Effects of Protease Inhibitors on Growth of Hamster Tumor Cells in Culture

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SUMMARY

Beef pancreas trypsin inhibitor has been shown to promote parallel alignment of hamster tumor cells in culture and to increase the adhesiveness of rounded cells. It has a slightly depressing effect upon cell proliferation. Soybean inhibitor depressed cell proliferation but had no effect upon cell alignment. Egg white inhibitor had no effect upon proliferation or cell alignment. The synthetic protease inhibitor N-α-p-tosyl-L-lysine chloromethyl ketone HCl had no effect upon cell alignment but depressed cell proliferation. It was toxic in concentrations in excess of 20 μM. Beef pancreas trypsin inhibitor had no effect upon cell proliferation or cell interaction of nontumorigenic hamster embryo cells in culture. The results suggest that some aspects of the morphology and social behavior of the tumor cells studied may be associated with protease activity that can be inhibited by beef pancreas trypsin inhibitor.

INTRODUCTION

Proteolytic enzymes have been shown to play a role in the initiation of division of cells in culture. When quantities of trypsin too small to cause disaggregation were added to confluent cultures of normal chick embryo cells, there was a rise in incorporation of thymidine-3H and a subsequent increase in mitotic rate (11). Similarly, cell division and escape from contact inhibition of growth occurred when trypsin, ficin, and Pronase were added to confluent cultures of nonmalignant mouse fibroblasts (3T3) in amounts too small to produce detachment (2). An overgrowth-stimulating factor isolated from the culture medium of chick embryo cells infected with Rous sarcoma virus stimulated thymidine uptake and mitosis in cultures of normal chick embryo cells (9). The similarity between the activity of this factor and the activity of trypsin led to the hypothesis that the factor might be a proteolytic enzyme. Proteolytic activities have been found to be associated with epithelial (6), mesenchymal (1), and nerve growth (3) factors. Protease inhibitors were found to inhibit the promotion by croton oil or phorbol ester of tumorigenesis in mouse skin initiated by dimethylbenzanthracene (13).

Treatment with phorbol ester resulted in an increase in protease activity in skin.

In view of the above results, we reasoned that the inhibition of protease activity might affect the growth rates and growth patterns of malignant cells. In this report we present the results of a study of the effects of 3 types of protease inhibitors on the growth of transformed hamster fibroblasts in culture. The following substances were tested: the chloromethyl ketone of TLCK2, a substance that inhibits proteases by forming covalent bonds with histidine residues (12) and/or sulfhydryl groups; p-toluene sulfonyl-L-arginine methyl ester HCl, a substrate and competitive inhibitor for trypsin and papain; and natural trypsin inhibitors obtained from soybean, egg white, and bovine pancreas.

MATERIALS AND METHODS

Tumor Cells. The cells used for these studies were derived from 1 of several fibrosarcomas that developed in golden hamsters following s.c. injection of embryonic cells previously treated in vitro with 9,10-dimethylbenzanthracene. The cells from the tumor routinely were grown in Falcon plastic tissue culture dishes (Falcon Plastics Co., Los Angeles, Calif.) in an enriched Eagle's medium at 37° in a humidified gas phase of 3% CO₂ in air. The ingredients for the enriched medium were obtained from Grand Island Biological Co., Grand Island, N. Y. and consisted of: Earle's balanced salt solution, twice the original concentration of Eagle's minimum essential medium amino acids, 4 times the original concentration of Eagle's basal medium of vitamins, 0.3 mM nonessential amino acids, 4 mM glutamine, 0.1 mg of streptomycin sulfate per ml, 50 units of penicillin G per ml, and 10% fetal calf serum. The pH was adjusted to 7.4.

The cells were cloned twice by growing a single cell that had been sucked up into a microcapillary. The final clone was tested for Mycoplasma. For this purpose cells and medium were cultured on enriched Mycoplasma medium (8) from the Baltimore Biological Laboratory, Baltimore, Md., in an atmosphere of 5% CO₂ and also in the absence of CO₂. No Mycoplasma contamination was detected in the cells or medium.

The present studies were performed about 6 months after the 2nd cloning. At this time the clone was no longer homogeneous. Chromosome numbers ranged from 32 to 96.

