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PROTEASES STIMULATE MOUSE ERYTHROLEUKEMIA CELL DIFFERENTIATION AND MULTIPLICATION

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SUMMARY: Seventeen different commercially available proteases stimulate both mouse erythroleukemia (MEL) cell differentiation and multiplication. These are the first enzymes shown to stimulate these processes in Friend leukemia virus-infected MEL cells. The induction of differentiation by proteases can be synergistically enhanced by the addition of low concentrations of dimethyl sulfoxide or other low molecular weight inducers. Since proteases are the first inducers of differentiation with a known biochemical function, their study should facilitate the understanding of the molecular mechanism of this process.

INTRODUCTION: Friend leukemia virus-infected MEL* cells can be induced to differentiate along the erythroid pathway *in vitro* by approximately 100 different agents (1,and see 2 for references). The mechanisms(s) by which these agents influence differentiation is unknown. It has not been possible to unambiguously identify a unique molecular target for any of them in the cell.

In the present study a variety of proteases have been shown to stimulate MEL cells to both multiply and differentiate. The presence of active proteolytic function appears to be required for these stimulations to occur. This is the first report of the stimulation of MEL cell differentiation by molecules of known, easily assayable, enzymatic activity.

MATERIALS AND METHODS: MEL cell line DS-19 and line 5-86 were routinely passed every 3 or 4 days in Glasgow-MEM medium (GIBCO, Grand Island, NY) supplemented with 15% (v/v) fetal bovine serum (Flow Laboratories, Inc., McLean, VA) in sealed bottles flushed initially with 5% CO_2 in air as previously described (3,4). One and/or 2 days prior to adding test agents the cells were subcultured at 0.5-1.0 x 10^5 cells/ml and at 10^5 cells/ml at the time of adding the test agents. The proteases were dissolved in medium, sterilized by passage through 0.45μ filters (Millipore Corp., Bedford, MA), and added directly to the medium just prior to the addition of the cells. Except for time course studies, the cultures were examined after 5 days of growth without a medium change. The cells were counted (with the aid of a hemocytometer or a Coulter Electronics, Inc., Model 5725, Zf Particle Counter, Hialeah, FL) and the extent of differentiation was determined by a benzidine-H_2O_2 staining method (3,4). Three hundred cells were examined for each sample. Hemoglobin was also identified in cell lysates after converting it to cyanomethemoglobin

* The abbreviations used are: B^+ cells, benzidine-stained, hemoglobin-containing cells; DMSO, dimethylsulfoxide; MEL cells, murine erythroleukemia cells; PMSF, phenyl methyl sulfonylfluoride; TIU, trypsin-inhibition units; TPCK, 1-tosylamide-2-phenylethyl chloromethyl ketone.

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by a method utilizing Drabkin's solution (5). The percentage of viable cells used to initiate experiments was determined by counting the percent of cells that excluded 1% (v/v) trypan blue in 0.9% NaCl. Proteolytic activity was determined by a standard spectrophotometric method using casein as a substrate (6). Collagenase (clostridiopeptidase A), pronase, proteinase K, protease V8, and trypsin were from GIBCO; Calbiochem-Behring Corp., La Jolla, CA; Beckman Instruments, Inc., Palo Alto, CA; Miles Laboratories, Inc., Elkhart, IN; and Worthington Biochemistry Corp., Freehold, NJ, respectively. Other proteases, protease inhibitors, protease precursors, benzidine $(HCl)_2$, and DMSO were from Sigma Chemical Co., St. Louis, MO.

