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Growth Acceleration of Ehrlich Ascites Tumor Cells Treated by Proteinase *In Vitro*

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Abstract—*Since the cytotoxic effect of ionizing radiation increases in faster proliferating cell populations, the effects of a mild trypsin and bromelain treatment on the growth rate of Ehrlich ascites tumor (EAT) cells cultured* in vitro *were studied. A continuous exposure to proteinase started at the time of cell plating caused a temporary block of DNA synthesis that was followed by an accelerated growth rate* 48 h *later* (≈1.5-*fold*). *EAT cells exposed to bromelain and trypsin after completed adaptation to the substratum demonstrated a similar increase of growth* 2 *days after the beginning of the enzyme treatment. The acceleration of growth was also observed when exposure to proteinase was interrupted after* 24 h *but the stimulation effect was reversible and continued only* 2 *days. It is concluded that bromelain and trypsin are able to modify reversibly the growth rate of EAT cell population cultured* in vitro.

INTRODUCTION

STIMULATION of eukaryotic cell growth and division *in vitro* after a mild proteinase treatment has been described by several authors [1]. A mitogenic effect of proteinases was first reported by Mazzei *et al.* [2]. The same phenomenon was subsequently observed in other non-transformed cell lines *in vitro* [3, 4]. This effect is irreversible, but it is not inheritable like the stimulation of chemical or viral transformation [5]. Observations on mouse erythroleukemia cells demonstrated that proteinases cause an accelerated proliferation also in transformed cells cultured *in vitro* [6].

Since the cytotoxic effect of ionizing radiation increases in faster proliferating cell populations, a proteinase stimulation may enhance this effect. Therefore, the studies of a reversible growth stimulation of transformed cells are of special importance for radiotherapy. Ehrlich ascites tumor (EAT) cells are a convenient model for such investigations. This cell line can be grown either in the peritoneal cavity of the mouse [7] or in monolayer culture *in vitro* [8]. A review of results reported in the literature reveals that there is no experience about the influence of proteinases on the growth of Ehrlich ascites tumor cells. Therefore we studied the influence of bromelain and trypsin on EAT cells cultured *in vitro*.

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MATERIALS AND METHODS

The cells used were Ehrlich ascites tumor cells (EAT cells) grown on plastic Petri dishes (Nunc) in monolayer culture at 37°C in a humidified, 12.5% CO_2 atmosphere. The stock culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM, Gibco, U.K.) supplemented with 10% newborn calf serum (NCS, Gibco, U.K.), streptomycin (100 μg/ml, Boehringer, Mannheim), penicillin (100 U/ml, Sigma) and 3.7 g $NaHCO_3$. Cultures between the 4th and 10th passage were used. Observations of growing cells were performed with an inverted microscope (Leitz, Wetzlar). The cells were received from the Department of Physiological Chemistry, Hannover Medical School. The proteinases used for cell stimulation in this study were crystalline trypsin (EC 3.4.21.4, 3.5 U/mg, Merck) and lyophilized bromelain (EC 3.4.22.4, 5 FIP-U/mg, Merck). Proteolytic activity was measured according to methods described by Ruyssen and Lauwers [9]. For inhibition studies a purified, lyophilized soybean trypsin inhibitor (SBI, Merck) was used. Proteinase and inhibitor solutions in NCS containing medium used for growth stimulation were prepared and filtered (Millipore, Molsheim, pore size 0.22 μm) before each experiment. The concentration range used was 1–75 μg/ml for bromelain and 5–750 μg/ml for trypsin. Heat inactivation of proteinases was performed by warming up to 90°C for 3 min. For studies of trypsin inhibition 1–200 μg/ml of sterile filtered SBI were added to the DMEM and DMEM + NCS containing a

