THE ROLE OF BESTATIN, AN INHIBITOR OF CELL SURFACE PROTEASES, IN THE INTERACTION OF SERUM WITH UNTRANSFORMED CELLS IN CULTURE

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Bestatin is an inhibitor of cell surface-associated aminopeptidase B and leucine aminopeptidase. This microbial product simulates the role of serum as an activator of uridine uptake in quiescent BHK cells. The compound significantly stimulates the incorporation of labelled thymidine into the acid-insoluble fraction of serum-starved Nil 8 cells in the presence of low concentration of serum. The possible mechanisms of these interactions are discussed.

Recent studies had revealed the fact that several exopeptidases are associated with the outer membrane of cells from various origins[1]. The biological role of these enzymes has not been elucidated. A series of compounds of microbial origin were isolated and purified by Umezawa et al.[2], and found to inhibit the activity of some of these cell surface-associated proteases[2,3]. Among these inhibitors, bestatin, [(2S, 3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine, has been studied in greatest detail. Bestatin is an inhibitor of aminopeptidase B and leucine aminopeptidase[4]. The mode of inhibition was found to be competitive with respect to the substrate[3]; the Ki was found to be around 10^{-8} M[3]. It has been found that bestatin stimulates DNA synthesis in T-type lymphocytes both in vivo[3,5] and in vitro[3,7]; the concentration range for these effects was shown to be in the order of 100 μM. It is not known whether the stimulatory influence of bestatin is due to the specific inhibition of aminopeptidases or to stimulation of some other biological activity in the lymphocyte[6].

Untransformed cells growing in a monolayer reduce their multiplication rate after the cells become "confluent". This phenomenon has been called "density dependent inhibition of growth" or "contact inhibition of growth"[8,9]. Many mammalian cells also stop growing when the culture medium is depleted of growth factors. Such cells are arrested in the G_1/G_0 or A state of the cell cycle[10,11]. Both density inhibited and growth factor depleted cells are stimulated to synthesize DNA and proliferate when fresh serum[12,13,14] or purified growth factors (review: Reference 15) are supplied.

The correlation between the uptake rate of uridine and the rate of growth of untransformed cells in culture is an established phenomenon (review: Reference 16). The rate of uridine uptake decreases as cells reach confluency and is one of the first processes to be activated after the addition of fresh serum to quiescent cultures[16].

Due to the fact that the existence of cell surface proteases seems to be a general phenomenon[1], and the evidence of the "mitogenic-like" effect of the specific protease inhibitor bestatin[5,6] it was intriguing to look for its possible role in the growth regulation of untransformed cells in culture. This was done by assaying both uridine uptake and thymidine incorporation into DNA.

Materials and Methods

Cell culture

Nil-8 and BHK cell cultures were restarted every 6 weeks from frozen stocks. The cells were grown and maintained in DULBECCO's modified EAGLE's medium (DMEM) to which 10% new born calf serum, antibiotics and glutamine (Bio-Lab Jerusalem) had been added. The cells were grown at 37°C in a CO_2 enriched humidified atmosphere on 90 mm plastic petri dishes (Nunc, Denmark) and sub-cultured twice weekly at a density of 5×10^5 cells/90 mm dish. For uptake and incorporation experiments cells were subcultured in 35 mm dishes at a density of approximately 10^5 cells/dish.

Quiescent BHK cells for the uridine uptake experiments were prepared in the following manner:

Medium was replaced $2 \sim 3$ days after seeding by a medium containing 0.25% serum; the experiments were performed $72 \sim 96$ hours after medium change. Serum-starved Nil 8 and BHK cells were seeded at a similar density and propagated for $3 \sim 4$ days at 10% serum. Twenty four hours prior to the experiment, medium was replaced by serum free DMEM. At confluency, the cells reach a density of approximately 10^6 cells/35 mm dish.

Uridine uptake experiments

Prior to the uptake experiments culture medium was sucked off and the cells were washed in PBS (phosphate buffered saline) containing NaCl, 8 g/liter; KCl 0.2 g/liter; $Na_2HPO_4 \cdot 12 H_2O$, 2.9 g/liter; KH_2PO_4, 0.2 g/liter; 0.5 mM $MgCl_2$ and 0.7 mM $CaCl_2$; pH 7.4. Uptake of 5 μM [³H]uridine was followed as previously described[17]. Briefly, cells were exposed to 0.5 ml of labelled uridine (Israel Nuclear Research Centre, Negev) solution (0.5 μCi/ml) and placed on an enamel tray into a 33°C water bath for the desired time period. Uptake was terminated by 5 quick washes in ice cold PBS. 0.75 ml of cold trichloroacetic acid (TCA) was added to the dish and acid-soluble material extracted at 4°C for 20 minutes; 0.5 ml of the TCA extract was used for scintillation counting in a Triton based scintillation fluid.

