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Selective Inhibition of Growth of Transformed Cells by Protease Inhibitors*

(contact inhibition/mouse/hamster)

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ABSTRACT **Five protease inhibitors with different modes of action were found to reduce the growth of transformed mouse (Py3T3, SV3T3, and 3T12) and hamster (PyBHK) cells. Some of these inhibitors caused the transformed cells to cease growth at saturation densities characteristic for nontransformed cells.**

The protease inhibitors were strikingly selective with regard to the transformed cells; they had essentially no effect on the growth of the nontransformed cells. From this result, it is concluded that the inhibitors block a protease-like activity that is required for the unrestrained growth of transformed cells.

The inhibitors exerted their effect directly on the cells; they did not affect growth by interacting with serum components of the medium.

Normal cells in tissue culture cease to divide upon formation of a monolayer. This phenomenon, which has been termed contact inhibition (1) or density-dependent inhibition of growth (2), is observed to a much lesser degree in virally or spontaneously transformed cells.

Proteolytic treatment temporarily releases normal fibroblasts from contact inhibition (3, 4), and induces the appearance of surface properties characteristic of transformed cells (5–9). This finding implies the existence of a proteolytic activity in transformed cells that might be responsible for some of the cell surface alterations associated with the release from contact inhibition of growth (5, 10). If this speculation were correct, inhibition of this proteolytic activity should lead to a decreased growth of transformed cells and, possibly, to an "induction" of contact inhibition.

This report describes the strikingly selective inhibition of growth of transformed cells by five commercially available protease inhibitors, and suggests that these cells require a protease-like activity for escape from contact inhibition.

MATERIALS AND METHODS

3T3-mouse fibroblasts and their polyoma virus-transformed (Py3T3), Simian virus 40-transformed (SV3T3), and spontaneously transformed (3T12) derivative lines were a gift from Dr. H. Green, M.I.T., Cambridge, Mass. Baby hamster kidney cells (BHK/C_{13}) and transformed PyBHK were kindly supplied by Dr. M. P. G. Stoker, ICRF, London. All cells were grown in Dulbecco's modified Eagle's Medium (Gibco No. H-16) containing 10% fetal-calf serum (Microbiological Assoc., Bethesda, Md.). Stocks were passaged two to three times weekly, and care was taken to keep normal cells at low densities. Cells were checked for pleuropneumonia-like organisms regularly and were free of contamination throughout the study.

Cell growth curves in the presence or absence of protease inhibitors were obtained as follows: the cells were plated in 3.5-cm petri dishes (Falcon) 48 hr before addition of the inhibitors. At the beginning of each experiment, and every 24 hr thereafter, the medium with or without inhibitors (1.5 ml per plate) was changed in each plate.

Five cell counts were done on each of two petri dishes for each point. To do this, the cells were trypsinized in 1 ml of trypsin–EDTA (Gibco No. 530), and were counted in a Neubauer Hemocytometer.

The following protease inhibitors were used: Tosyl-arginine methylester (TAME), Tosyl-phenylalanyl-chloromethylketone (TPCK) and Tosyl-lysyl-chloromethylketone (TLCK) all from Calbiochem; soybean trypsin inhibitor 1-S (Sigma), ovomucoid II O (Sigma), and Trasylol injectible (5000 units/ml) from Bayer. Trasylol was dialyzed against phosphate-buffered saline, pH 7.4 to remove a toxic bactericidal additive before use.

RESULTS AND DISCUSSION

As an example of the cell growth curves obtained in the presence or absence of protease inhibitors, the effect of TLCK on the proliferation of SV3T3 and 3T3 cells is shown in Fig. 1. A severe inhibition of growth of the SV3T3-cells was produced by 50 μg/ml of TLCK (0.135 mM). The shape

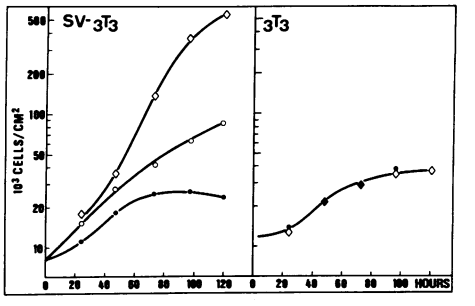

FIG. 1. Growth curves of SV3T3 and 3T3 cells in the presence and absence of TLCK. ◇——◇, control; ○——○, 25 μg/ml of TLCK; ●——●, 50 μg/ml of TLCK. The inhibitor was added at 0 hr.

Abbreviations: TAME, *N*-α-tosyl-L-arginine methylester; TLCK, *N*-α-tosyl-L-lysyl-chloromethane; often called Tosyl-lysyl-chloromethylketone; TPCK, *N*-α-tosyl-L-phenylalanyl-chloromethane, often called Tosyl-phenylalanyl-chloromethylketone.

* The experiments reported herein were performed independently in two laboratories; since the results agree closely, we report them here together.

TABLE 1. *Viability of TLCK-treated SV3T3 cells*

	Untreated control (%)	50 μg/ml of TLCK (%)
Trypan blue-excluding cells	88	90
Plating efficiency	86	84

SV3T3 cells were treated for 72 hr with 50 μg/ml of TLCK exactly as in growth experiments (e.g., Fig. 1, *left*). The cells were trypsinized and used for dye exclusion and plating efficiency tests. For plating efficiency tests, 100 cells were plated in normal medium in a 6-cm dish, and colonies were counted after 7 days (means of four independent determinations).

and the plateau (saturation density) of this curve resemble those of 3T3 growth curves. Microscopic examination revealed that the TLCK-treated transformed cells were much flatter than the untreated cells, but that they still overlapped and grew in irregular patterns.