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2The abbreviations used are: TLCK, N-α-p-tosyl-L-lysine; BPTI, beef pancreas trypsin inhibitor.
and peaked at 42 to 45. Mass cultures showed an overall irregular crisscross pattern of cell arrangement. In 2 to 10% of colonies the cells were, to some degree, aligned in parallel. Nearly all colonies contained some polynucleated cells (Fig. 1).

Proteolytic Inhibitors. The nature, specifications, and source of the inhibitors used in these experiments are shown in Table 1.

All inhibitors were dissolved in double-distilled water, and, except for TLCK, were sterilized by filtering through Millipore filters which had been washed with double-distilled water. TLCK rendered the filter impassable. The unfiltered solution was tested for bacteria and fungi, was found to be uncontaminated, and was used without further treatment. All solutions were stored at 4° for the duration of the experiments.

Each inhibitor was prepared at 10 times the concentration needed for the particular experiment, and prior to each feeding the solution was diluted 10-fold with culture medium. For the control dishes, double-distilled water was diluted 10-fold with culture medium. The growth and appearance of cells in these control dishes were identical with those of cells in the undiluted medium.

Experimental Procedure. Culture dishes (50-mm diameter) were plated with 500 cells/dish in 5 ml of medium. On the 3rd day the cultures were fed with normal medium. On Days 6, 8, and 10, pairs of dishes were fed with medium containing proteolytic inhibitor as specified in each experiment, and control pairs were fed with medium containing 10% double-distilled water.

It is a characteristic of this cell line that some cells, particularly those in mitosis, temporarily detach and float in the medium. In order not to prejudice the results in favor of cells adhering to the dish, floating cells were centrifuged out of the medium removed on Days 8 and 10 and added back to the culture with fresh medium.

The development of colonies was observed with an inverted microscope. Before change of medium on Day 6 and on Day 12 or Day 13, the colonies per dish were counted and the number of cells per dish was determined with a hemocytometer after trypsinization. Photomicrography was carried out on additional cultures stained with Giemsa.

## Results

**Effect of TLCK.** Eight pairs of dishes were plated with 500 cells/dish. On the 6th day, 1 pair of dishes was examined and found to contain, respectively, $4.44 \times 10^3$ and $4.9 \times 10^3$ attached cells. These cells were distributed in 165 and 181 colonies, respectively, which contained between 8 and 80 cells/colony. The cells in the majority of colonies were loosely and irregularly arranged. In the 1st dish examined, 1 colony was observed to have cells in parallel alignment; in the 2nd dish, 3 colonies showed parallel alignment. A few floating cells were observed in each culture. On this day and on Days 8 and 10, pairs of dishes were fed with medium containing 0.0, 2.5, 5, 10, 20, 40, 80, and 160 μM TLCK, respectively. All dishes were examined on Day 12. The results show a rapid decline in cell yield per dish when TLCK was present in excess of 20 μM (Chart 1, open circles), which indicates that this inhibitor becomes cytotoxic at higher concentrations, possibly by virtue of its alkylating properties which are not, of course, specific for trypsin. The percentage of cells that were floating in the

![Chart 1. The effect of TLCK and BPTI on yield of cultured hamster tumor cells. Results are expressed as percentage of yields of cells grown in the absence of proteolytic inhibitors, and each point represents the average of counts from 2 dishes, after 6 or 7 days of growth in the presence of the inhibitor.](chart1.png)

### Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>Lot</th>
<th>Specific activity</th>
<th>M.W.</th>
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<tr>
<td>TLCK</td>
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<td>90.3</td>
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<tr>
<td>p-Tosyl-L-arginine methyl ester HCl</td>
<td>Sigma</td>
<td>81C-0550</td>
<td>1.5</td>
<td>379.0</td>
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<td>BPTI, Type I-P</td>
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<td>81C-8200</td>
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<td>6,000</td>
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<tr>
<td>BPTI</td>
<td>Sigma</td>
<td>120C-8140</td>
<td>1.3</td>
<td>24,000</td>
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<td>1C-8110</td>
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<td>Ovomucoid (egg white) trypsin inhibitor, Type II-O</td>
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<td>1GB-9020</td>
<td>1.3</td>
<td>27,000</td>
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<td>5301</td>
<td>27,000</td>
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* a Sigma Chemical Co., St. Louis, Mo.; Nutritional Biochemicals Corp., Cleveland, Ohio.
  b Milligrams of trypsin inactivated by 1 mg of inhibitor.
  c The molecular weights of this and the subsequent natural inhibitors are approximate.
medium declined with concentrations up to about 40 μM TLCK, and with higher concentrations increased again (Chart 2, open circles). Within this population, the proportion of cells in mitosis decreased from 48% at 0.0 μM TLCK to 7% at 40 μM and 0% at 80 and 160 μM; and cells that showed signs of degeneration, such as vacuolation or excessive granulation, increased in number.

