BESTATIN, A MICROBIAL AMINOPEPTIDASE INHIBITOR, INHIBITS DNA SYNTHESIS INDUCED BY INSULIN OR EPIDERMAL GROWTH FACTOR IN PRIMARY CULTURED RAT HEPATOCYTES

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Bestatin, a microbial aminopeptidase inhibitor, inhibited insulin- or epidermal growth factor-induced DNA synthesis and cell proliferation in primary cultured hepatocytes of rats. The aminopeptidase inhibitor also affected the growth of FM3A or L_{OBN} cells of mice, when it is included in the culture media at the concentration of 0.5 mg/ml.

These results suggest the important role of the bestatin-sensitive aminopeptidase(s) in the process of DNA synthesis and proliferation of animal cells.

It is well established that insulin or epidermal growth factor (EGF) induces DNA synthesis and cell division in many cultured cells.[1~4] These peptides have been shown to be bound to their receptors on the cell surface,[5,6] internalized and degraded probably in lysosomes.[7~10] Their ability to induce DNA synthesis or cell division is transmitted to the nucleus during these intracellular events. However, the mechanisms of transmission of the signal to the nucleus is not elucidated. Recently, WIDELITZ *et al.*[11] showed that lysosomotropic amines inhibit the formation of the final product of the intracellular processing of EGF and, at the same time, inhibited EGF-induced DNA synthesis in rat fibroblasts. Their results suggest that the transmission of the biological signal of EGF is coupled with the completion of the degradation of EGF. Since lysosomotropic amines affect many intracellular reactions,[12] application of more specific inhibitors of the degradation pathway of the internalized peptides will be helpful to confirm the possible coupling of the biological activity and the degradation of the peptides.

Leupeptin and antipain are presumed to be more specific inhibitors of lysosomal proteolytic enzymes than lysosomotropic amines,[13] although their actions are not restricted to lysosomal proteases.[14] Contrary to the expectations derived from the work of WIDELITZ *et al.*,[11] SAVION *et al.*[15] demonstrated that leupeptin and antipain inhibit the degradation of internalized EGF but do not affect the activity of EGF to induce DNA synthesis or mitosis. These discrepancies have not been explained yet.

In our previous studies, we showed that a microbial aminopeptidase inhibitor, bestatin, induces accumulation of intermediate peptides of cellular protein degradation[16,17] as well as of the breakdown of the internalized insulin in cultured rat hepatocytes.[18] However, bestatin showed little effect of the insulin-dependent enhancement of the transport of α-aminoisobutyric acid or 3-*O*-methylglucose into cultured hepatocytes. The results show that some effects of insulin are not inhibited by bestatin although

this aminopeptidase inhibitor affects the completion of the intracellular degradation of internalized insulin (S.-I. TAKAHASHI *et al.*, in preparation). These observations prompted us to investigate the effect of bestatin on the induction of DNA synthesis by insulin as well as EGF. The present paper describes that bestatin inhibits insulin- or EGF-induced DNA synthesis and mitosis in primary cultured hepatocytes of rats. Bestatin also affected proliferation of other cultured cells, namely FM3A and L_{OBN} cells, which multiply in the absence of added growth factor in the medium.

Materials and Methods

Materials

Insulin, EGF, and dexamethasone were from Sigma. The media for cell cultures were obtained from Nissui Co. (Tokyo, Japan). [*methyl*-^{3}H]Thymidine (54.2 Ci/mmol) was purchased from Amersham.

Isolation and Monolayer Culture of Parenchymal Cells of Rat Liver

Parenchymal cells were isolated from male Wistar strain rats, weighing 150~200 g, by a method to perfuse the liver with collagenase.[19] The isolated cells were cultured according to TANAKA *et al.*[19] as follows. They were cultured in WILLIAMS' E medium at 37°C under 5% CO_2 and 95% air. For 1 hour after the isolation, 10% of calf serum was included in the medium in order to promote the cells to attach to the bottom of the culture dishes.

In the experiment to study the incorporation of [^{3}H]thymidine into DNA of the cells, 2.5×10^5 or 1×10^6 cells were inoculated to 35 mm Corning tissue culture dishes. After 1 hour incubation with calf serum (as described above), the medium was changed to the fresh one without calf serum and with 1 μM dexamethasone and 10 μM insulin. After 24 hours, the medium was changed again to the fresh one. This time, dexamethasone and insulin was not included in the medium. The cells were incubated in this medium for 24 hours and the medium was changed again to that with bestatin (0.5 mg/ml), insulin (0.1 μM) and/or EGF (0.1 μg/ml).

