Brief Definitive Report

CONVERSION OF MONOCYTES TO CELLS CAPABLE OF ANCHORAGE-INDEPENDENT GROWTH IN VITRO*

By HSIU-SAN LIN,‡ SAIMON GORDON, DIEN-MING CHEN, AND MICHAEL KURTZ

From the Division of Radiation Oncology, Mallinckrodt Institute of Radiology and the Department of Microbiology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110; and the Sir William Dunn School of Pathology, Oxford University, Oxford, England

It has been shown that most of the macrophages present in the peritoneal cavity after a local stimulation are freshly recruited from the blood (1, 2). It is the freshly recruited cells that are primarily responsible for the increase in plasminogen activator secretion (3) and in responsiveness to lymphokine (4). Aside from small numbers of committed stem cells for both granulocytes and macrophages (5), the only known class of mononuclear phagocytes present in blood is the monocyte, which means that blood monocytes are the most likely antecedent of the majority of exudate macrophages. However, monocytes freshly isolated from peripheral blood are not known to be as metabolically active as exudate macrophages (6). Thus, if blood monocytes are indeed the precursors of exudate macrophages, they must undergo a process of activation or conversion when they are recruited into the peritoneum.

Recently, we have observed another difference between resident and exudative peritoneal mononuclear phagocytes. The incidence of mononuclear phagocyte colony-forming cells (CFC) is much higher in exudate cells (10%) than in resident peritoneal cells (<0.1%) (7). The CFC themselves are macrophage-like cells (8) and their source has not been determined. The two most likely sources of CFC are resident peritoneal cells and blood monocytes. However, the majority of resident peritoneal cells do not replicate and form colonies in vitro and blood monocytes can grow well in liquid culture but poorly in agar (anchorage-dependent growth) (7, 9). This is in sharp contrast to CFC in inflammatory exudate, which grow equally well in liquid and agar (anchorage-independent growth) (10).

In this report, we took advantage of these phenotypic differences in growth characteristics in culture and fibrinolytic activity among blood monocytes and resident and exudative peritoneal macrophages to determine the source of exudate CFC. We showed that blood monocytes, but not resident macrophages, could be converted to cells similar to peritoneal exudate mononuclear phagocyte CFC in vitro.

Materials and Methods

Mice. Both sexes of 6- to 8-wk-old C3D2F1 mice were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Cells. Blood was obtained by cardiac puncture and mononuclear cells were separated by

* Supported by grants AI-15542 and HL-19746-04 from the U. S. Public Health Service.

‡ Address reprint requests to Dr. Hsiu-San Lin, Mallinckrodt Institute of Radiology, Washington University School of Medicine St. Louis, Mo. 63110.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/81/02/0488/06 $1.00
Volume 153 February 1981 488-493

the Ficoll-Hypaque technique described by Böyum (11). Peritoneal exudates were induced by intraperitoneal injection of 1.5 ml of sterile thioglycollate medium (TM; Difco Laboratories, Detroit, Mich.). 3 d later, we collected peritoneal exudates by washing out the peritoneal cavities with 5 ml of alpha medium (Flow Laboratories, Inc., Rockville, Md.) containing 10% fetal calf serum (FCS) and 5 U/ml of heparin. Peritoneal cells washed out from unstimulated mice were considered as resident peritoneal cells.

Culturing of Cells in Gelatin-coated Flasks. Blood mononuclear cells and peritoneal cells in complete growth medium were cultured in gelatin-coated 25-cm^2 Falcon tissue culture flasks (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) (12). The growth medium contained 10% FCS, 5% horse serum (HS) (Flow Laboratories, Inc.) and 10% L cell-conditioned medium (LCM) in alpha medium. At different times after culture, both nonadherent (NA) and adherent (AD) cells were harvested and assayed for CFC. NA cells in medium were harvested, and the flasks were then washed once with alpha medium. To obtain AD cells, we added 3 ml of 15 mM lidocaine in alpha medium supplemented with 10% FCS to each flask (13). After 20 min at room temperature, these flasks were shaken vigorously to dislodge AD cells. They were washed once by centrifugation.

