Vol. 90, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 12, 1979 Pages 158-163

CHANGES IN PROTEASE DURING DIFFERENTIATION OF MOUSE MYELOID LEUKEMIA CELLS[1)]

Genichiro Oshima*, Michiyuki Yamada**, and Takashi Sugimura**

*School of Pharmaceutical Sciences, Kitasato University
Minato-ku, Tokyo 108

and

**Biochemistry Division, National Cancer Center Research Institute
Tsukiji, Chuo-ku, Tokyo 104, Japan

Received July 26,1979

SUMMARY: The protease activities of mouse myeloid leukemia cells Ml were examined using fluorescein isothiocyanate-labeled albumin as substrate. Protease activity in Ml cells was greatest at alkaline pH values with a maximum at pH 11.0, and only slight activity was seen at neutral and acidic pHs. When Ml cells were induced to differentiate into mature cells by lipopolysaccharide, their alkaline protease activity decreased greatly with marked increase in acid protease activity. Moreover, in a variant cell line Mml with the properties of differentiated Ml cells, no protease activity was found at alkaline pH values.

INTRODUCTION

Mouse myeloid leukemia cells Ml can be induced to differentiate into macrophages or granulocytes by various kinds of inducers, such as proteins, steroids, lipopolysaccharide and poly (ADP-Ribose) (1-4). This differentiation of Ml cells into macrophages is accompanied by induction of various physiological characters, such as phagocytosis, Fc receptors, and lysozyme and by change in cell morphology. A variant cell line Mml established from Ml cells showed similar physiological properties and cell morphology to those of induced, differentiated Ml cells (5).

We are interested in the role of protease in the differentiation of myeloid cells into macrophages or granulocytes. The main protease in mature macrophages is the acid protease cathepsin D (6-9), but it is unknown what proteases are present in the progenitor cells of macrophages.

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

0006-291X/79/170158-06$01.00/0

This paper shows that alkaline protease is the main protease in myeloid leukemia cells Ml, and that on differentiation of Ml cells into mature cells, the alkaline protease disappears with concomitant induction of acid protease.

MATERIALS AND METHODS

Materials: FITC-CM-BSA[2] was prepared by the reaction of CM-BSA (1 g/50 ml) containing 3% $NaHCO_3$ and 4 M urea with fluorescein isothiocyanate (50 mg/10 ml acetone) overnight at room temperature. CM-BSA was prepared by the method of Crestfield *et al.* (10). The FITC-CM-BSA used here had about 4 mol of fluoresceinthiocarbamyl residues per mol protein. Chymostatin, pepstatin, elastatinal and leupeptin were obtained under the resources program of Grants-in-Aid for Cancer Research from the Ministry of Education, Science, and Culture. These compounds were dissolved in dimethylformamide at 2 mg/ml. PMSF and TPCK were obtained from Sigma Chemical Co., and dissolved in methanol at 10 mM. Lipopolysaccharide of *Salmonella typhosa* was from DIFCO Laboratories.

Cell cultures: The Ml and Mml cell lines of mouse myeloid leukemia cells were grown as described previously (4). Harvested cells were washed with $CaCl_2$- and $MgCl_2$- free phosphate buffered saline and kept frozen until use.

Preparation of cell lysate: The cells were suspended in 0.15 M NaCl and sonicated for 15 sec in ice, and the cell lysate was used as enzyme. Protein was determined by the method of Lowry *et al.* (11) with bovine serum albumin as standard.

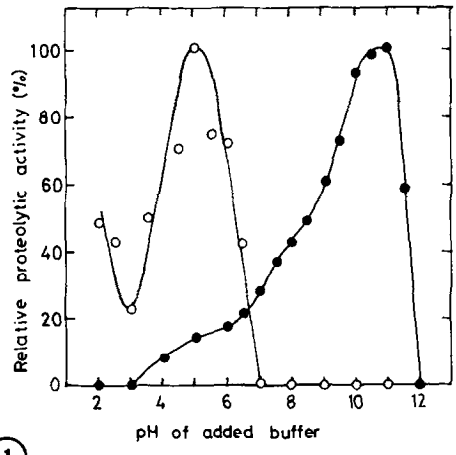
Assay of proteases: The reaction mixture for alkaline protease, containing 40 µg of FITC-CM-BSA and enzyme in 0.1 ml of 0.1 M boric acid-NaOH (pH 10.5), was incubated for 30 min at 37°C. The reaction was stopped by adding 0.1 ml of 0.4% sodium dodecylsulfate in 8 M urea. The remaining FITC-CM-BSA was separated by sodium dodecylsulfate polyacrylamide gel electrophoresis at 5 mA/tube for 1 h by the method of Weber and Osborn (12) and determined fluorometrically with emission and excitation wavelengths of 515 and 495 nm, respectively. One unit of protease was defined as the amount hydrolyzing 50% of the substrate in 1 min under these conditions.