Incorporation of labelled thymidine into acid insoluble fractions

Cells were exposed to a solution of 1 μM [³H] thymidine (0.25 μCi/ml) in DMEM for 24 hours at 37°C in a CO_2 enriched humidified atmosphere. Uptake was then terminated as in the uridine uptake experiments. After 20 minutes extraction with TCA, the residue was washed twice with TCA once with ethanol and acid insoluble fractions were extracted in 0.6 ml of 0.5 M KOH for 30 minutes at room temperature. 0.4 ml was taken for scintillation counting.

Bestatin was isolated from a fermentation broth of *Streptomyces olivoreticuli* and determined to be chromatographically pure[18].

Results

The effect of bestatin on the rate of uridine uptake by quiescent BHK cells is shown in Fig. 1. Quiescent cells were incubated for 90 minutes at 37°C in DMEM in presence or absence of 10% serum and/or 10 nM bestatin. Uptake of 5 μM uridine was assayed subsequently in phosphate buffered saline (PBS). It is apparent that bestatin, at a concentration of 10 nM, exerts the same effect on the rate of uridine uptake as do the effectors present in growth medium containing 10% fresh serum. The effects of serum and bestatin are not additive, indicating that the same "activated" state is reached as a result of bestatin or serum action.

Fig. 2 shows the results of an experiment in which the activation and uptake of uridine were assayed simultaneously. Quiescent BHK cells were exposed to PBS containing 5 μM [³H] uridine in presence or absence of 50 nM bestatin. As exposure to the labelled nucleoside and the activator occurred at the same time, the time course of the activation process could be assayed directly. The effect of bestatin is easily detectable after 10-minute incubation, indeed, a least square analysis of the data revealed no lag time in the action of bestatin on the uridine uptake system of BHK cells (a lag time in the order of $1 \sim 5$ minutes could not be detected in this experiment).

Fig. 1.   The effect of bestatin on the uptake of uridine by quiescent and serum activated BHK cells.
Radioactivity (cpm/assay) accumulated in acid soluble pools of serum treated and untreated cells.
A: Experiment carried out in absence of bestatin.   B: Experiment carried out in presence of 10 nM
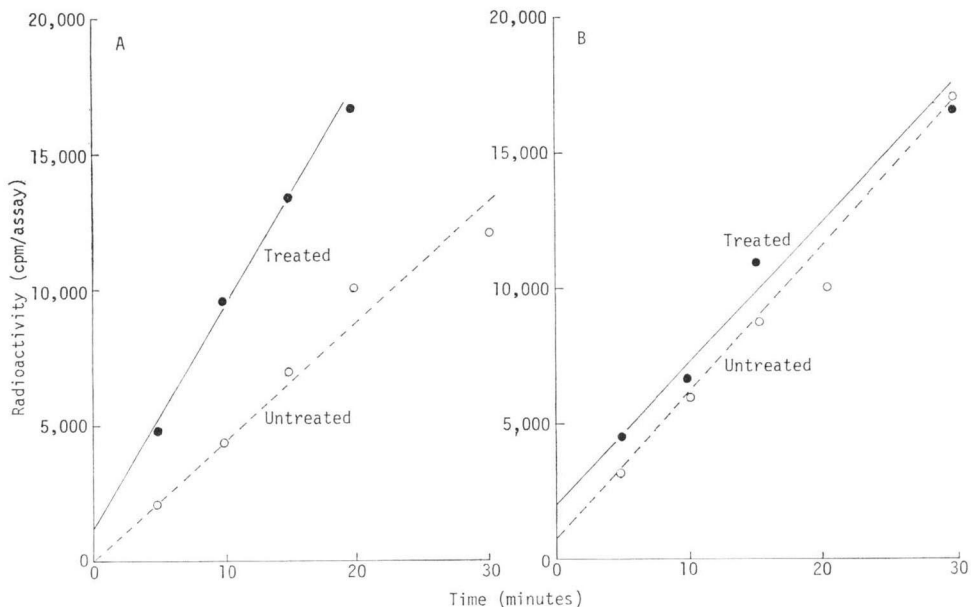bestatin.   (1,000 cpm is equivalent to 15 pmol/assay dish).

Fig. 2.   The time course of activation of uridine uptake into quiescent BHK cells by bestatin.
Radioactivity (cpm/assay) accumulated in acid soluble pools in presence and absence of 50 nM
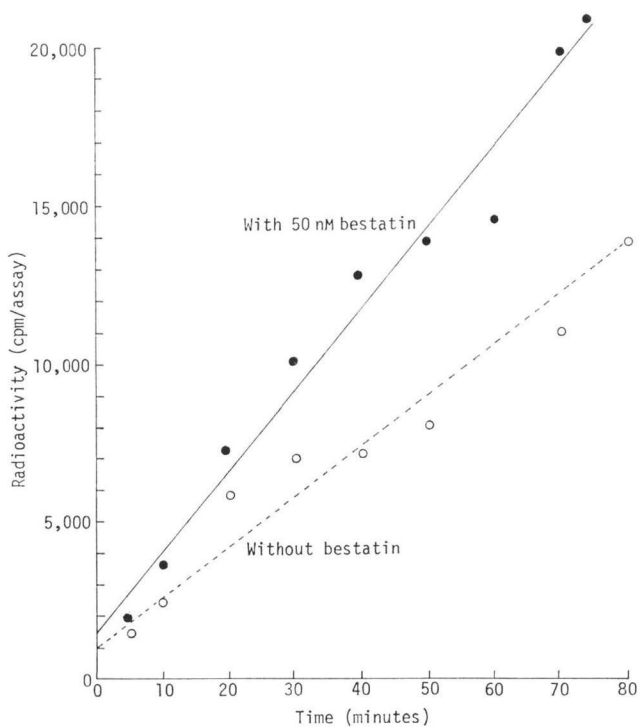bestatin.   (1,000 cpm is equivalent to 15 pmol/assay dish).