The number of colonies per dish declined rapidly at TLCK concentrations higher than 20 μM. At concentrations below 20 μM, there was no observable effect on the morphology of the cells. The general arrangement within colonies did not differ from that seen in untreated dishes.

**Effect of p-Tosyl-L-arginine Methyl Ester HCl.** In concentrations below 500 μM this substance had no effect on cell cultures. Above this concentration, there was a linear decrease in cell yield which reached 30% at 1.3 mM, the highest concentration tested. There were no observable effects upon cell arrangement within colonies.

**Effects of BPTI.** The experimental design was the same as that used in the studies with TLCK. On Day 6, 1 pair of dishes was examined and was shown to contain 5.04 × 10^5 and 5.84 × 10^5 cells, respectively, with 175 and 199 colonies of between 8 and 80 cells. Only 3 or 4 colonies in each dish showed some parallel arrangement of cells. On this day and on Days 8 and 10, pairs of dishes were treated with 0.0, 8.3, 16.6, 33.2, 49.8, 66.4, and 83.0 μM BPTI, respectively, and the dishes were examined on Day 13.

BPTI had a marked effect upon the arrangement of the attached cells. In the dishes without BPTI, the cells were loosely and irregularly arranged in 88% of the colonies (Chart 1) and showed some parallel alignment in 12%, only in the colony center in 9%, and in the colony center and periphery in 3%. In the dishes with increasing BPTI concentrations, the proportion of colonies with parallel cell alignment increased and reached 73% at 83 μM BPTI (Chart 3), including 57% in which the cells were aligned both in the colony center and periphery, as shown on Fig. 2. For these photomicrographs, rounded cells adhering to the colonies were partly washed off before staining in order to show the arrangement of the underlying layer, which is typical of the whorled pattern of normal fibroblastic growth. Since the number of colonies per dish remained practically the same from 0.0 to 83 μM BPTI, the observed shift in colony morphology was not attributable to preferential growth of aligning cells that were present in the original population, but rather to conversion of nonaligning cells to ones showing cell interaction typical of nontumorigenic fibroblasts.

The effect of this inhibitor upon cell yield was interesting because in concentrations up to 33 μM a small but steady decline was observed (Chart 2, solid circles). In higher concentrations (in contrast to the effect of TLCK) there was increasingly less effect, although at the highest concentration tested (83 μM) the cell yield still remained slightly below the control level. The percentage of floating cells decreased with increasing concentrations of BPTI (Chart 2, solid circles) and the proportion of mitotic cells in this population decreased from 60% at 0.0 μM BPTI to 23 and 29% at 49.8 and 83.0 μM, respectively. There was no obvious change in the proportion of cells with signs of degeneration. The observed reduction (Chart 2) in the overall number of floating cells was attributable to an increased adhesion of nonmitotic and mitotic rounded cells to those cells at the base of the colonies that were attached to the culture dish.

In order to determine whether the effect of BPTI upon cell alignment was reversible, cells grown in the presence of 83 μM BPTI were mechanically dispersed and replated. Medium containing 83 μM BPTI was added to one-half of the dishes and medium without BPTI was added to the remainder. After 6 days the dishes were examined. Cells grown in the presence of BPTI and replated with BPTI showed the same orderly arrangement within colonies that was observed previously. Cells replated without BPTI displayed the characteristic crisscross pattern typical of the control tumor cells.

In experiments with a 2nd batch of BPTI, slightly higher concentrations were required to produce the same effects, presumably because of variation in the specific activity of the product.