In the experiment to study the effect of bestatin on the proliferation of the isolated hepatocytes, 8×10^5 cells were inoculated to 60 mm Corning culture dishes. After 1 hour incubation with calf serum, the medium was replaced with serum-free one supplemented with insulin (0.1 μM) and/or EGF (0.1 μg/ml) and with or without bestatin (0.5 mg/ml). Cultures received daily the fresh medium of the same composition. At 48 hours after the first medium change (namely 1 hour after inoculation), phase-contrast photomicrographs were taken.

[^{3}H]Thymidine Incorporation into DNA

The activity of DNA synthesis was measured essentially as described by NAKAMURA *et al.*[20] At 20 hours after the addition of the peptide hormone, [^{3}H]thymidine (5 μCi/dish) was added to the medium and incubated with or without added hydroxyurea (final concentration was adjusted at 25 mM employing 250 mM hydroxyurea). After 2 hours incubation, the cells were washed with cold phosphate buffered saline and steeped in 10% trichloroacetic acid (TCA) at 4°C overnight. The cells were solubilized in 2 ml of 0.5 N NaOH followed by reprecipitation with 10% TCA as the final concentration. The pellet was washed twice with 5% TCA at 4°C. And then, DNA was hydrolyzed by heating at 100°C for 15 minutes in 1 ml of 10% TCA. The radioactivity solubilized by this hydrolysis was measured using Nonion-Toluene scintillator.[21] DNA synthesis was culculated as the difference of the radioactivity between the cells incubated without hydroxyurea and those with hydroxyurea.

Protein content of the cells was measured by the method of LOWRY *et al.*[22]

Mouse Cells in Culture

Mouse FM3A and L_{OBN} cells were kindly donated by Dr. Y. NAGATA, Institute of Applied Microbiology, The University of Tokyo. L_{OBN} cells were cultured in EAGLE's minimum essential medium containing 10% calf serum and FM3A cells in ES medium with 2% calf serum. These cells were cultured in 35 mm Corning tissue culture dishes at a density of 2.5×10^4 cells for L_{OBN} and 2.5×10^5

for FM3A in 2 ml of the media with or without bestatin (0.5 mg/ml). When the cells were inoculated, they were proliferating logarithmically. The number of cells per dish was counted every 24 hours during the experimental cultures.

Results

Fig. 1 shows that bestatin inhibits DNA synthesis induced by insulin or EGF. When the population of the cells in the dishes is low, DNA synthesis and proliferation of the cells were enhanced as reported by NAKAMURA *et al.*[20] In both of the populations, namely high (1×10^6 cells/dish) and low (2.5×10^5 cells/dish), the synthesis of DNA induced by insulin or EGF was depressed. The phase-contrast photomicrographs in Fig. 2 show that bestatin also inhibits cell division as well as DNA synthesis induced by EGF in cultured rat hepatocytes. The mean numbers of nuclei per the area shown in the photographs (for 4 dishes and 10 experiments a dish) ± SEM were shown in Table 1. These results suggest that bestatin inhibits the cell cycle at G_1 phase, although the possibility of inhibition of multiple points is not excluded.

Fig. 1. The effect of bestatin (shaded bars) on insulin- or EGF-induced DNA synthesis in primary cultured hepatocytes of rats.

Insulin and/or EGF were added to the monolayer cultures of rat hepatocytes in the presence or absence of bestatin. At 20 hours after the addition of these peptide hormones, incorporation of [^{3}H]-thymidine into DNA was measured. The concentrations of bestatin, insulin and EGF were 0.5 mg/ml, 0.1 μM and 0.1 μg/ml, respectively. Data are the mean±SEM for 4 dishes.

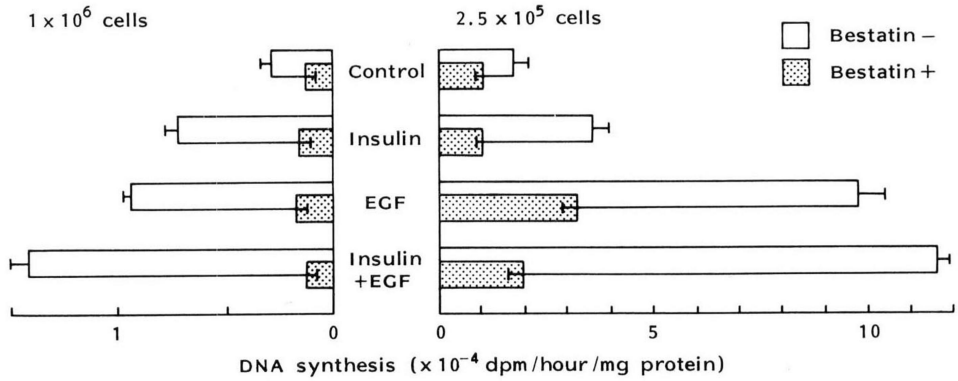

Table 1. The effect of bestatin in the culture medium on cell proliferation of primary cultured hepatocytes of rats.