Assay for CFC. The techniques for culturing cells in both agar and liquid cultures has been described in full elsewhere (7, 9, 10). The agar medium consisted of 10% FCS, 5% HS, 10% LCM, and 0.3% Noble agar (Difco Laboratories) in alpha medium. Cultures were incubated at 37°C in a fully humidified atmosphere of 10% CO_2 in air. Colonies were counted after 28 d. For liquid culture, cells were grown in 1 ml of growth medium containing all ingredients present in agar medium except agar. The number of colonies were counted 14 d after culture (10).

Preparation of LCM and Colony-stimulating Factor (CSF). LCM was prepared as described previously (7). Highly purified CSF was prepared by the method of Stanley and Heard (14). The sp act of this CSF preparation was 6.8×10^7 colonies/mg of protein.

Assay for Fibrinolytic Activity. Fibrinolytic activity was assayed on ^{125}I-fibrin-coated Linbro plates (Linbro Chemical Co., Hamden, Conn.) prepared as described (15, 16). We added cells to ^{125}I-fibrin-coated wells and incubated these plates at 37°C for 30 min for cells to adhere. To each well, we added 25 to 50 μl of acid-treated dog serum (ATDS, pH 2, for 30 min at room temperature) as a source of plasminogen (final concentration, 5% vol:vol). We incubated the plates and then withdrew 100-μl samples of medium to assay for release of radioactivity. Assays were performed in duplicate, and appropriate controls were included in all experiments.

Results

Activation of Monocytes In Vitro. At various times after culture, blood mononuclear cells grown in gelatin-coated flasks were separated into NA and AD cell fractions. The use of gelatin-coated flasks greatly facilitated the detachment of AD cells by lidocaine. The viability of harvested AD cells, as determined by the trypan blue dye exclusion test, was usually >90%. Cells from both NA and AD cell fractions were then assayed for their ability to form colonies in liquid and agar cultures. We performed 12 separate experiments, and Table I shows the results of a representative experiment. The majority of CFC were present in the AD cell fraction. The number of CFC capable of forming colonies in liquid culture stayed about the same as or slightly less than that in the original unfractionated cell suspension. However, the number of cells capable of forming colonies in agar increased rapidly with time and by day 3 (72 h), it usually equalled the number of CFC in liquid culture. The ratio of the number of CFC in agar and in liquid cultures was also calculated for all 12 separate experiments and pooled. The values of the ratio after 0, 24, 48, and 72 h in culture were 0.13, 0.55, 0.92, and 0.96, respectively. That means, by 48 h after culture, all CFC acquired an ability to grow in agar.

We next investigated whether resident peritoneal cells could be activated to form

TABLE I

*Conversion of Various Mononuclear Phagocytes In Vitro**

Source of cells	Time in culture (h)	Cells recovered		CFC/10^5 NA cells		CFC/10^4 AD cells		Total CFC in AD fraction		Ratio§
		NA	AD	Liquid C‡	Agar C	Liquid C	Agar C	Liquid C	Agar C	
	h									
Blood mononuclear cells	0	(2.0×10^6)‖		(58 ± 8)	(4 ± 3)			(116,000)	(8,000)	(0.07)
	24	1.10×10^6	0.52×10^6	0.4	0.2	201 ± 14	45 ± 3	104,520	23,400	0.22
	48	0.69×10^6	0.28×10^6	0.1	<0.1	405 ± 29	328 ± 18	113,400	91,840	0.81
	72	0.43×10^6	0.17×10^6	0.2	<0.2	689 ± 51	653 ± 32	117,130	111,010	0.95
Resident peritoneal cells	0	(2.00×10^6)		(1.1)	(0.2)			(2,200)	(400)	
	48	0.21×10^6	0.92×10^6	<0.1	<0.1	3 ± 2	1 ± 1	2,760	900	
	72	0.10×10^6	0.85×10^6	<0.1	<0.1	2 ± 2	2 ± 1	1,700	1,700	
TM-elicited peritoneal cells	0	(2.00×10^6)		(98 ± 12)	(89 ± 16)			(196,000)	(178,000)	(0.91)
	48	0.3×10^6	1.1×10^6	1	<0.1	171 ± 29	184 ± 20	118,100	202,400	1.08

* 2×10^6 cells were grown in gelatin-coated flask containing 5 ml of growth medium.

