EFFECT OF INHIBITORS OF PROTEOLYTIC ENZYMES ON THE GROWTH OF NORMAL AND POLYOMA TRANSFORMED BHK CELLS

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SUMMARY

This paper reports the effect of tosyl-phenylalanyl-chloromethyl ketone, Pepstatin, Antipain, Leupeptin and Trasylol, all inhibitors of proteolytic enzymes, on the growth and morphology of normal and polyoma transformed baby hamster kidney cells in culture. Above 1 μM, tosyl phenylalanyl chloromethyl ketone inhibited the growth of both normal and transformed cells to the same extent, and also inhibited the incorporation of radioactive leucine into protein. Pepstatin and Antipain also had no differential effect on the growth of normal or transformed cells, while Leupeptin and Trasylol inhibited the growth of normal cells more than transformed cells.

INTRODUCTION

When normal fibroblasts are transformed by oncogenic viruses their morphology and growth characteristics are profoundly altered. These changes include decreased adhesiveness to plastic and a more rounded shape, increased agglutinability by lectins, absence of a 250,000 mol. wt. glycoprotein from the surface membrane, lower intracellular ATP levels and growth to a higher cell density. There are now many examples of some or all of these changes being induced transiently in normal fibroblasts by treatment with low concentrations of proteolytic enzymes such as trypsin, chymotrypsin and pronase. On the basis of these observations Burger and others have suggested that transformed cells may have higher levels of proteolytic activity in, on, or secreted from their surface membranes, in such a way as to 'autocatalytically' maintain a higher growth rate than normal cells (for references and reviews see 1,2).

There is some evidence to support such an idea. For example, the 250,000 mol. wt. glycoprotein disappears from the surface membrane

of normal hamster Nil cells when virus transformed Nil cells are brought into contact with them (2), and SV40 transformed hamster fibroblasts produce larger quantities of a factor (presumably a protease) which activates serum plasminogen to plasmin (3). Schnebli and Burger (4) have reported that a number of inhibitors of proteolytic enzymes specifically slow the growth of Py3T3, SV3T3 and PyBHK cells compared to their normal counterparts. Their original claim that one of these inhibitors (tosyl-lysyl-chloromethyl ketone (TLCK)) also restored density dependent inhibition of growth to SV3T3 cells has since been retracted (5). The inhibitors tosyl phenylalanyl chloromethyl ketone (TPCK), TLCK and Leupeptin have also been reported to inhibit the formation of tumours in mouse skin by carcinogenic hydrocarbons (6,7).

In this paper we show that a number of inhibitors of proteolytic enzymes have little or no differential effect on the growth of normal or polyoma transformed baby hamster kidney (BHK) cells in culture. Moreover, one of these inhibitors, TPCK, used in previous studies (4), also inhibits protein synthesis in the same concentration range as it blocks cell growth. These results do not support the hypothesis that transformed cells maintain their higher growth rate by means of increased proteolytic activity.

MATERIALS AND METHODS

Normal and polyoma transformed BHK/21 cells were obtained from Dr. G. Clarke, I.C.R.F., London. The BHK cells were recloned and taken up from stock every month and were free from PPLO. For measuring cell growth, 1×10^4 cells were seeded into each well of a LINBRO tissue culture multi-dish in 1 ml of Dulbecco's modified Eagle's medium containing 10% calf serum (Flow Labs, Scotland) and incubated at 37°C in 5% CO_2/95% air. Under these conditions the BHK and PyBHK cells had doubling times of approximately 24 and 14 hr respectively. Inhibitor

was added after about 18 hr, and 48 hr later the cells were trypsinised and counted in a Coulter counter. Each point is the average of 4 wells.

To measure amino acid incorporation, BHK and PyBHK cells were seeded into the wells so that 48 hr later the cell concentration was about 0.8 and 1.5 x 10^5 respectively. 0.5 μCi/ml [^{3}H]-L-leucine (51 Ci/mmole) was added with various concentrations of inhibitor and 2 hr later the cells were washed with phosphate buffered saline and lysed in 1 ml of 0.3N NaOH containing 1 mg/ml of cold leucine. The samples were incubated at 37°C for 20 min, precipitated with 10% CH_3COOH, collected onto glass fibre filters and counted in toluene scintillator. Each point is the average of 2 wells.

To measure the efficiency of plating (e.o.p.), 100 BHK cells were seeded into 5 ml of medium containing 10% foetal calf serum. Inhibitor was added to duplicate dishes after 18 hr and 7 days later clones were stained and counted.

TPCK was from Serva Feinbiochemica and was dissolved in methanol, an equal volume of which was added to control cultures. It reacts irreversibly with the histidine in the active site of chymotrypsin. Leupeptin, Antipain and Pepstatin were a gift from Professor Sigimura, Department of Medical Oncology, University of Tokyo. The first two were dissolved in water and the last in methanol. They are naturally occurring peptides isolated from Actinomycetes, which competitively inhibit a range of proteolytic enzymes (8). Trasylol (10,000 units/ml), a purified naturally occurring proteolytic enzyme inhibitor from bovine lungs, was from Bayer Pharmaceuticals and was dialysed against serum-free medium before use.