The growth of the 3T3 cells was unaffected at concentrations up to 50 μg/ml of TLCK (Fig. 1, *right*). At higher concentrations (above 200 μg/ml) growth of untransformed cells begins to be affected also; the cells round up and show signs of general toxicity.

At 50 μg/ml (the concentration producing severe inhibition of SV3T3 cells; Fig. 1, *left*), TLCK is not toxic as judged by dye exclusion and plating efficiency tests (Table 1).

TLCK acts on the cells directly and does not affect growth by interacting with serum components of the medium (Table 2): TLCK-treated, dialyzed serum supported growth as well as did the untreated serum. Furthermore, transformed cells treated with TLCK for 1 hr, then washed and grown in control medium, remained inhibited for 24 hr (Table 2).

With most of the other inhibitors, solubility problems limited the range of concentrations tested. The inhibitions thus obtained were less dramatic, and the curves typically resembled that of the partially inhibited SV3T3 culture (25 μg/ml) in Fig. 1, *left*.

TABLE 2. *Effects of TLCK on serum and cells*

	Cells per cm² after 72 hr	% of control
Control	276,000	(100)
TLCK, 100 μg/ml	18,400	7
Dialyzed serum	247,000	90
TLCK-treated, dialyzed serum	258,000	94
Cells, TLCK-treated, but grown on control medium	33,200	12

SV3T3 cells were plated 48 hr before the experiment; media were changed at the beginning and every 24 hr during the experiment. Cells were counted 72 hr after the first medium change. All media contained 10% fetal-calf serum; where noted, the serum was dialyzed extensively against phosphate-buffered saline (pH 7.4) or treated with 1 mg/ml of TLCK for 1 hr at 37° before exhaustive dialysis. In the last experiment the cells were exposed to TLCK (100 μg/ml) for only 1 hr at the beginning of each day of the experiment; after this exposure, the cells were washed twice and grown in control medium.

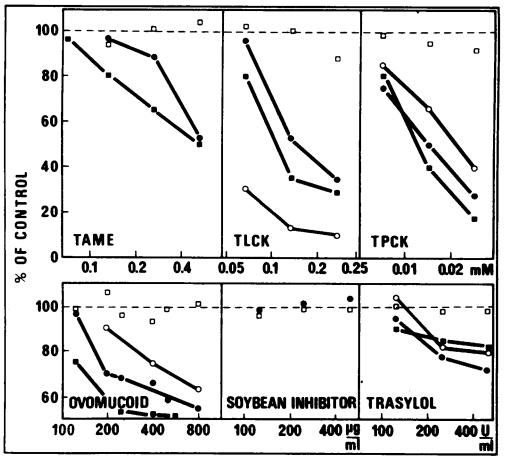

FIG. 2. Effect of protease inhibitors on the growth of 3T3 and transformed 3T3 cells. Growth curves, such as the ones shown in Fig. 1, were determined. This graph represents the ratio of the number of cells in a treated culture to the number of cells in a control culture (%) at 72 hr after addition of the inhibitors. Inhibitors and concentrations are indicated in the graph. ⊡, 3T3 cells; ■, 3T12 cells; ●, Py3T3 cells; ⊙, SV3T3 cells.

The effects of six protease inhibitors on the growth of 3T3, Py3T3, SV3T3, and 3T12 cells are summarized in Fig. 2. Transformed cells were inhibited in a dose-dependent manner by all inhibitors, except for the soybean trypsin inhibitor. In contrast, growth of 3T3 cells was not impaired by any of these compounds. TLCK, TPCK, and ovomucoid similarly inhibited the growth of PyBHK cells without affecting the growth of the parental BHK-cells (not shown).

The inhibitors used are of different types: TAME is a substrate analogue and acts competitively on proteases and esterases (11); TLCK and TPCK are "active-site titrants," reacting irreversibly with the enzyme (12); and ovomucoid forms a poorly dissociating macromolecular complex with proteases of the trypsin family (13). Since it is improbable that inhibitors with such different mechanisms of action would all have similar side effects, it is likely that the inhibition of growth observed in this study is, in fact, due to the inhibition of a protease.

Transformed cells could hydrolyze Chlorella [^{14}C]protein more efficiently than their normal counterparts under conditions where phagocytosis was negligible (14). In fact, transformed cells, when brought into contact with stationary 3T3 cells, were able to remove surface material from the stationary cells (14) and caused overgrowth analogous to a trypsin treatment (15). This result suggested that transformed cells contain more of a protease-like activity on their surface than do normal cells.

Ovomucoid covalently linked to Sepharose beads is effective in inhibiting growth of transformed cells, as is soluble ovomucoid (M. M. Burger, unpublished), again indicating that the target of the protease inhibitors might be pericellular.

It is concluded that an inhibitor-sensitive, protease-like activity, which appears to be surface located, is required by the transformed cells for unrestrained growth. If contact inhibition depends on intercellular interactions through specific receptor structures, one is tempted to speculate (although there is no evidence for this) that the protease-like

activity destroys or modifies surface structures involved in normal cell-to-cell contacts.

Indeed, TLCK treatment of Py3T3 cells (50 μg/ml, 96 hr) renders these cells less agglutinable with wheat-germ agglutinin (5-fold) and with concanavalin A (3-fold), indicating that this inhibitor prevents some of the surface modifications characteristic of transformed cells.

In the context of the present experiments, it is interesting that TAME, TLCK, and TPCK have successfully been used to suppress dimethylbenzanthracene-induced and phorbol-ester-promoted tumorigenesis in mouse skin (16). This finding is compatible with the suggestion above that a protease-like activity is required for the malignant expression of a cell.

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