‡ C, culture.

§ Ratio = $\frac{\text{CFC in agar culture in AD cell fraction (or in original unfractionated cell suspension)}}{\text{CFC in liquid culture in AD cell fraction (or in original unfractionated cell suspension)}}$.

‖ Unfractionated cells.

TABLE II

Fibrinolytic Activity of Various Mononuclear Phagocyte Populations Cultured In Vitro

Time in culture*	Fibrinolysis‡/10^5 adherent cells		
	Blood mononuclear cells	Resident peritoneal cells	TM-elicited peritoneal cells
h			
2	1.2	0.8	33.7
24	3.6	1.1	37.2
48	16.5	5.2	38.1
72	38.4	6.5	31.5

* Time when adherent cells from these cultures were harvested.

‡ Radioactivity released (percentage of total) 2.5 h.

colonies in both liquid and agar cultures. The incidence of CFC in resident peritoneal cells was very small and there was no significant increase in the number of colony forming cells even after 3 d of culture (Table I). For comparison, we also studied TM-elicited peritoneal exudate cells which contained a high proportion of CFC capable of forming colonies equally well in both liquid and agar cultures (Table I). After 3 d of culture, there was no significant change in the total number of CFC. Almost all CFC were recovered in the AD cell fraction.

One might argue that blood mononuclear cell CFC which form colonies in agar might be completely different from those which form colonies in liquid culture. The increase in the number of CFC in agar culture could be a result of the proliferation of preexisting CFC. To rule out this possibility, we added cytosine arabinoside (100 μg/ml) to the culture flask from the time of culture to the time cells were harvested for assay 48 h later. In three such experiments there was no inhibition in the increase of CFC in agar (data not shown), indicating that the increase in CFC in agar is not a result of the proliferation of preexisting CFC.

Increase in fibrinolytic activity. To study fibrinolytic activity of blood mononuclear

TABLE III

*Differential Enhancement by CSF of Fibrinolysis by Various Mononuclear Phagocytes Cultured In Vitro**

AD cells‡	Fibrinolysis§	
	Control	With CSF‖
Resident peritoneal cells (4 h)	1.0	1.3
(48 h)	3.2	4.0
TM-elicited peritoneal cells (4 h)	19.2	37.3
(48 h)	20.1	35.4
Blood mononuclear cells (4 h)	0.8	1.1
(24 h)	5.4	7.6
(48 h)	18.0	37.5

* AD cells were harvested and 1×10^5 cells/well were plated on ^{125}I-fibrin in alpha medium. CSF or control medium was then added and they were incubated for 4 h at 37°C. The cells were then washed twice and fibrinolysis assayed in 5% ATDS.

‡ The length of time in culture is in parentheses.

§ Radioactivity released (percentage of total)/2.5 h.

‖ The amount of CSF was 10 ng/well.

cells cultured in vitro, we harvested NA and AD cells at various times and investigated their fibrinolytic activity. For comparison, both resident and TM-elicited peritoneal cells were cultured under identical conditions. Although the results of eight such experiments varied in detail, the trend was similar and results of a representative experiment is shown in Table II. Original blood mononuclear cells and NA cells obtained after as much as 72 h in culture had very little fibrinolytic activity (data not shown). Fibrinolytic activity of AD cells from blood mononuclear cells culture increased with culture time. After 72 h in culture, the fibrinolytic activity of AD cells from blood mononuclear cell cultures was as great as that of AD cells from TM-elicited peritoneal cell cultures. However, the increase in fibrinolytic activity was much less for AD cells from resident peritoneal cell cultures.