trypsin concentration of 10 and 100 μg/ml, respectively. Cells used in growth assays were released from stock cultures with 0.125% trypsin solved in balanced salt solution (BSS). Trypsin was removed by centrifugation and the cells were resuspended in DMEM containing 10% NCS. The cell number prior subculturing was estimated by counting in a hemocytometer. In some experiments the cells were suspended in proteinase containing DMEM and plated out. Subsequently, the culture medium with proteinase was renewed on day 1, 3 and 5. In other experiments cells were grown 24 h and culture medium was then replaced by DMEM with bromelain or trypsin. The medium with proteinase was changed on days 3 and 5. Measurements of cell growth and DNA content were carried out in cell suspensions obtained from four separate Petri dishes with the Folin–Ciocalteau phenol reagent according to a method described by Oyama and Eagle [10] and a fluorometric method with diaminobenzoic acid (DABA, Aldrich) described by Thomas and Farquar [11], respectively. Every measurement was performed twice. Extinction was measured at a wavelength of 660 nm by a Beckman DU-7 Spectrophotometer (Beckman, U.S.A.). Measurements of fluorescence were performed with an Eppendorf-Photometer 1101 M provided with an accessory 1030 (Netheler & Hinz, F.R.G.) using a primary mercury filter 405–436 nm and a secondary filter 500–3000 nm. A growth rate acceleration under exposure to proteinase was expressed by growth increase factor (GIF) obtained by division of protein increments of stimulated by control cell population. The statistical analysis of results was performed using the *t*-test for matched samples.

RESULTS

Subculturing of EAT cells in proteinase containing medium caused within the first 24 h of culture time an inhibition of cell adhesion to the substratum. The restriction of cell attachment was dependent on proteinase concentration (Table 1). A complete inhibition of cell adaptation was observed at lower concentrations of bromelain (75 μg/ml) than trypsin (750 μg/ml).

EAT cells that adapted to the substratum within 24 h after plating despite the exposure to active proteinase grew exponentially till the late growth phase. The growth of stimulated cells between the 24th and 72nd hour after subculturing was comparable to control cells. In contrast, a growth acceleration of cells exposed to proteinase was observed between the 72nd and 120th hour of culture growth. This effect continued till the late growth phase (120–168th hour of culture time). A significant acceleration of growth was found at bromelain concentrations of 1–50 μg/ml and trypsin concentrations of 50–500 μg/ml (Table 1). A correlation between the extent of growth stimulation and proteinase concentration was found neither for bromelain nor for trypsin.

The DNA/protein ratio in EAT cells treated by proteinase decreased 24 h after plating suggesting a partial block of DNA synthesis. Compared with cells suspended in culture medium without proteinase the decrease of DNA/protein ratio was highly significant ($P < 0.01$) for bromelain and trypsin concentrations of 1–50 μg/ml and 50–500 μg/ml, respectively. The DNA/protein ratio estimated on days 3, 5 and 7 did not demonstrate any differences between treated cells and controls. If proteinase exposure was interrupted after 24 h, the extent of the stimulation effect was the same but it continued only 2 days and was followed by a growth rate comparable with control cells. The same reverse effect was observed in cultures treated with trypsin at a concentration of 100 μg/ml after addition of SBI 24 h after treatment beginning in concentration inhibiting completely its enzymatic activity (200 μg/ml). The acceleration of growth was absent if a heat inactivated proteinase was used.

An addition of bromelain or trypsin in concentrations of 1–50 μg/ml and 50–500 μg/ml, respectively, to monolayer culture 24 h after subculturing between the 24th and 72nd hour of culture time caused no measurable acceleration of growth. Nor was a decrease in DNA/protein ratio in adapted cells exposed to proteinase found. In contrast, after the 72nd hour of culture time the growth rates of stimulated cell populations were significantly higher than controls (Table 2). If EAT cells released from the substratum by high proteinase concentrations were harvested after 24 h exposure and plated out in a fresh culture medium, they demonstrated a decreased DNA/protein ratio for 24 h and grew subsequently faster than controls. The acceleration of growth was comparable to cells treated by proteinase at the time of plating.