The reaction mixture for assay of acid protease was the same as that for alkaline protease, except that 0.1 M sodium acetate buffer (pH 5.0) was used instead of boric acid buffer.

RESULTS

Effect of pH on the protease activities in Ml and Mml cell lysates: As shown in Fig. 1, protease activity in a lysate of Ml cells was observed between pH 4 and 11.5, with a maximum at pH 11 and broad trailing at neutral and acidic pHs.

2. Abbreviation used: CM-BSA, S-carboxymethyl bovine serum albumin; FITC-CM-BSA, N-fluoresceinthiocarbamyl-S-carboxymethyl bovine serum albumin; PMSF, phenylmethylsulfonylfluoride; TPCK, L-1-tosylamide-2-phenylethyl-chloromethyl ketone; PCMB, p-chloromercuribenzoate; NEM, N-ethylmaleimide.



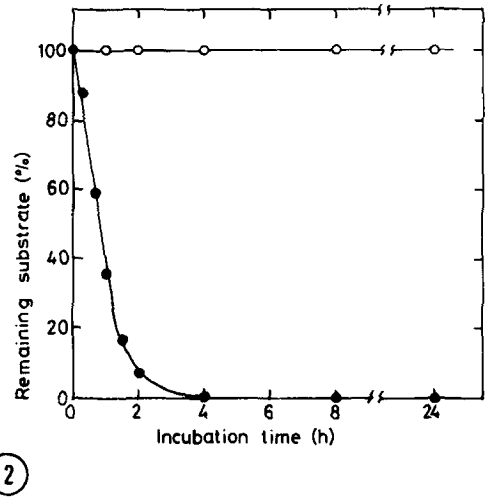


Fig. 1. Effects of pH on proteolytic activities in lysates of mouse myeloid leukemia cells Ml and Mml. Buffers from pH 2.0 to 7.0 were prepared by adding 1 N NaOH to a solution of 0.1 M phosphoric acid, 0.1 M acetic acid and 0.1 N HCl. Buffers from pH 7.5 to 12 were made by adding various amounts of 1 N NaOH to a solution of 0.1 M Tris-HCl and 0.1 M boric acid. Activities were assayed as described in MATERIALS AND METHODS, except that 18 μg protein of Ml cell lysate (•) and 25 μg protein of Mml cell lysate (o) were incubated for 0.5 and 3 h, respectively.

Fig. 2. Time courses of substrate hydrolysis by alkaline proteases in lysates of Ml and Mml cells. Activity was assayed in 0.1 M boric acid-NaOH (pH 10.5) at 37°C using 18 μg protein of Ml cell lysate (•) or 33 μg protein of Mml cell lysate (o).

Protease activity in a lysate of Mml cells was seen between pH 2 and 6.5 with an apparent maximum at pH 5.0 (Fig. 1). In contrast to Ml cells, the lysate of Mml cells did not have any protease activity at above pH 7.0. In confirmation of this, as shown in Fig. 2, at pH 10.5, the lysate of Ml cells hydrolyzed more than 90% of the added FITC-CM-BSA within 2 h, whereas the lysate of Mml cells did not hydrolyze any detectable FITC-CM-BSA within 24 h. Thus alkaline protease was present in Ml cells, but not Mml cells. Moreover, alkaline protease activity in Ml cells was not inhibited by the addition of the lysate of Mml cells (data not shown), indicating that the lysate of Mml cells did not contain an inhibitor of alkaline protease.

Effects of protease inhibitors on alkaline and acid proteases: As shown in Table I, the alkaline protease activity in the lysate of Ml cells was inhibited by chymostatin, TPCK, and PMSF, but was not affected by $CaCl_2$, EDTA, PCMB,

Table I

Effects of Protease Inhibitors on the Proteases[a)]

Addition		Activity	
		Alkaline protease in Ml cell lysate	Acid protease in Mml cell lysate
		(% of the control)	
None		100	100
$CaCl_2$	1 mM	127	112
EDTA	50 μM	115	104
PCMB	0.1 mM	109	86
NEM	1 mM	145	75
PMSF[b)]	1 mM	16	100
TPCK	1 mM	0	N.D.[c)]
Chymostatin	0.1mg/ml	0	60
Pepstatin	0.1mg/ml	111	15
Elastatinal	0.1mg/ml	107	86
Leupeptin	0.05mg/ml	120	107

a) Ml cell lysate (14 μg protein) as alkaline protease and Mml cell lysate (25 μg protein) as acid protease were preincubated for 10 min at 37°C with or without the additions indicated, and then incubated with substrate for 0.5 and 3 h for measurement of alkaline and acid proteases, respectively.

b) Assayed at pH 8.0 and expressed as % of the control.

c) Not determined.