Dish	Treatment			Average No.	
	Insulin	EGF	Bestatin	Nuclei	SEM
A	–	–	–	22.1	1.4
B	–	–	+	20.0	2.1
C	+	–	–	32.2	2.9
D	+	–	+	22.4	2.4
E	–	+	–	60.1	2.6
F	–	+	+	32.5	1.4
G	+	+	–	85.0	2.0
H	+	+	+	51.5	2.0

The mean numbers of nuclei per the area shown in Fig. 2 were counted. Data are the mean± SEM for 4 dishes (10 experiments a dish).

Fig. 2. The effect of bestatin on cell proliferation of primary cultured hepatocytes of rats.

Rat hepatocytes were cultured for 48 hours in the presence or absence of bestatin in the medium. The cell density is higher when the cells were cultured in the absence of bestatin in the medium.

For the concentrations of the substances employed, see Fig. 1. Dishes A ~ H correspond to those shown in Table 1.

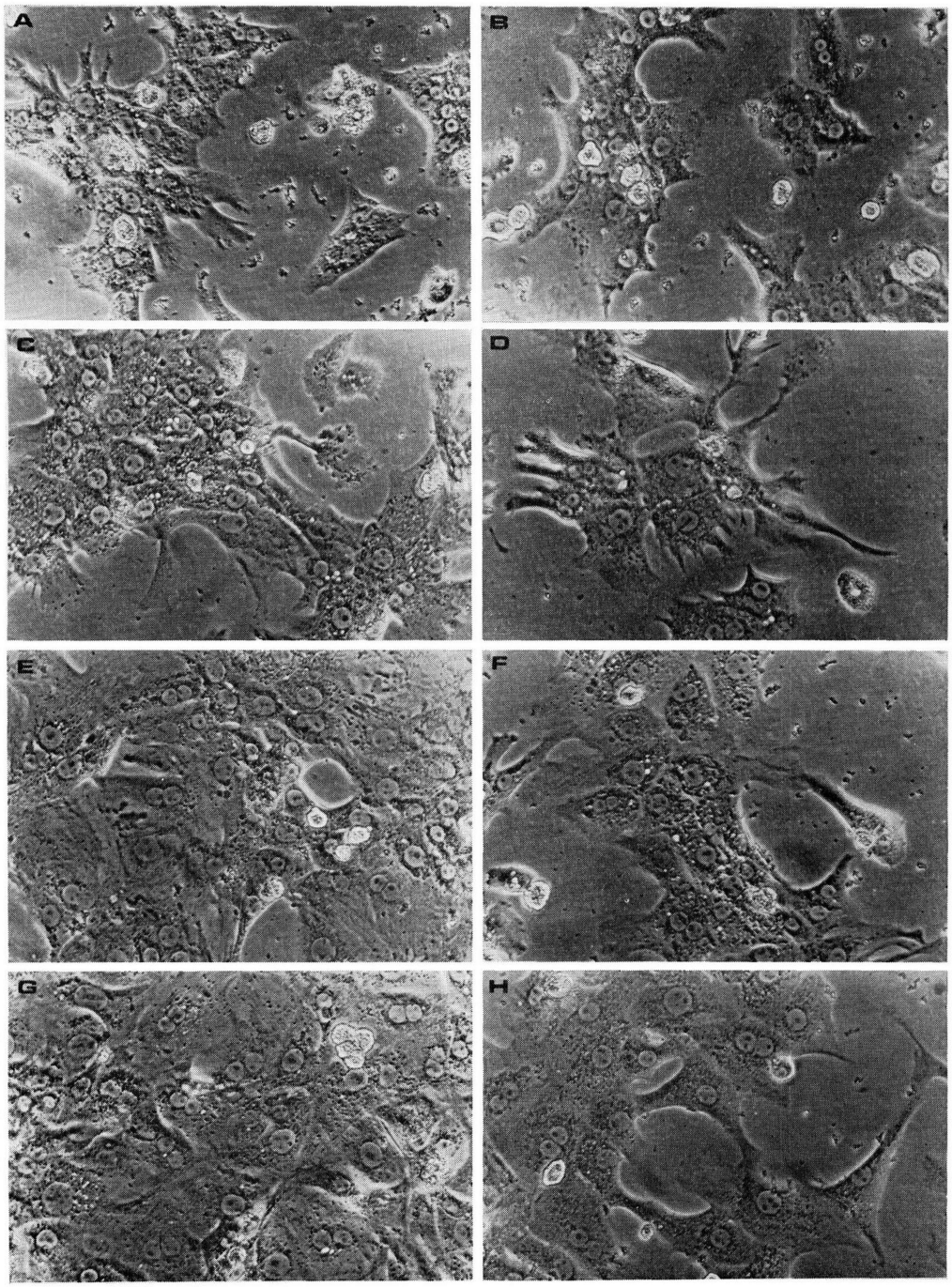

Fig. 3. The effect of bestatin on the proliferation of L_{OBN} and FM3A cells of mice.