A particularly interesting finding was that BPTI (83 μM) had no effect whatsoever upon cell interaction or cell proliferation of nontumorigenic hamster embryo cells in culture. Typically, these cells show parallel alignment within colonies.
Soybean Trypsin Inhibitor. Under the same experimental conditions as those described above, soybean trypsin inhibitor in concentrations up to 42 μM had no effect upon the growth of cultures. At a concentration of 84 μM there was a 50% decrease in cell yield but no change in cell alignment within colonies or in the number of colonies showing parallel alignment.

Egg White Trypsin Inhibitors. Both samples of egg white inhibitors used had no effect upon cell yield and cell arrangement in concentrations up to 75 μM, the highest concentration tested.

DISCUSSION

Our results demonstrate that protease inhibitors can modify both the rate of growth and the morphology of cultured tumor cells.

The inhibition of tumor cell growth observed when TLCK, p-tosyl-L-arginine methyl ester HCl, soybean trypsin inhibitor, and, to a lesser extent, BPTI are added to the culture medium would appear to be the reverse of the effect seen when trypsin is added to a confluent sheet of cells (2, 11), or when overgrowth factor is added to a crowded culture (9). Our results, taken together with the work cited, lend support to the idea that a proteolytic enzyme may be involved in the series of events that leads to cell replication. Evidence for this supposition was adduced by the demonstration that treatment of isolated lymphocyte nuclei with trypsin resulted in increased template activity (5). Moreover, TLCK and p-tosyl-L-arginine methylester HCl inhibited several parameters of the stimulation of lymphocytes by phytohemagglutinin (4). Trasylol, a natural polyvalent inhibitor with properties similar to BPTI, had no effect.

In our experiments, p-tosyl-L-arginine methyl ester HCl, which is a substrate for trypsin and inhibits this enzyme by competing with the natural substrate, was effective only at 5 times the molar concentration required for TLCK. Progressively increasing concentrations of both TLCK and p-tosyl-L-arginine methyl ester HCl produced increasing reduction in cell growth. Such was not the case with BPTI. In fact, a maximal reduction in growth was observed at a concentration of approximately 30 μM, and higher concentrations of BPTI resulted in progressively less effect. One possible explanation of our findings, which cannot be ruled out by our present experiments, might be that TLCK and p-tosyl-L-arginine methyl ester HCl were acting at some intracellular site that was not readily accessible to the larger BPTI molecule. The degree to which BPTI entered the cells could conceivably have decreased as intercellular adhesion and parallel alignment of cells increased because of decrease in area of the cell surface left in direct contact with the medium. It also is possible that the change in cell morphology brought about by the presence of BPTI in the medium modified the permeability of the membrane to BPTI.

It would appear from our results that the morphological characteristics of our tumor cells are attributable at least in part to a proteolytic enzyme which is either absent or inactive in normal hamster embryo cells. The nature of this enzyme has not been elucidated in these experiments. However, it seems unlikely that it has properties similar to trypsin which, in addition to being inhibited by BPTI, is also inhibited by TLCK, p-tosyl-L-arginine methyl ester HCl, egg white inhibitor, and soybean inhibitor (10); none of these except BPTI had any effect upon the morphology of our cells. Similarly, it seems unlikely that the enzyme is similar to chymotrypsin which is inhibited by soybean inhibitor as well as BPTI (10).

Among other known properties of BPTI are its inhibitory effects upon kallikreins (10). Such enzymes do not appear to have immediate relevance to fibroblasts in culture. However, their general property, which is the release of biologically active peptides, provides the basis for a speculation that such an enzyme might exist in tumor cells and release a substance which modifies cell properties. A precedent for this idea is the existence of leukokininase in the outer membrane of polymorphonuclear leukocytes (7).

An alternative possibility is that this enzyme may act on the cell periphery or on a microexudate to destroy proteins required for cell adhesion or cell flattening.

REFERENCES

Fig. 1. Hamster tumor cells from dishes not treated with trypsin inhibitors showing typical loose, irregular arrangement of cells within colonies. Giemsa stain. a, c, e: X 40; b, d, f: X 230.
Fig. 2. Hamster tumor cells grown in the presence of 83 μM BPTI, showing alignment of cells within colonies in 3 separate experiments. As many as possible rounded cells adhering to these colonies were carefully removed to uncover the structure of the underlying layer of cells. Giemsa stain. a, c: × 40; b, d, f: × 230.
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