

## Effects of bestatin (Ubenimex) on human T-cell colony formation

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**The antitumor action of bestatin is considered to be an indirect action mediated by T-cells. Therefore, we investigated the effects of bestatin on the differentiation and proliferation of human precursor T-cells using a colony formation technique. Bestatin did not increase the overall number of T-cell colonies, but it significantly increased in CD4<sup>+</sup> cell and significantly decreased in CD8<sup>+</sup> cell subpopulations. It also induced CD4<sup>+</sup> · 8<sup>+</sup> cells.**

**These findings indicated that bestatin acts on precursor T-cells to induce the differentiation of these cells into CD4<sup>+</sup> cells.**

**Key words:** Bestatin, CD4<sup>+</sup> cells, CD4<sup>+</sup> · 8<sup>+</sup> cells, T-cell colony.

### Introduction

Bestatin (Ubenimex (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutyryl-L-leucine) is a dipeptide that inhibits leucine aminopeptidase,<sup>1</sup> a membrane enzyme.

Bestatin increases delayed-type hypersensitivity reaction to SRBC in mice.<sup>2</sup> Moreover, this agent has been demonstrated to possess antitumor activity against myeloid leukemia in mice,<sup>3</sup> to inhibit the growth of fibrosarcoma in rats,<sup>4</sup> to prevent progression of nitrosoguanidine-induced stomach cancer in rats,<sup>5</sup> and to inhibit lymph node metastasis of P388 leukemia in mice.<sup>6</sup> All of these activities of bestatin have been shown experimentally to be mediated by host T-cells.<sup>7</sup> Therefore, bestatin is regarded as an immunopotentiator.

Furthermore, bestatin increases hemopoietic stem cells<sup>8</sup> as well as nucleated cells in human bone marrow.<sup>9</sup> Since bestatin loses the ability to increase hemopoietic stem cells after removal of T-cells,<sup>10</sup> it is postulated that this agent activates T-cell functions.

Therefore, we examined the effects of bestatin on the differentiation and proliferation of human precursor T-cells using an *in vitro* T-cell colony forming technique.

### Materials and methods

#### Materials

About 10 ml of venous blood was collected in a heparinized syringe from eight healthy volunteers (18–40 years old, average 27.8 years old). None of the samples was kept longer than 5 h prior to the start of culture.

#### Separation of peripheral blood mononuclear cells

The blood collected by the above-mentioned method was diluted two-fold with pH 7.4 Tyrode's solution. Lymphoprep (sodium metrizoate–Ficoll solution, specific gravity 1.077, Nygaard) was then placed in small test tubes. The diluted blood was gently laid over the Lymphoprep at a Lymphoprep-to-diluted blood ratio of 1:3. The test tubes were then centrifuged at 20°C, 450 *g* for 30 min, to separate the mononuclear (MN) cell fraction. Thus, (7–10) × 10<sup>6</sup> MN cells were obtained and over 95% of them were confirmed to be viable under trypan blue dye exclusion.

#### T-cell colony formation

The one-step monolayer method was employed for the human T-cell colony formation.

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$5 \times 10^5$  MN cells separated by the above method were plated into semisolid gel consisting of pH 7.4 Eagle's minimum essential medium (MEM) 1.0 ml containing phytohemagglutinin-P (Difco Laboratories) 30  $\mu$ g, 20% autologous plasma, 2-mercaptoethanol  $5 \times 10^{-5}$  M, penicillin-G 100 U, streptomycin 100  $\mu$ g, and 1.0% methylcellulose (4000 cps, Wako Junyaku Chemical & Co, Ltd, Tokyo).