The cells were cultured in the presence and absence of bestatin in the medium. The cell number was counted every 24 hours after the addition of bestatin to the medium.

The concentrations of bestatin in the medium was 0.5 mg/ml. The cell numbers were the mean of 3 experiments for 4 dishes. In both cells, the differences in the cell numbers of the corresponding dishes were statistically significant by Student's t-test ($P < 0.01$) except the differences at 0 time.

— Control, --- bestatin, ▲ total, ● viable.

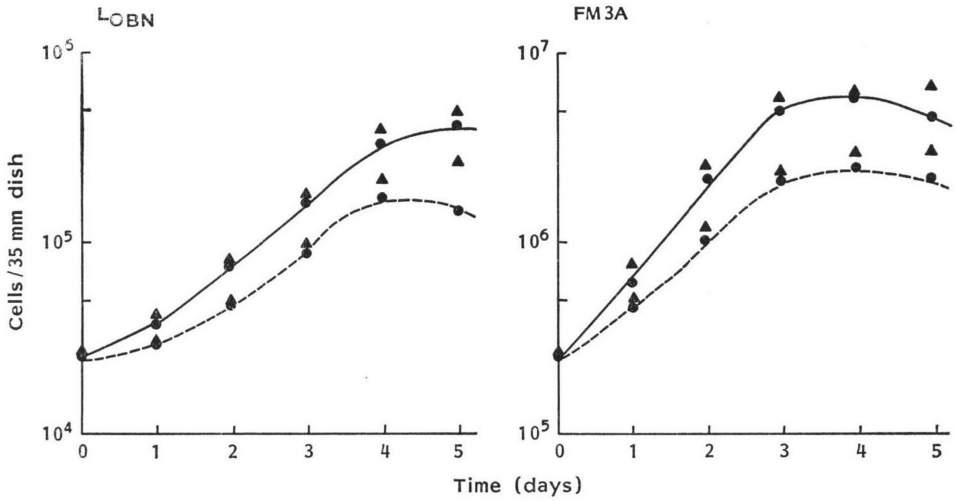

Fig. 3 shows that bestatin affects the cell division which proceeds independently of insulin or EGF in such cells as L_{OBN} and FM3A. Dishes incubated without bestatin for more than 1 day contained significantly more numbers of cells than those cultured with bestatin, especially in the case of FM3A cells. These results mean that the effect of bestatin is not confined to primary cultured hepatocytes.

Discussion

Bestatin is a microbial aminopeptidase inhibitor produced by *Streptomyces olivoreticuli*.[23] This inhibitor inhibits leucine aminopeptidase and arylamidase in various animal cells and organs.[14] When bestatin is injected into rats, a marked accumulation of acid soluble peptides (ASP) in liver has been observed.[16] Furthermore, bestatin induces urinary excretion of ASP,[16] induces ASP accumulation in perfused skeletal muscle,[17] and brings about accumulation of intermediates of insulin degradation in primary cultured hepatocytes of rats.[18] These observations suggest that bestatin acts on the final step of peptide hormone or protein degradation, namely the step to degrade small peptides to free amino acids.

From these properties of bestatin, there is enough possibility that this inhibitor inhibits the production of the second messenger of insulin or EGF, which has been proposed to be a peptide derived from membrane glycoprotein(s)[25~27] or their receptors.[28]. The effect of low molecular weight protease inhibitors on the production of possible second messenger has already been proposed.[29] The results presented in this paper favor those observations and give further information on the nature of the putative peptidase(s). In other words, the second messenger possibly is produced by a trypsin-like protease(s) followed by bestatin-sensitive aminopeptidase(s).

Recent studies on the mechanism of insulin's action show that phosphorylation of tyrosine residue of the insulin receptor is of prime importance in the action of the hormone.[1,3,29] After the tyrosine phosphorylation, phosphorylation of serine or threonine residues follows, then bringing about pleiotropic action of insulin on the metabolic processes of the target cells.[30] However, further informations

on the transmission of insulin's signal to the nucleus have not been obtained.

The mechanism of action of EGF to induce DNA synthesis of many cells[30] or that of transforming growth factors on transformed cells[2,3,31] has been considered to be similar to that of insulin's action on DNA synthesis of some kinds of cells, although no precise explanation of the mechanism has been proposed. Recently, KOHNO and KANNO reported the increase in cytosol aminopeptidase activity in mitogen-stimulated lymphocytes.[34] Their results also suggest the importance of aminopeptidase in cellular DNA synthesis.

The present results, demonstrating the inhibition of DNA synthesis by bestatin, may also contribute to the development of a new type of anti-mitosis chemicals.

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