Fig. 3. The effect of bestatin and serum on the incorporation of thymidine into acid insoluble fractions of serum starved Nil 8 cells.

A: [Bestatin]=0.0; B: [Bestatin]=10^{-7} M; C: [Bestatin]=10^{-6} M; D: [Bestatin]=5×10^{-6} M. (1,000 cpm are equivalent to 15.7 pmol/assay).
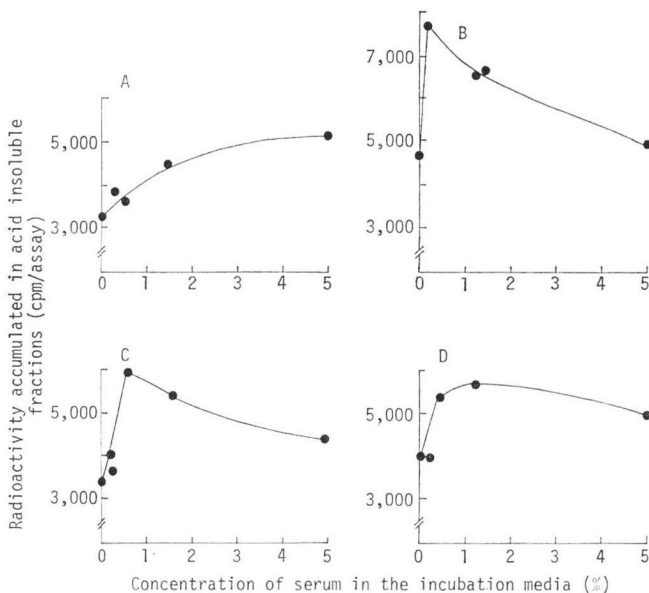

Fig. 3 shows the results of a 24-hour exposure of serum-starved Nil 8 cells to bestatin and labelled thymidine. The incorporation of [³H]thymidine into acid insoluble fractions increased significantly in those cells which were exposed to bestatin. The effect of bestatin is most striking in the presence of low concentration of serum. The stimulating effect of bestatin is most apparent in the presence of low concentrations of serum. The stimulating effect decreases as the concentration of either serum or bestatin increases, indicating a possible involvement of more than 1 site for bestatin.

A similar pattern is observed with BHK cells. Serum-starved cells were exposed for 24 hours to a solution containing 1 μM [³H]thymidine, serum and/or bestatin. In the absence of either serum or bestatin the incorporation of thymidine into acid insoluble fractions was 30±1 pmol/assay; in presence of 10^{-6} M bestatin the incorporation was: 38±1 pmol/assay. This stimulation was eliminated in presence of 5% serum. The incorporation in the presence of 5% serum was 46±1 pmol/assay, but when bestatin at the concentration of 10^{-7} M was added to the same incubation medium incorporation increased to 55±0.5 pmol/assay. As stated above this enhanced incorporation was eliminated when the bestatin concentration was increased to 10^{-6} M.

Discussion

We have been able to show that bestatin completely simulates the role of serum as an activator of uridine uptake into quiescent BHK cells. This effect in intact cells occurs at the same concentration range (10 nM) as was reported to be the effective inhibitory concentration of protease in isolated form[4]. Although this cannot be considered as a direct proof it is least possible that cell membrane proteases are involved at the initial phase of interaction between serum growth factors and quiescent cells.

After 24-hour incubation, bestatin at a concentration of 10^{-7} M potentiates the effect of serum on DNA synthesis in serum-starved cells. This effect does not occur at high bestatin and/or serum

concentrations. It would seem that bestatin exerts its effect only in a limited concentration range in the presence of low concentrations of serum. It is well established that externally added proteases have a stimulatory effect on confluent cells[19,20]. The results reported here may indicate the presence of a cells surface-associated protease that has to be inhibited when cells are stimulated to grow. On the other hand it cannot be ruled out that bestatin acts directly upon a membrane receptor of a serum growth factor in a mechanism which is distinct from its action as an enzyme inhibitor. Investigation of the molecular nature of the interaction between bestatin and cell surface proteases should enable us to distinguish between these two mechanisms.

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