no significant effect on the adipose conversion of H-1/A cells.

CSA levels in the supernatants from H-1/A cell cultures whose adipose conversion was modulated with BrdU or HCD. Control cultures: The production of CSA increased quickly after 3 days of culture, peaking on Day 9 and decreasing by the 12th day (Fig. 2). After 15 days of culture, the production of CSA remained constant at about 60% of the maximum level. There was no significant difference between the CSA levels of nondialyzed supernatants and dialyzed supernatants

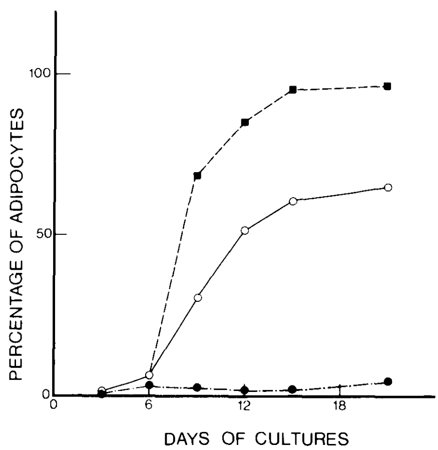

FIG. 1. Adipose conversion of H-1/A cells. Mean percentage of adipocytes ($n = 4$) in H-1/A cells cultured with 10^{-6} *M* hydrocortisone sodium succinate (HDC, ■) and 2×10^{-5} *M* 5-bromo-2′-deoxyuridine (BrdU, ●). (○) Control cultures.

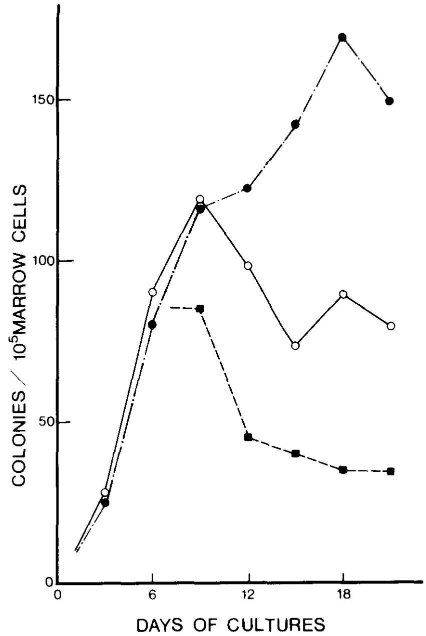

FIG. 2. Colony-stimulating activity (CSA) in the supernatants from H-1/A cultures treated with 2×10^{-5} *M* BrdU (●), 10^{-6} *M* HDC (■), and control cultures (○). CSA determinations are expressed as mean numbers of colonies/10^5 marrow cells ($n = 4$).

from the control cultures (Table I). When supernatants from Days 9, 15, 18, and 21 of H-1/A cultures were added to CFUc cultures stimulated by 9-day H-1/A CM, the number of CFUc-derived colonies was not significantly different in each group (Day 9: 168 ± 24, Day 15: 148 ± 17, Day 18: 152 ± 18, Day 21: 158 ± 13). The CFUc-derived colonies stimulated by PWM-SCM were not inhibited either.

Cultures with BrdU: CSA production did not decline during the 21-day observation period in the cultures to which 2×10^{-5} *M* BrdU was added (Fig. 2). Before the 10th day of culture, the production of CSA was practically the same in the 2×10^{-5} *M* BrdU-treated cultures and the control cultures. Thereafter, CSA levels in the BrdU-treated cultures gradually increased and reached a constant level after 15 days of culture, while CSA levels in the control cultures declined to approximately 60% of the maximum level. The production of CSA in BrdU-treated cultures increased dose dependently when the

TABLE I. COLONY-STIMULATING ACTIVITY (CSA) OF NONDIALYZED AND DIALYZED SUPERNATANTS FROM CONTROL H-1/A CULTURES

Days of culture	CSA	
	Nondialyzed*	Dialyzed*
3	36 ± 13	28 ± 7
6	63 ± 14	90 ± 10
9	92 ± 10	119 ± 14
12	68 ± 15	98 ± 8
15	66 ± 19	73 ± 11
18	75 ± 9	89 ± 8
21	76 ± 8	79 ± 13

Note. CSA determinations are expressed as number of colonies/10^5 marrow cells (M ± SD, $n = 4$).