Enhancement of Fibrinolytic Activity. Previous studies have shown that CSF can modulate the fibrinolytic activity of mononuclear phagocytes (12). Moreover, mononuclear phagocytes obtained from different sources vary in their response to CSF. In this study, we incubated AD cells obtained from different mononuclear phagocyte cultures with highly purified CSF for 4 h before assaying for fibrinolytic activity. Table III shows a representative experiment from four experiments. AD cells from TM-elicited macrophage cultures almost doubled their fibrinolytic activity after a short exposure to CSF. AD cells obtained from the early (4 h) blood mononuclear cell culture did not respond to CSF, but their sensitivity to the enhancing effect of CSF increased with time and by 48 h they were as sensitive as TM-elicited cells. In contrast, the fibrinolytic activity of AD cells from resident peritoneal cell culture increased only slightly even after 48 h.

Discussion

These experiments have shown that blood monocytes can be converted in vitro to cells that resemble TM-elicited peritoneal macrophages. Within 2–3 d of culture, they acquire an ability to proliferate and form colonies in soft agar (anchorage-independent growth) and produce and secrete high levels of plasminogen activators, levels com-

parable to those of TM-elicited macrophages. In sharp contrast, resident peritoneal macrophages do not acquire the ability to grow in agar medium or even in liquid culture (anchorage-dependent growth) under similar culture conditions. Furthermore, only a slight increase in fibrinolytic activity by cultured resident macrophages was noted. It thus appears that the majority of so-called activated or stimulated macrophages present in inflammatory exudate elicited by various irritants are most likely activated newly arrived blood monocytes rather than activated resident macrophages. Our finding is consistent with the observations of others (3, 4). Newly arrived blood mononuclear phagocytes are primarily responsible for the increased production of plasminogen activators (3) and they are more responsive to lymphokines than are macrophages from the resident cell population (4). Thus, the influx of blood monocytes to the site of inflammation is not just to increase the number of macrophages but to provide mononuclear phagocytes that can be converted to highly active macrophages. When monocytes are converted, they not only retain the ability of anchorage-dependent growth but also acquire the ability to undergo anchorage-independent growth. The physiologic significance of the latter function is not clear at present but it may serve to provide more active macrophages locally. In fact, the replication of macrophages at the site of inflammation has been well documented (17) and inflammatory exudates have been shown to contain the growth factor required for the replication of mononuclear phagocytes (18, 19).

The time-course of monocytes in vitro to gain anchorage-independent growth appears to be roughly parallel to the kinetics of appearance of CFC capable of anchorage-independent growth in peritoneum after a local injection of TM or other irritants (20). In addition, there is a close correlation between the incidence of CFC capable of growing in agar and the level of fibrinolytic activity. The reason for the close correlation between these two phenotypes remains to be elucidated. A similar relationship has been observed in other mononuclear phagocyte populations (12) and in transformed fibroblasts (21, 22).

Summary

We investigated the time-course involved in the conversion of mouse blood monocytes in vitro to cells capable of anchorage-independent growth. Two criteria were used to define when monocytes were fully converted to cells similar to mononuclear phagocytes present in inflammatory exudate, such as thioglycollate medium (TM)-elicited peritoneal exudate. They were the production of high levels of plasminogen activators and an ability to undergo anchorage-independent growth. Resident peritoneal macrophages were used as controls and for comparison. Our studies indicated that monocytes, but not resident peritoneal macrophages, could be converted to cells similar to TM-elicited mononuclear phagocytes after 2 d in culture.

Received for publication 22 September 1980 and in revised form 11 November 1980.

References

1. Volkman, A., and J. L. Gowans. 1965. The production of macrophages in the rat. *Br. J. Exp. Pathol.* **46**:50.
2. Spector, W. G., M. N.-I. Walters, and D. A. Willoughby. 1965. The origin of the

mononuclear cells in inflammatory exudates induced by fibrinogen. *J. Pathol. Bacteriol.* **90:** 181.