RESULTS: Protease V8 stimulated both MEL cell differentiation and multiplication (Fig. 1). Cells were grown with several concentrations of protease V8 in the absence and presence of added 0.25% (v/v) DMSO. Protease V8 at 30-40 μg/ml, maximally stimulated hemoglobin synthesis. Over 40% of the cells became B^+ in this representative experiment (Fig. 1 lower panel). A relatively low concentration of DMSO, which by itself resulted in cultures containing only 0 to 7% B^+ cells (see Fig. 1 for the value obtained with no added protease), acted synergistically with protease V8 in increasing the percentage of B^+ cells. Protease V8 at 25 μg/ml added with 0.25% DMSO was expected to result in about 45% B^+ cells based upon their individual inductive capacities, but when added together, about 75% B^+ cells were noted. The synergistic effect of the two agents was most marked at protease V8 concentrations between 25 and 40 μg/ml. At higher concentrations of protease V8 this synergism did not occur. At the concentrations tested, neither protease V8 nor DMSO was cytotoxic (Fig. 1 upper panel). Protease V8 actually was growth-promoting and increased the cell yield about one third. The final concentration of hemoglobin-containing cells following DMSO and/or protease V8 treatment is shown in the middle panel.

Another protease, α-chymotrypsin, was found to be even more powerful than protease V8 in terms of stimulating an increase in B^+ cells, however, it was cytotoxic at some concentrations at which it was an effective inducer (Fig. 2). α-Chymotrypsin was growth-stimulatory when used at concentrations up to 0.4 mg/ml. α-Chymotrypsin treatment resulted in 75% B^+ cells when added alone (Fig 2 lower panel). DMSO (0.25%), which by itself induced only 4% B^+ cells in this experiment, was synergistic with α-chymotrypsin at almost all of the concentrations tested as shown by the increase in B^+ cells due to DMSO over the "EXPECTED" percentage of B^+ cells (Fig. 2 lower panel). The greatest synergism with DMSO noted, in terms of the absolute yield of hemoglobin-containing cells was at approximately 0.3 mg/ml α-chymotrypsin (Fig 2 middle panel).

Seventeen of the different commercially available proteases tested were shown to markedly stimulate MEL cell multiplication and differentiation in cell line DS-19 (Table 1) ,and all of the ones tested acted similarly in cell line 5-86 (not shown). The protease-induced cellular accumulation of hemoglobin was documented not only cytochemically with a benzidine stain, but

