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INHIBITORY EFFECT OF BESTATIN ON THE GROWTH OF HUMAN LEUKEMIC CELLS

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Abstract

We examined the effect of bestatin (Ubenimex) on the growth of human leukemic cells (i.e., HL-60, K562, MT-1, MT-2, Molt-4, and Raji cells). The growth of each cell line was inhibited by the cocultivation with bestatin at higher concentrations than employed for clinical use in Japan. [^3H]TdR incorporation was also inhibited in MT-1 and MT-2 cells by treatment with bestatin. Degenerated cell-to-cell adhesion was observed among the treated cells. These findings suggest that the inhibitory effect on some leukemic cells, especially on MT-1 cells, results from the inhibition of DNA synthesis.

Key words: Bestatin, leukemic cell lines, [^3H]TdR incorporation.

Bestatin, (Ubenimex), which is a dipeptide and specifically inhibits the activity of aminopeptidase B and leucine aminopeptidase of the cell membrane, has been found by Umezawa et al. in *Streptomyces olivoreticuli* (1, 2). Recently, it has been reported that bestatin has immunomodulating activity as a biological response modifier (3), stimulates B-cell colony formation (4), and serves as immunomodulator for anti-tumor activity in vitro and in vivo (5, 6). In clinical medicine, it has been used at the stage of complete remission of acute non lymphocytic leukemia (ANLL) in Japan in order to prolong the survival (7). It has also been used in patients with various cancer and AIDS-related conditions with favorable response (8, 9).

The mechanism of the anti-tumor activity is thought to be mediated by the immunomodulating activity, but the exact mechanism for the anti-tumor activity of bestatin is not yet clear.

The purpose of this study was to examine the effect of

bestatin on the growth of human leukemic cell lines in vitro, including the effect of bestatin on the incorporation of [^3H]TdR.

Materials and Methods

Human leukemic cell lines, including HL-60 and K562 as myeloid cell lines, MT-1 (10), MT-2 (11), and MOLT-4 as T-cells lines and Raji as B cell line, were cultured in RPMI 1640 medium with 10% FCS in the presence of bestatin (Ubenimex), at various concentrations (0, 1 $\mu\text{g}/\text{ml}$ to 4.5 mg/ml) and harvested at 12 h, 24 h, 48 h and 72 h after the culture. The initial culture was started with $1 \times 10^5 - 1 \times 10^6$ cells in the culture dish. The viable cells were counted by trypan blue dye exclusion test. The morphological feature of the cultured cells at various culture periods were examined by staining with May-Grünwald solution on light microscopy, by scanning electron microscopy (Hitachi S450) and by transmission electron microscopy (Hitachi HU-12A). DNA synthesis was studied by [^3H] thymidine ([^3H]TdR) incorporation at 24 h, 48 h, and 72 h after the culture in the presence of bestatin.

Results

Growth of leukemic cell lines. As shown in Fig. 1a, the number of viable HL-60 cells decreased with time at a high concentration of bestatin (4.5 mg/ml) with time. The

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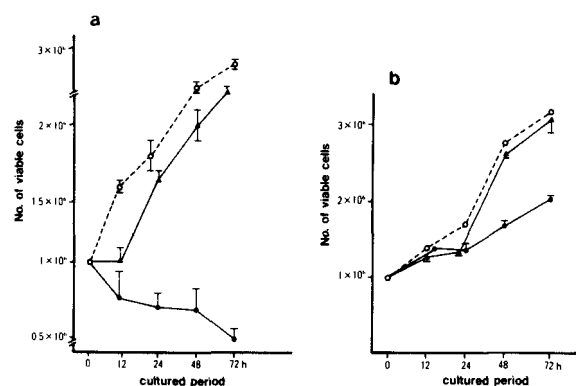


Fig. 1. Effect of bestatin on the growth of myeloid cell lines, a) HL-60 and b) K562 cells. Each cell line (1×10^6 cell/well) was cultured in the presence of bestatin (0.450 mg/ml, 4.5 mg/ml) in the culture plate (Costar, Cambridge, MA, USA) at 37°C . The cultured cells were harvested at each cultured period and the viability of the cells was determined by trypan blue dye exclusion test. $\circ$ — $\circ$ control; $\blacktriangle$ — $\blacktriangle$ 450 $\mu\text{g/ml}$; $\bullet$ — $\bullet$ 4.5 mg/ml.

decrease of the number of viable K562 cells was less pronounced at the same concentration of bestatin (Fig. 1b). However, the growth of both cell types was unaffected at lower concentrations (4.5, 45, and 450 $\mu\text{g/ml}$).