To verify whether the observed stimulation effect can be related to a direct proteinase effect and not to serum digestion products, the culture was exposed 24 h after plating to the enzyme dissolved in serum-free culture medium. After 24 h of treatment the medium was replaced by medium containing serum but without proteinase. Bromelain in concentrations of 1–50 μg/ml caused, between the 72nd and 120th hour of culture time, a measurable increase in growth (Table 3). Trypsin in concentrations of 5–50 μg/ml also increased the growth rate. Higher trypsin concentrations than 50 μg/ml released the adapted cells from the substratum. The growth increase, however, was lower in comparison to the stimulation produced by the proteinases in the presence of serum. The growth stimulating effect of trypsin was abolished by an addition of soybean trypsin inhibitor. However, the SBI concentration

Table 1. Stimulation of growth of EAT cells under continuous exposure to bromelain and trypsin started simultaneously with plating in dependence on enzyme concentration

Protease	Concentration of enzyme in DMEM + 10% NCS (μg/ml)	Percentage of adapted cells 24 h after subculturing	Percentage increase in cell population between 72nd and 120th hour of culture growth	GIF
Bromelain	0 (control)	105.4	133.4	1.00
(5 FIP-U/ml)	1	67.9	191.2	1.43
	5	47.4	202.8	1.52
	10	26.5	188.1	1.41
	50	20.6	185.4	1.39
	75	0		
Trypsin	0 (control)	105.4	133.4	1.00
(3.5 U/mg)	5	101.3	136.6	1.02
	10	84.7	132.1	0.99
	50	95.5	148.1	1.11
	75	90.3	150.4	1.13
	100	88.9	149.4	1.12
	500	44.2	146.7	1.10
	750	0		

Proteinase was added simultaneously with plating at a cell density of 2.4×10^5. Culture medium containing proteinase was renewed 24 and 72 h after subculturing. The protein content of the cell population adapted to the substratum was assayed 24, 72 and 120 h after plating. The data are an average of results obtained in three separate experiments.

Table 2. Growth stimulation of EAT cells under continuous exposure to bromelain or trypsin started 24 h after plating in relation to enzyme concentration

Proteinase	Concentration of proteinase in DMEM + 10% NCS (μg/ml)	Percentage increase in cell population between 72nd and 120th hour of culture growth	GIF
Bromelain	0 (control)	100	1.00
(5 FIP-U/ml)	1	135	1.35
	5	131	1.31
	10	149	1.49
	50	140	1.40
Trypsin	0 (control)	100	1.00
(3.5 U/mg)	5	99	0.99
	10	103	1.03
	50	120	1.20
	75	116	1.16
	100	125	1.25
	500	123	1.23

Culture medium was replaced by medium containing proteinase after 24 h of culture growth and renewed 48 h later. The cell density just prior to addition of proteinase was 2.6×10^5. The protein content of cell population adapted to the substratum was assayed 72 and 120 h after beginning of exposure to proteinase. The data are an average of results obtained in three separate experiments.

of 5 μg/ml that blocked approximately 50% of trypsin activity did not inhibit the stimulation of growth. The growth activation was absent at SBI concentrations (20 μg/ml) causing a complete inhibition of trypsin activity.

DISCUSSION

Our observations demonstrate that the proliferation of EAT cells growing in monolayer can be stimulated by an exposure to bromelain and trypsin and this effect can be maintained if the enzyme

Table 3. Growth stimulation of EAT cells after an interrupted exposure to bromelain and trypsin in serum-free culture medium started 24 h after plating in relation to enzyme concentration