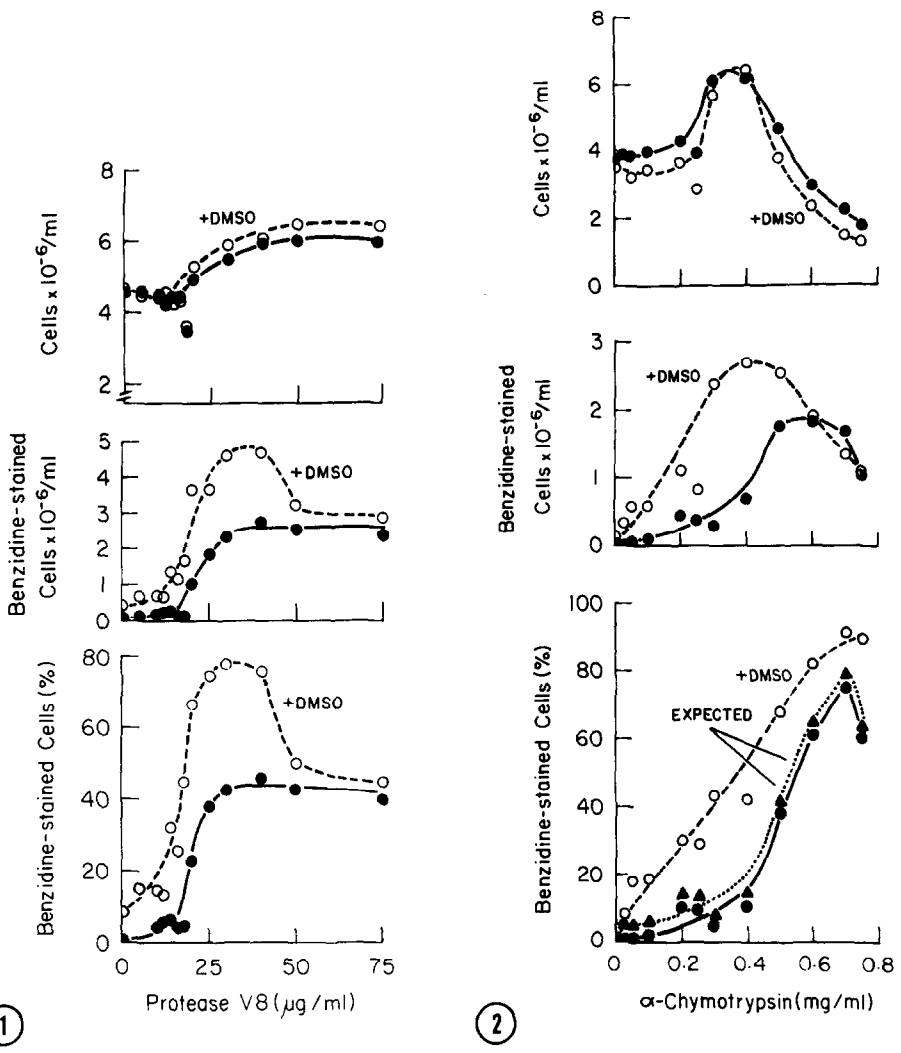

Fig. 1. Effect of protease V8 on cell differentiation and multiplication. Cells were grown for 5 days with protease V8 at the concentrations indicated and with 0.25% (v/v) DMSO where indicated. The percentage of B^+ cells and the cell concentrations were then determined as described in Materials and Methods. Protease V8 (●——●), protease V8 plus 0.25% DMSO (o----o).

Fig. 2. Effect of α-chymotrypsin on cell differentiation and multiplication. Cells were grown for 5 days with α-chymotrypsin at the concentrations indicated and with 0.25% (v/v) DMSO where indicated. The methods were the same as in Fig. 1. The expected percentage of B+ cells was calculated by adding the percentage of B^+ cells observed when 0.25% DMSO was the sole inducing agent to the percentage of B+ cells observed for each concentration of protease used in the absence of DMSO. α-Chymotrypsin (●——●), α-chymotrypsin plus DMSO (o- - -o), expected percentage for chymotrypsin plus DMSO (▲·····▲).

also spectrophotometrically by a cyanomethemoglobin method (5, not shown). The proteases tested that effectively enhanced cell differentiation and multiplication were obtained from protista, plants, and mammals. Proteases having different substrate specificities and acting via metal, serine, or thiol active sites were all effective. Of the proteases tested the ones obtained from groups of lower biological forms were optimally effective at lower con-

TABLE 1: Effect of Proteases on Differentiation and Cell Multiplication of MEL cells.[1]

PROTEASE			OPTIMAL PROTEASE CONCENTRATION FOR DIFFERENTIATION[3]	B^+ CELLS	OPTIMAL PROTEASE CONCENTRATION FOR GROWTH	GROWTH[5]
NAME	SOURCE	TYPE	(μM)	(%)	(μM)	(% OF CONTROL)
NONE	-	-	-	0-7.7[4]	-	100
	MAMMALS					
α-Chymotrypsin	bovine pancreas	serine	31.0	82	17.2	180
Kallikrein	porcine pancreas	serine	14.9	69	14.9	197
Trypsin	bovine pancreas	serine	12.5	54	16.7	171
Elastase	porcine pancreas	serine	7.72	52	11.6	161
γ-Chymotrypsin	bovine pancreas	serine	17.4	34	21.7	153
β-Chymotrypsin	bovine plasma	serine	13.0	25	21.7	146
Plasmin	porcine plasma	serine	9.95	9	0.663	109
Thrombin	human plasma	serine	2.0	9	1.33	116
	PLANTS					
Papain	papaya	thiol	2.14	64	4.27	177
Bromelin	pineapple stem	thiol	2.27	56	4.54	136
Ficin	fig latex	thiol	4.2	42	12.6	136
	FUNGUS					
Proteinase K	*Tritrachium album*	metal	0.476	61	0.476	193
	BACTERIA					
Protease V8	*Staphylococcus aureus*	serine	3.33	82	3.33	167
Pronase[2]	*Streptomyces griseus* K-1	mixture	-	76	-	247
Nagarse	*Bacillus subtilis* N'	serine	0.363	55	0.908	148
Collagenase	*Clostridium histolyticum*	metal	4.0	51	5.0	131
Thermolysin	*B. thermoproteolyticus* rokko	metal	2.13	41	0.533	188
Clostripain	*C. histolyticum*	thiol	6.0	33	6.0	143
Subtilisin	*B. subtilis* Carlsberg	serine	1.83	30	0.183	176