RESULTS AND DISCUSSION

Effect of TPCK on cell growth

Fig. 1 shows that TPCK inhibits the growth of both normal and polyoma transformed BHK cells, although at concentrations above 10 μM

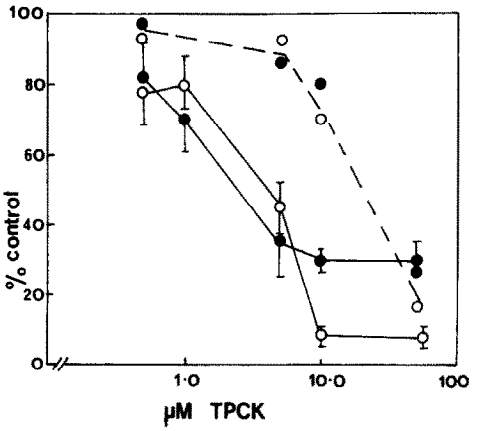

Fig. 1. Effect of TPCK on the growth and protein synthesis of BHK and PyBHK cells.
Various concentrations of TPCK were added to normal (●—●) and Py (○—○) BHK cells 18 hr after plating out at 1 x 10^4/well. 48 hr later the cells were trypsinised and counted and the number expressed as percent of the control without inhibitor (11.7 x 10^4 BHK cells and 85.9 x 10^4 PyBHK). For measuring protein synthesis, normal (●--●) and Py (○--○) BHK cells were incubated for 2 hr with 0.5 μCi/ml [^{3}H]-L-leucine and varying concentrations of TPCK and the incorporation of radioactivity into TCA precipitable material expressed as a percent of the control.

the normal cells are somewhat more resistant. In the experiments reported by Schnebli and Burger (4) higher concentrations of TPCK (about 25 μM) inhibited the growth of Py3T3 cells by 70% but of normal cells by only 10%.

In other studies (9) we have shown that 50 μM TPCK inhibits intracellular protein degradation (as measured by the release of acid soluble radioactivity from pre-labelled general cell proteins) in rat hepatoma (HTC) cells by 66%. TPCK also inhibits the incorporation of amino acids into protein in intact HTC cells and rabbit reticulocytes, as well as in haem supplemented reticulocyte lysates (9). (In these lysates, which continue to synthesize globin for more than 20-25 min, TPCK specifically blocks initiation and in the absence of haem only a small inhibition of chain elongation is seen (McIlhinney and Sampson, unpublished observations)). We therefore tested the effect of varying

concentrations of TPCK on the incorporation of leucine into protein in both normal and PyBHK cells and Fig. 1 shows that above 10 μM TPCK, protein synthesis is inhibited equally well in both cases. 1,10 and 50 μM TPCK reduced the e.o.p. of normal BHK cells by 12,36 and 100% respectively.

Taken together these results suggest that TPCK may be slowing the growth of BHK and PyBHK cells by inhibiting protein synthesis and degradation rather than by inhibiting proteolytic enzymes specifically acting on the outer cell membrane, although such an effect is not entirely excluded. However, we observed no differential effect of TPCK on the growth or morphology of the transformed BHK cells, as might be expected if they were producing more proteolytic activity than normal cells.

Effect of Pepstatin and Antipain on cell growth

Pepstatin at 0.1-0.5 μg/ml had no differential effect on normal or transformed BHK cells (Fig. 2). (The concentrations of Pepstatin reported to inhibit pepsin, proctase B and cathepsin half-maximally *in vitro* are one or two orders of magnitude lower than this (Sigimura,

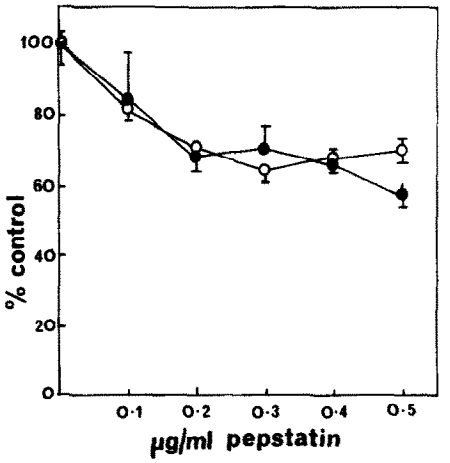

Fig. 2. Effect of pepstatin on the growth of BHK and PyBHK cells. Conditions as in the legend to Fig. 1.

personal communication (10))). 0.5 μg/ml Pepstatin reduced the e.o.p. of BHK cells by 20%. A similar kind of result was obtained with Antipain in the range 10-50 μg/ml. (Again, these concentrations are much higher than those reported to inhibit cathepsins A and B.) The effect of these inhibitors on protein synthesis was not studied, and no change in morphology of BHK cells was seen.

Effect of Leupeptin and Trasylol on cell growth

Leupeptin at 10-50 μg/ml (much higher doses than are needed to inhibit trypsin, papain and cathepsin B *in vitro*) appeared to have a preferential inhibitory effect on the growth of normal BHK cells compared with PyBHKs (Fig. 3). This difference was still observed in the concentration range 1-5 μg/ml. 30 μg/ml leupeptin reduced the e.o.p. of BHK cells by 34%.

Trasylol at 100-1,000 units/ml also inhibited the growth of normal cells more effectively than PyBHKs; at 600 units/ml the former were inhibited by about 50% while transformed cells were only inhibited 20%. This is in direct contrast to the results of Schnebli and Burger (4) using Trasylol and 3T3 cells, but was consistently observed. Whatever the significance of these results, they do not support the

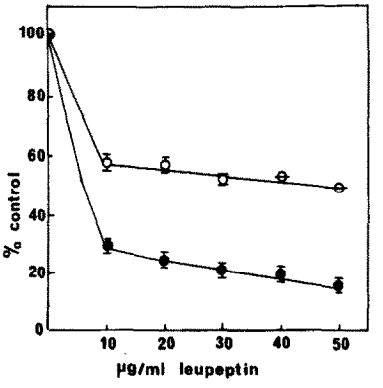

Fig. 3. Effect of leupeptin on the growth of BHK and PyBHK cells. Conditions as in the legend to Fig. 1.

hypothesis that the growth of transformed cells is stimulated autocatalytically by proteolytic enzymes.

ACKNOWLEDGEMENTS

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