N-ethylmaleimide, pepstatin, elastatinal or leupeptin. The acid protease activity in the lysate of Mml cells was inhibited 85% and 40% by pepstatin and chymostatin, respectively, but was not significantly affected by $CaCl_2$, EDTA, PCMB, N-ethylmaleimide, elastatinal or leupeptin. The acid protease may be cathepsin D, which is strongly inhibited by pepstatin (13).

Change in protease activity on differentiation of Ml cells: When Ml cells were induced to differentiate into mature cells by lipopolysaccharide, the specific activity of alkaline protease decreased to 10% of that in control cells, while the specific activity of acid protease increased to 5 times that in control cells (Table II). In this experiment phagocytic cells in the cultures with

Table II

Changes in Protease Activities in Leukemia Cells on Differentiation by Lipopolysaccharide[a)]

Culture	Specific activity[b)]	
	Alkaline protease	Acid protease
	FITC-CM-BSA hydrolyzed (unit/mg protein)	
Control	2.53	0.10
Lipopolysaccharide	0.23	0.46

a) Ml cells were seeded at 5×10^5 cells/ml and incubated with or without 2 μg/ml of lipopolysaccharide for 70 h. Then the cells were collected and alkaline and acid protease activities in their lysates were measured as described in the MATERIALS AND METHODS.

b) Alkaline protease activity in 30 min was assayed with lipopolysaccharide-treated cell lysate (97 μg protein) and control cell lysate (25 μg protein). Acid protease activity in 2.5 h was assayed with lipopolysaccharide-treated cell lysate (23 μg protein) and control cell lysate (92 μg protein).

or without lipopolysaccharide amounted to 94% and 1%, respectively, of the total cells.

DISCUSSION

Lotem and Sachs demonstrated that differentiation of mouse myeloid leukemia cells *in vitro* follows the same course as differentiation of normal myeloid cells into macrophages in the bone marrow on the basis of inductions of phagocytosis, Fc receptors and lysozyme, and changes in morphology (14). Our experiments show that alkaline protease is the main protease in myeloid leukemia cells and that it decreases with marked increase of acid protease during maturation of myeloid leukemia cells into macrophages. Thus, the changes in alkaline and acid proteases observed during differentiation of the myeloid leukemia cells *in vitro* must also occur during *in vivo* differentiation of normal cells. The alkaline protease present in Ml cells may be the main protease in the progenitor cells of macrophages *in vivo*.

The presence of acid protease as the main protease in Mml cells and differentiated Ml cells is compatible with the fact that the main protease in

macrophages from rabbits and rats is cathepsin D (6,7). It has been reported that acid protease activity in Ml cells increases slightly during differentiation of the cells (15).

The alkaline protease found in the cytoplasm of Ml cells is most active at pH 11.0 and is inhibited by chymostatin. The protease also exhibits maximum activity at pH 10.5 with casein as substrate instead of FITC-CM-BSA. The enzyme is not released into the culture medium of Ml cells and is not found in unbroken cells. We do not know any reports of a mammalian protease with an optimum at such a higher pH value. Further studies are required on the characters and functional roles of this protease in myeloid cells.

ACKNOWLEDGEMENT

The authors thank Professor Kinzo Nagasawa, Kitasato University for his encouragement.

REFERENCES

1. Sachs, L. (1978) Nature 274, 535-539.
2. Ichikawa, Y. (1969) J. Cell. Physiol. 74, 223-234.
3. Kasukabe, T., Honma, Y., and Hozumi, M. (1977) Gann 68, 765-773.
4. Yamada, M., Shimada, T., Nakayasu, M., Okada, H., and Sugimura, T. (1978) Biochem. Biophys. Res. Commun. 83, 1325-1332.
5. Maeda, M., and Ichikawa, Y. (1973) Gann 64, 265-271.
6. Dannenberg, A.M., and Bennett, W.E. (1964) J. Cell Biol. 21, 1-13.
7. Kato, T., Kojima, K., and Murachi, T. (1972) Biochim. Biophys. Acta 289, 187-193.
8. Unkeless, J.C., and Gordon, S. (1975) in Proteases and Biological Control (Reich, E., Rifkin, D.B., and Shaw, E., eds.) pp. 495-514, Cold Spring Harbor Lab.
9. Cohn, Z.A. (1975) in Proteases and Biological Control (Reich, E., Rifkin, D.B., and Shaw, E., eds.) pp. 483-493, Cold Spring Harbor Lab.
10. Crestfield, A.M., Moore, S., and Stein, W.H. (1963) J. Biol. Chem. 238, 622-627.
11. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
12. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
13. Aoyagi, T., and Umezawa, H. (1975) in Proteases and Biological Control (Reich, E., Rifkin, D.B., and Shaw, E., eds.) pp. 429-454, Cold Spring Harbor Lab.
14. Lotem. J., and Sachs, L. (1977) J. Cell. Physiol. 92, 97-108.
15. Nagata, K., Takahashi, E., Saito, M., Ono, J., Kuboyama, M., and Ogasa, K. (1976) Experimental Cell Res. 100, 322-328.