* No statistical difference between the CSA levels of nondialyzed supernatants and dialyzed supernatants at these time points ($P < 0.05$).

cultures were treated with 10^{-6} to 2×10^{-5} *M* BrdU. Nondialyzed conditioned media from BrdU-treated cultures showed a reduction of about 60% in CSA compared to the dialyzed conditioned media.

Cultures with HDC: After 9 days of culture, CSA levels were constantly lower in the dialyzed supernatants from cultures with 10^{-6} *M* HDC than in the control culture group (Fig. 2). This reduction was dose dependent and 10^{-10} *M* HDC had no practical effect on the production of CSA in H-1/A cells. There was a 70% reduction in CSA levels in the culture on Day 18, when cultures containing 10^{-6} *M* HDC reached nearly 100% adipose conversion.

Cultures with indomethacin: During the 21-day observation period there was no significant difference between CSA levels in nondialyzed and dialyzed supernatants from H-1/A cultures with or without 10^{-7} to 10^{-5} *M* indomethacin (data not shown).

Types of CFUc-derived colonies in agar cultures stimulated by supernatants from different groups of H-1/A cultures. When supernatants from control H-1/A cultures were used as a source of CSA in agar cultures, types of colonies were 6% G, 12% G/M, and 82% M, irrespective of the age of the H-1/A cell cultures. This variation in colonies was unaffected by supernatants from H-1/A cultures when different treatments were used.

Discussion. Several investigators observed that levels of CSA declined after the adipose conversion of fibrocytic marrow stromal cell lines but the precise nature of the reduction of CSA has remained unresolved (9, 10). The present studies showed that adipose conversion of marrow preadipocyte line (H-1/A) reduces the production of CSA, although these cells are avid producers of CSA during the fibrocytic stage. When adipose conversion was completely inhibited by BrdU, CSA levels remained maximal in the supernatants even after 15 days of culture. Furthermore, the acceleration of such adipose conversion by HDC resulted in further reductions of CSA levels.

The H-1 cell line produced labile inhibitors of CFUc (4), but these inhibitory substances were not detected in the culture supernatants of H-1 cells. Our results suggested that no inhibitory effect of prostaglandin E was detected on the formation of CFUc-derived colonies stimulated by conditioned medium of the adipocyte stage of H-1/A cells. It was also unlikely that H-1/A cells produced other factors inhibitory to CSA during their adipocyte stage, because there was not a substantial reduction in the number of colonies when the supernatant medium in the adipocyte stage was added to the CFUc cultures stimulated by either PWM-SCM or 9-day H-1/A CM. The types of colonies were constant, irrespective of the different treatments or different ages of H-1/A cultures from which the supernatants were obtained. These indicate that the reduction of colony-stimulating activity is not due to the production of inhibitors, but that the production of CSA declines in the adipocyte stage of H-1/A cells.

It is well known that adventitial reticular cells become fatty when hematopoiesis is reduced in the marrow (11), yet adipocytes are suggested to contribute to the proliferation and differentiation of immature granulocytes (1, 12). Indeed, granulocyte–macrophage precursors are more closely associated with peripheral preadipocytes than with central maturing adipocytes in preadipocyte colonies (13). This observation is in accord with our notion that preadipocyte or reticular cells in the bone marrow contribute to white cell proliferation and differentiation, and they are hematopoietically inactive when they are converted to adipocytes.

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Received December 26, 1984. P.S.E.B.M. 1985, Vol. 179.
Accepted March 8, 1985.