The culture medium was put into plastic Petri dishes (#3001, Falcon) and cultured at 37°C in a CO<sub>2</sub> incubator in fully humidified air containing 5% CO<sub>2</sub> for 7 days. A cell aggregate exceeding 50 cells was identified as a T-cell colony under an inverted microscope. The total number of colonies formed per Petri dish was then calculated as colony forming capacity at the seventh culture day. The samples were cultured in triplicate. Details of T-cell colony formation have been reported elsewhere.<sup>11</sup>

#### Effects of bestatin on T-cell colony formation

Bestatin (Lot X70003, Nippon Kayaku Co, Ltd, Tokyo, Japan) was used dissolved in pH 7.4 Eagle's MEM. Bestatin was added to each semisolid culture system at a ratio of 0–20  $\mu$ g/ml to examine the effects on T-cell colony formation

#### Analysis of T-cell colony component cells by monoclonal antibodies

Monoclonal antibodies employed were anti-CD3, 4, and 8 antibodies (peroxidase-conjugated anti-Leu 4, PE-labeled anti-Leu 3<sub>a</sub>, and FITC-labeled anti-Leu 2<sub>a</sub> antibodies, Becton-Dickinson).

Under an inverted microscope, colonies were gently picked up using a Pasteur pipet, and were suspended in a small test tube containing pH 7.4 Eagle's MEM. Colonies were washed three times, then re-suspended in pH 7.2 phosphate buffered saline containing 10% human AB serum. Colonies were collected on the glass slides using Cytospin 2 (Shandon).

Colonies were fixed on the glass slides with buffered formalin-acetone and the samples were washed three times in pure water. Then, pH 7.2 PBS containing 2% bovine serum albumin (BSA) was laid on the glass slides, and both FITC-labeled anti-CD8 and PE-labeled anti-CD4 antibodies were reacted at 4°C for 24 h.

The CD3<sup>+</sup> cells were stained with immunoper-

oxidase method. After anti-CD3 antibody was reacted for 24 h, the samples were treated with anti-mouse biotinylated goat serum. Then peroxidase-conjugated streptoavidin was reacted. Lastly, the samples were colored with DAB solution.

The calculation of CD4<sup>+</sup> and CD8<sup>+</sup> cells was made by photographing them under a fluorescent microscope, projecting the pictures on a screen, and counting the positive cells.

Since CD4<sup>+</sup> cells were colored in red and CD8<sup>+</sup> cells in green, they were easily distinguishable. The calculation of CD3<sup>+</sup> cells was performed under a microscope and the number of peroxidase-positive cells was counted. In each sample, at least 20 or more colonies were usually counted, and the mean value was considered to be the number of the positive cells.

#### Statistical analysis

Statistical analysis was performed by Student's *t*-test.

## Results

#### T-cell colony formation and effects of bestatin

Figure 1 shows the relationship between the culture days and T-cell colony growth in one case. Colony formation was noted on the third culture day, and a nearly linear increase was observed up to the seventh culture day.

Colonies broke down gradually after 7 days.

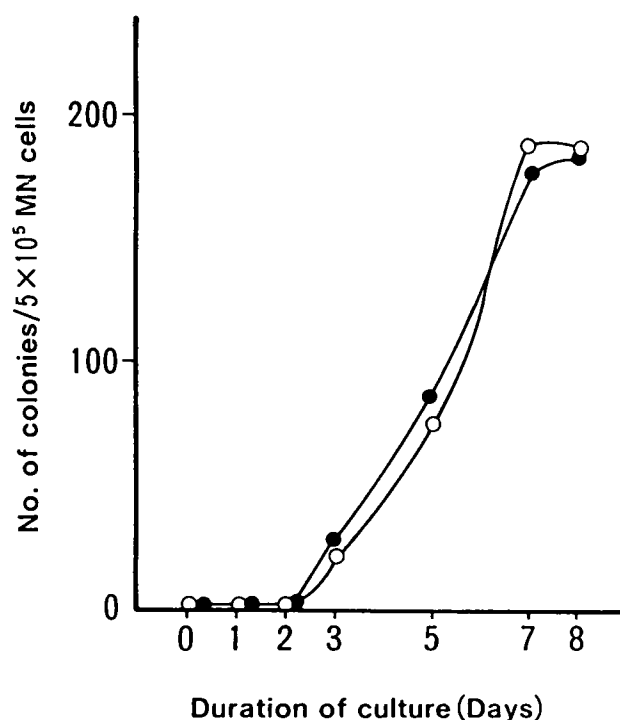
As shown in this figure, no changes were seen in the number of colonies irrespective of addition of bestatin (1  $\mu$ g/ml).