[1]Cultures were grown for 5 days with the proteases listed (at 7 or more concentrations) plus 0.25% DMSO and examined for B^+ cells and cell concentration as described in Materials and Methods. DMSO, at this concentration did not affect cell growth.

[2]Pronase contains at least 9 peptidases: 2 metallo-aminopeptidases, a metallo-carboxypeptidase, 3 metallo-neutral proteases, and 3 serine alkaline proteases (20). The optimal concentration for stimulating cell differentiation and multiplication were 200 μg/ml and 50 μg/ml, respectively. Bovine pancreas α-chymotrypsinogen A and human plasma plasminogen were ineffective in stimulating either cell differentiation or multiplication. Human urine urokinase was also inactive in both respects at the concentrations tested (up to 125 nM).

[3]The concentrations are maximum values based on an assumed 100% purity of each preparation and the followng molecular weights in thousands: α-chymotrypsin, 22.6; β-chymotrypsin, 23.0; γ-chymotrypsin, 23.0; clostripain, 50.0; collagenase, 100.0; elastase, 25.9; ficin, 23.8; kallikrein, 33.5; nagarse (subtilisin BPN'), 27.5; plasmin, 75.4; thrombin, 37.5; trypsin, 24.0; bromelin, 33.0; papain, 23.4; proteinase K, 21.0; thermolysin, 37.5; protease V8, 12.0; subtilisin, 27.3; and urokinase (plasminogen activator), 54.0.

[4]The % B^+ cells due to 0.25% DMSO (generally 2.5%) has been subtracted from all of the other values in this column.

[5]Growth stimulation values are expressed as the percent of the number of cells per ml found in cultures treated with 0.25% DMSO alone (approximately 4.0 x 10^5/ml) compared to those treated with a protease plus DMSO.

centrations; cf. chymotrypsin and protease V8. Of the proteins tested, 2 protease precursors (chymotrypsinogen and plasminogen, which have little, if any, proteolytic activity) were ineffective as inducers, while 2 proteases (plasmin and thrombin) were only weak inducers.

A marked stimulation in cell yield occurred following treatment with each of the proteases that were effective inducers of differentiation. This stimulation was not dependent upon DMSO (Fig. 1,2). The stimulation of growth by proteases was not unexpected since exogenous proteases have been shown to stimulate cell multiplication in several systems (e.g., 7-9). The cell yield following pronase treatment was, however, remarkable. Under the influence of pronase, with or without added DMSO, cultures achieved cell densities of greater than 10^7/ml in 5 days. The optimal protease concentration for stimulating cell multiplication was not necessarily the optimal one for inducing differentiation. The rates of increase in cell differentiation and multiplication due to increasing concentrations of many of the proteases were not the same. Therefore, although a stimulation of cell multiplication sometimes occurred concomitantly with differentiation, it was not required.