Morphological examination showed more degenerated cell-to-cell adhesion in cells treated with bestatin than in control cells. Also, abnormal agglutination of chromatin and giant cell formation was observed more frequently in the cells cultured with bestatin (data not shown).

In the T-cell lines (Fig. 2), the number of viable MT-1 cells was also decreased at the concentrations 4.5 mg/ml and 450 $\mu\text{g/ml}$ bestatin, but not observed at 4.5 $\mu\text{g/ml}$ and 45 $\mu\text{g/ml}$. Similar effect on the cell growth was observed in MT-2 cells treated with bestatin (data not shown). On the other hand, the number of viable MOLT-4 cells did not decrease even at high concentrations of bestatin. (Fig. 2b).

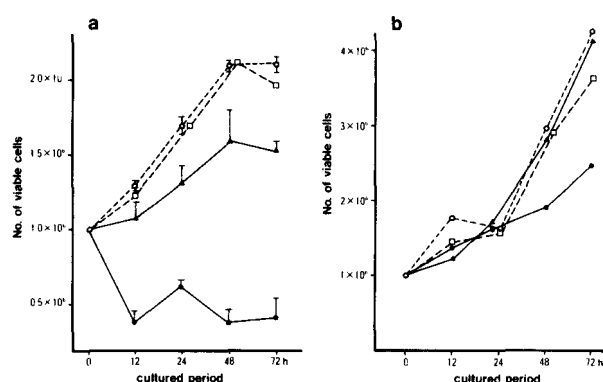


Fig. 2. Effect of bestatin on the growth of T-cell lines, a) MT-1 and b) MOLT-4 cells. The culture condition was same as in Fig. 1. Data of MT-1 cells represents mean $\pm$ SD (standard deviation) of triplicate culture. Data of MOLT-4 cells represents mean of two independent experiments. $\circ$ — $\circ$ control; $\square$ — $\square$ 45 $\mu\text{g/ml}$; $\blacktriangle$ — $\blacktriangle$ 450 $\mu\text{g/ml}$; $\bullet$ — $\bullet$ 4.5 mg/ml.

The number of viable Raji cells also decreased at 4.5 mg/ml of bestatin, but was not affected at 450 $\mu\text{g/ml}$ in contrast to the MT-1 cells (Fig. 3). These results suggested that the MT-1 cells were most sensitive to bestatin. Therefore, we also investigated other effects of bestatin on MT-1 cells.

The morphological features of MT-1 cells. As shown in Fig. 4, the bestatin treated cells showed degenerated

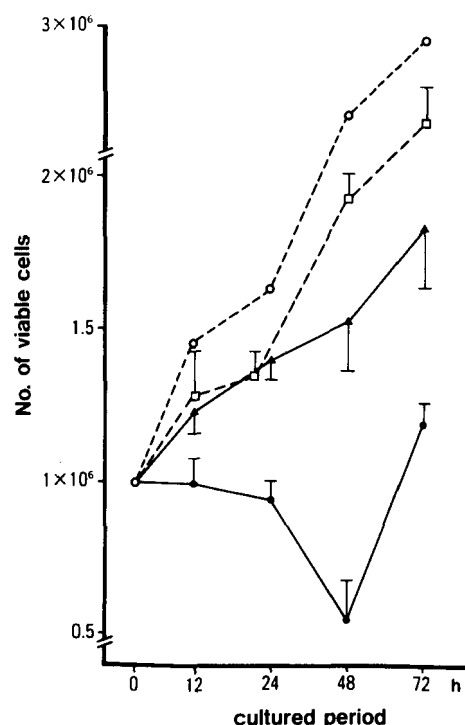


Fig. 3. Effect of bestatin on the growth of a B-cell line, Raji cells. The culture condition was same as in Fig. 1. Data represents mean $\pm$ SD of triplicate culture. $\circ$ — $\circ$ control; $\square$ — $\square$ 45 $\mu\text{g/ml}$; $\blacktriangle$ — $\blacktriangle$ 450 $\mu\text{g/ml}$; $\bullet$ — $\bullet$ 4.5 mg/ml.