3. Vassalli, J.-D., J. Hamilton, and E. Reich. 1976. Macrophage plasminogen activator. Modulation of enzyme production by anti-inflammatory steroids, mitotic inhibitors, and cyclic nucleotides. *Cell.* **8:**271.
4. Ruco, L. P., and M. S. Meltzer. 1978. Macrophage activation for tumor cytotoxicity: increased lymphokine responsiveness of peritoneal macrophages during acute inflammation. *J. Immunol.* **120:**1054.
5. Bradley, T. R., and D. Metcalf. 1966. The growth of mouse bone marrow cells *in vitro. Aust. J. Exp. Biol. Med. Sci.* **44:**287.
6. Bennet, W. E., and Z. A. Cohn. 1966. The isolation and selected properties of blood monocytes. *J. Exp. Med.* **123:**145.
7. Lin, H.-S., and C. C. Stewart. 1974. Peritoneal exudate cells. I. Growth requirement of cells capable of forming colonies in soft agar. *J. Cell. Physiol.* **83:**369.
8. Lin, H.-S., and P. G. Freeman. 1977. Peritoneal exudate cells. IV. Characterization of colony forming cells. *J. Cell. Physiol.* **90:**407.
9. Lin, H.-S. 1977. Colony formation *in vitro* by mouse blood monocytes. *Blood.* **49:**593.
10. Stewart, C. C., H.-S. Lin, and C. Adles. 1975. Proliferation and colony-forming ability of peritoneal exudate cells in liquid culture. *J. Exp. Med.* **141:**1114.
11. Böyum, A. 1968. Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* **21**(Suppl. 97):1.
12. Lin, H.-S., and S. Gordon. 1979. Secretion of plasminogen activator by bone marrow-derived mononuclear phagocytes and its enhancement by colony-stimulating factor. *J. Exp. Med.* **150:**231.
13. Rabinovitch, M., and M. J. DeStefano. 1976. Cell shape changes induced by cationic anesthetics. *J. Exp. Med.* **143:**290.
14. Stanley, E. R., and P. M. Heard. 1977. Factors regulating macrophage production and growth. *J. Biol. Chem.* **252:**4305.
15. Gordon, S., Z. Werb, and Z. Cohn. 1976. Methods for detection of macrophage secretory enzymes. *In In vitro* methods in cell mediated and tumor immunity. B. Bloom and J. David, editors. Academic Press, Inc., New York. 341.
16. Unkeless, J., S. Gordon, and E. Reich. 1974. Secretion of plasminogen activator by stimulated macrophages. *J. Exp. Med.* **139:**834.
17. Ryan, G. B., and W. G. Spector. 1970. Macrophage turnover in inflamed connective tissue. *Proc. R. Soc. Lond. B Biol. Sci.* **175:**269.
18. Adolphe, J., J. Fontagne, M. Pelletier, and J. P. Giroud. 1975. Induction of DNA synthesis in rat macrophages *in vitro* by inflammatory exudate. *Nature (Lond.).* **253:**637.
19. Wynne, K. M., W. G. Spector, and D. A. Willoughby. 1975. Macrophage proliferation *in vitro* induced by exudates. *Nature (Lond.).* **253:**636.
20. Lin, H.-S. 1974. Peritoneal exudate cells. II. Kinetics of appearance of colony-forming cells. *J. Cell. Physiol.* **84:**159.
21. Ossowski, L., J. P. Quigley, G. M. Kellerman, and E. Reich. 1973. Fibrinolysis associated with oncogenic transformation. *J. Exp. Med.* **138:**1056.
22. Pollack, R., R. Risser, S. Conlon, and D. Rifkin. 1974. Plasminogen activator production accompanies loss of anchorage regulation in transformation of primary rat embryo cells by Simian Virus 40. *Proc. Natl. Acad. Sci. U. S. A.* **71:**4792.