Cells induced to differentiate by proteases appeared morphologically similar to those observed after treatment with the classical inducer in this system, DMSO (1.8%, v/v). The increase in B^+ cells due to protease treatment was first observed 2-3 days after initiating treatment which was only shortly after the increase noted in DMSO (1.8%)-treated cultures (not shown). The protease-induced cellular accumulation of hemoglobin was documented, not only cytochemically with the benzidine-staining method, but also spectrophotometrically (see Materials and Methods, not shown).

Control experiments were performed using heat- or inhibitor-inactivated α-chymotrypsin or pronase and the remaining proteolytic activity was determined. When a heat-sensitive protease, such as α-chymotrypsin or pronase, was subjected to 90°C for 3 min it was no longer active in the proteinase assay and was no longer able to stimulate either cell differentiation or multiplication. After similar treatment, the relatively heat-stable enzyme, protease V8, retained both proteolytic activity by standard assay and the ability to stimulate both cell differentiation and multiplication (not shown). Aprotinin, at the non-cytotoxic concentration of 0.9 TIU/ml, completely abolished the stimulation of both differentiation and growth due to α-chymotrypsin at 0.25 mg/ml. In the presence of α-chymotrypsin alone the cultures grew to a cell density 146% that of an untreated culture and exhibited 26% B^+ cells. In the presence of α-chymotrypsin plus aprotinin the cultures grew to a cell density of 99% that of an untreated culture and exhibited 2% B^+ cells.

DISCUSSION: Of nineteen commercially available proteases tested in detail, seventeen have been found to markedly stimulate MEL cell multiplication and

differentiation. Inhibition of the proteolytic activity of several of these enzymes by heat or aprotinin-treatment completely abolished their ability to stimulate both cell differentiation and multiplication. Other protease inhibitors such as PMSF and TPCK were able to inhibit at least 50% of the protease-dependent stimulations of these processes when used at concentrations that were not cytotoxic (not shown). Proteases that had less catalytic activity on a molar basis, such as β- and γ-chymotrypsin, were not as efficient in stimulating differentiation as a related protease of higher activity such as α-chymotrypsin. Taken together these results strongly suggest that the ability of 17 proteases to markedly stimulate cell differentiation and multiplication was a function of their proteolytic activities and was not the consequence of casual contaminants in the various preparations. The effects of proteases observed in this report required the simultaneous presence of cells and active enzyme and were not due to proteolysis of a component of the medium such as serum (in preparation).

Concentrations of DMSO that were suboptimal for inducing differentiation were found to act synergistically with proteases. In the presence of DMSO (0.25%), differentiation induced by α-chymotrypsin or protease V8 was found to be stimulated by greater than 40% of the expected additive values. This type of synergism was found with all effective proteases tested and with several, but not all, low molecular weight inducers other than DMSO (in preparation). The synergistic effect of DMSO on the stimulation of MEL cell differentiation has been reported previously with estradiol, etiocholanolone, testosterone (10), ultraviolet light, erythropoietin (11-14) and possibly interferon (15,16).

Only a few previous investigations may be related to protease activity and MEL cell differentiation. One MEL cell acid-protease activity has been shown to decrease during DMSO-induced differentiation (17). In addition a series of apparently related fluoride-inhibitable, fatty acid ester esterases (which are active at neutral pH) present in both cytoplasm and lysosomal-like granules have been demonstrated to increase during differentiation of some (18), but not all (18,19), MEL cell lines. It is not known if these esterases also possess proteolytic activity.

The mechanism(s) by which proteases stimulate MEL cell differentiation and multiplication may be similar to those operating in protease induction of cell multiplication that has been studied in other systems (e.g., 7-9). These findings may present an opportunity to directly correlate the mechanism of induction of differentiation with specific biochemical changes. Using appropriately controlled conditions, unique biochemical targets may be isolated and characterized. Since both replication and differentiation can be stimulated by protease treatment of MEL cells this system also may prove to be a va-

luable model in which to study the regulatory relationship between the functions that govern replication and those that govern cell differentiation.

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