Fig. 4. Scanning electron microscopy of MT-1 cells. A drop of MT-1 cells cultured with 1 mg/ml bestatin for 0, 24 and 48 h was fixed in 2% glutaraldehyde in 0.1 mol/l cacodylate buffer (pH 7.4) for 30 min at room temperature. After fixation with 2% osmium tetroxide for 30 min, the specimens were dehydrated in graded series of ethanol followed by substitution with isoamyl acetate. The specimens were critical-point-dried in carbon dioxide and coated with gold in an ion coater. Thereafter, they were analysed by Hitachi S-450 scanning electron microscopy. The left picture, (A), shows the cells cultured for 0 h with bestatin (1 mg/ml). The middle picture, (B), shows cells cultured for 24 h with bestatin at the same concentration. The right picture, (C), shows cells cultured for 48 h. The length of each bar corresponds to 5 μm .

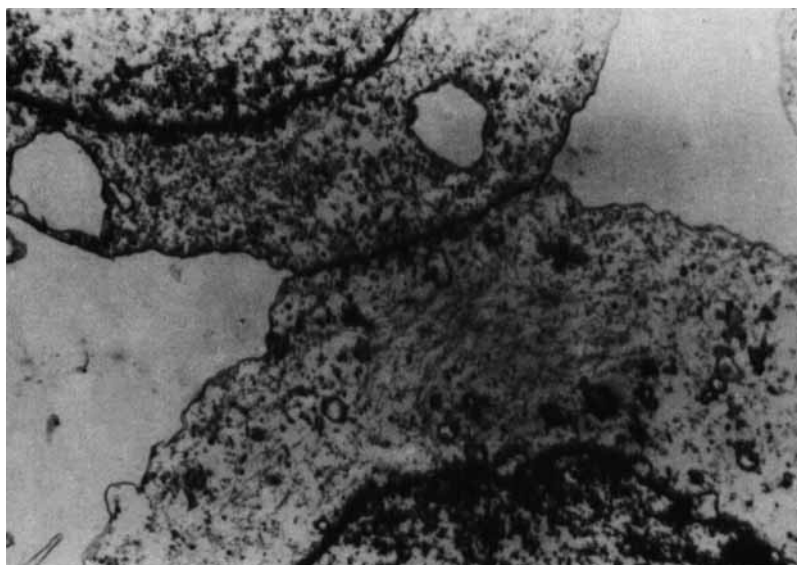


Fig. 5. Transmission electron microscopy of MT-1 cells. A drop of MT-1 cells cultured at same conditions as in Fig. 4 was fixed in 2% glutaraldehyde in 0.1 mol/l cacodylate buffer (pH 7.4) for 2 h at 4°C. After fixation, the specimen was treated in 2% osmium tetroxide in distilled water for 2 h at room temperature, followed by dehydration in Epok812 (Oken, Inc.). Ultra-thin sections were stained with lead citrate and observed with Hitachi HV-12A electron microscope. $\times 15\,000$.

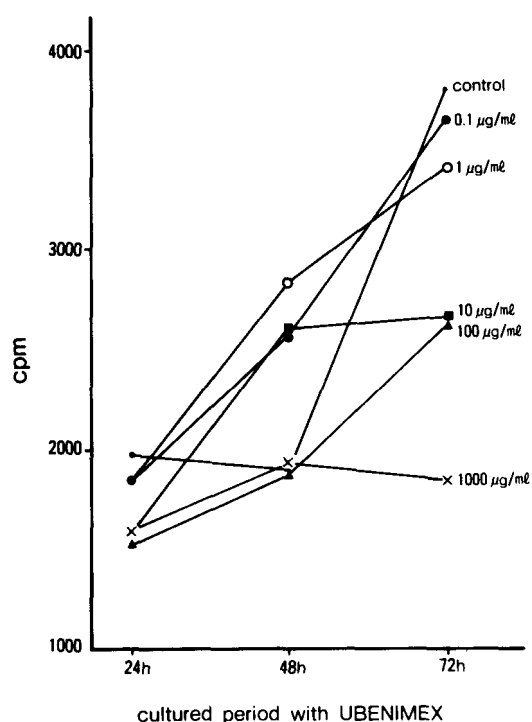


Fig. 6. DNA synthesis of MT-1 cells cultured with bestatin. The MT-1 cells cultured with bestatin at various concentrations were harvested in each culture period. Two days later, 37 kBq [^3H]TdR was added to each well. The [^3H]TdR incorporation of the treated cells was then measured by a scintillation counter. Data represent the mean of triplicate culture.