Protease	Concentration of enzyme or enzyme + SBI in DMEM (μg)ml)	Percentage of adapted cells 48 h after subculturing	Percentage increase in cell population between 72nd and 120th hour of culture growth	GIF
Bromelain	0 (control)	132.4	100.0	1.00
(5 FIP-U/ml)	1	117.6	129.3	1.19
	10	109.1	135.7	1.21
	50	119.2	125.5	1.14
Trypsin	0 (control)	132.4	100.0	1.00
(3.5 U/ml)	5	110.6	123.7	1.14
	10	113.7	131.3	1.21
	50	125.8	120.1	1.12
Trypsin + SBI	0 + 5	133.8	101.1	1.01
(3.5 U/ml)	0 + 20	130.4	98.5	0.99
	10 + 5	122.2	123.1	1.17
	10 + 20	129.8	99.1	0.99

Culture medium was replaced by medium containing proteinase but without NCS after 24 h of culture growth. The cell density just prior to addition of proteinase was 2.6×10^5. After 24 h the proteinase containing serum-free medium was replaced by medium with 10% NCS again. The protein content of the cell population adapted to the substratum was assayed 48, 72 and 120 h after plating. The data are an average obtained in three separate experiments.

treatment is continued. If the proteinase treatment was interrupted after 24 h the effect was reversible. It lasted approximately 2 days and was followed by a normal growth rate of cultured cells. The reversion of growth activation could also be achieved by an enzyme inhibitor added to the culture 24 h after the beginning of treatment. The stimulation effect could be related to a direct action of an active enzyme, the presence of serum was not necessary to produce the growth stimulation.

The response of EAT cells to proteinase exposure varied depending on the time of beginning the treatment. Proteinases added to the culture at the time of cell plating produced a block of DNA synthesis that was followed by an accelerated growth of treated cells. In contrast, proteinase exposure started 24 h after subculturing caused no inhibition of DNA synthesis but a growth acceleration 2 days later. A similar differential effect was observed in non-transformed fibroblasts growing in monolayer culture [12]. Therefore, this type of response of cells to proteinase exposure may be typical of cells growing in monolayer *in vitro*.

The mechanisms of increased cell growth after proteinase treatment were extensively studied in non-transformed cells. According to these data a modification of protein molecules on the outer cell surface in a manner inducing new membrane transport components facilitating the uptake of nutrients seem to be the possible mechanism of growth stimulation [1, 13]. Although these results were obtained in other systems, this hypothesis has also been applied to transformed cells [6]. According to this assumption the accelerated growth of EAT cells may be due to an increased uptake of amino acids and glucose by proteinase treated cells. On the other hand, the influence of factors contained in serum added to the culture medium cannot be excluded. Although in our experiments the stimulation of growth was brought about by proteinase in the absence of serum, the extent of the increase in growth was lower compared to increments observed after an enzyme treatment in serum containing medium. This suggests that in addition to the active proteinase the serum digestion products play a role in the growth acceleration.

A condition necessary for growth stimulation is an intact proteolytic activity of an enzyme. However, the extent of growth acceleration is not proportional to the amount of enzyme contained in the culture medium. The necessity of a complete block of enzyme activity to abolish the growth stimulation produced by a proteinase and no inhibition of growth acceleration if only a part of enzyme activity is blocked suggest that this phenomenon may be due to a form of a triggering mechanism on the cell surface.

The concentrations of trypsin that led to detachment of cells from the substratum or to the stimulation of cell growth were higher than bromelain, although both bromelain and trypsin possessed a comparable proteolytic activity against protein

substrates like casein. This was probably due to a stronger inhibition of trypsin by serum contained in the culture medium. Calf serum also contains, beside numerous unspecific proteinase inhibitors, specific trypsin inhibitors [14] that were responsible for a more effective enzyme activity block. This assumption is supported by the observation that trypsin concentrations necessary to produce a growth stimulation in serum-free medium were considerably lower than in the presence of serum.

Summarizing, it can be concluded that the growth of EAT cells in serum containing culture medium can be stimulated reversibly by bromelain and trypsin. The effect is sufficient to modify significantly the growth rate of an EAT cell population *in vitro*.

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