agglutination with time and degeneration (e.g. vacuolation) as compared with the control cells. With transmission electron microscopy (Fig. 5), many vacuoles were clearly observed in the cytoplasm. However, the cell membrane of the MT-1 cells appeared not to be damaged.

DNA synthesis of MT-1 cells. The effect of bestatin on the DNA synthesis was studied by [^3H]TdR incorporation. As shown in Fig. 6, [^3H]TdR incorporation of MT-1 cells was inhibited even at low concentrations, which did not inhibit the growth of MT-1 cells. A similar effect of bestatin on MT-2 cells was observed (data not shown). As shown in the Table, the [^3H] incorporation of normal human lymphocytes was not affected even at high concentration of bestatin (4.5 mg/ml).

Table

Effect of bestatin on proliferation of normal lymphocytes

Treatment	Dose	(^3H) TdR incorporation
None	0	884 $\pm$ 80**
Bestatin	4 500*	531 $\pm$ 52
Bestatin	1 000	713 $\pm$ 59
Bestatin	500	627 $\pm$ 69

* $\mu\text{g/ml}$

** Mean (cpm) $\pm$ SD

Discussion

We found bestatin to have an inhibitory effect on the growth of some leukemic cell lines, especially MT-1 (10), and MT-2 (11), which are infected by and produce HTLV-1 virus. Although the exact mechanism is not yet ascertained, it is reported that bestatin has anti-tumor activity in vitro and in vivo (5, 6). It has been shown that this peptide prolongs survival of ANLL patients in Japan (7). In this case, it has been assumed that the anti-tumor activity is mediated by some immunomodulating mechanism. In our present study the cell growth and [³H]TdR incorporation was markedly inhibited in a dose-dependent fashion by the in vitro treatment with bestatin. It is not clear how bestatin induced this effect or why especially MT-1 or MT-2 cells are sensitive to bestatin. However, it seems likely that factors specific to T-cells or T-cell leukemia are related to this phenomenon. Recently, Mathé et al. (8, 9) have reported that the target of bestatin may be the CD4 molecule on the cell membrane. MT-1 and MT-2 cells have the CD4 molecule on their cell membranes. We are at present examining whether treatment with anti-CD4 antibody is able to counteract the inhibitory activity of bestatin on MT-1 cells.

The bestatin-treated cells showed degenerated cell-to-cell adhesion. It is not yet clear whether this adhesion is related to the inhibition of cell growth. Several adhesion molecules including CD2 (12), CD4 (13), CD8 (13, 14), TCR-CD3 complex, LFA-1 (CD11a), MAC-1 (CD11b), P150, 95, (CD11c) or VLA-1 to VLA-5 have been reported (15). LFA-1, MAC-1, p150, 95, and VLA constitute superfamily of adhesive receptors, called integrins (16). It is likely that some of these adhesive molecules are involved in the cell-to-cell adhesion caused by treatment with bestatin. According to a preliminary experiment, the cell-to-cell adhesion of MT-1 cells caused by bestatin appeared to be blocked by pretreatment with anti-CD4 antibody but not by anti-LFA-1, anti-CD11a, anti-CD11b, anti-CD11c, or anti-CD18 antibody. Anti-CD8 and -CD2 antibodies showed an incomplete blocking effect. These findings may suggest that the CD4 molecule on MT-1 cells are the most important molecule for inducing the adhesion.

This cell-to-cell adhesion may play some role in the inhibition of cell growth since the growth of recultured MT-1 cells after removal of bestatin by washing were slightly inhibited. However, this result cannot exclude the possibility that bestatin inhibited the cell growth or [³H]TdR incorporation without mediating the adhesion via the CD4 molecule. The mechanisms by which bestatin inhibits the growth of MT-1 cells should probably be determined in the near future.

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