Figure 2 shows the results of T-cell colony formation and the effects of bestatin in one case on the seventh culture day.

Bestatin at 0.1–2  $\mu$ g/ml had no effect on colony formation. At concentrations higher than 4  $\mu$ g/ml or more, colony formation was inhibited remarkably. These results suggest that bestatin does not increase human T-cell colony formation.

#### Morphology of T-cell colony component cells

Colonies on the seventh culture day were picked up using a Pasteur pipet and were collected on the glass



**Figure 1.** Effect of bestatin on a normal human T-cell colony growth: ○, control; ●, bestatin (1 µg/ml).

slides using Cytospin 2. Wright-Giemsa staining was performed. Colony component cells were consisted of lymphocytes, lymphoblastoid cells, and a small number of macrophages.

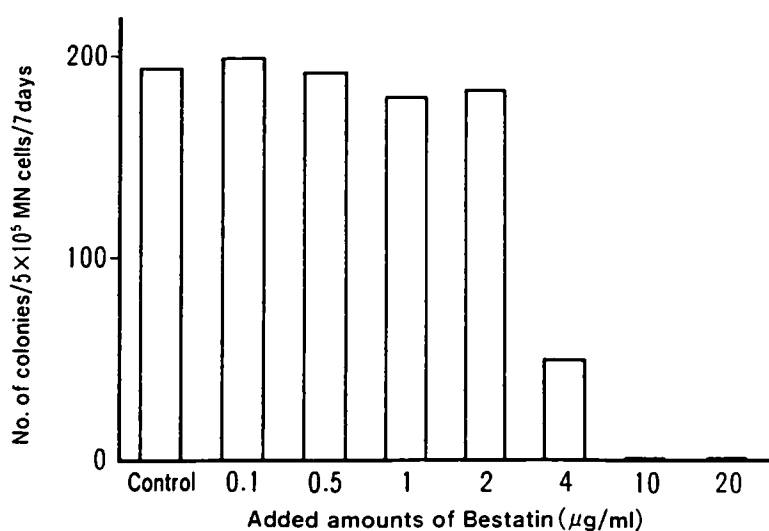
#### Analysis of T-cell colony component cells by monoclonal antibodies

Table 1 shows the result of analysis of normal human T-cell colony component cells by monoclonal antibodies (CD3, 4, and 8 antibodies). Almost 100% of the colony component cells were CD3<sup>+</sup> cells. CD4<sup>+</sup> cells accounted for 52.5%, and CD8<sup>+</sup> cells for 21.2% of all component cells. Cells expressing both CD4 and CD8 were not detected. Also, there was no colony expressing CD3 alone, CD3, 4 alone or CD3, 8 alone.

Surface markers on colony component cells formed in the presence of 1 µg/ml of bestatin were investigated using eight samples. As shown in Table 2, results similar to those shown in Table 1 were obtained. In this experiment, an increase in CD4<sup>+</sup> cells, a decrease in CD8<sup>+</sup> cells, and appearance of CD4<sup>+</sup>·CD8<sup>+</sup> cells were observed.

#### Discussion

In the present study, bestatin did not affect the number of formed colonies, but it increased CD4<sup>+</sup> cells, decreased CD8<sup>+</sup> cells and induced CD4<sup>+</sup>·CD8<sup>+</sup> cells. It has been reported that bestatin induces cytotoxicity to various tumor cells,<sup>12,13</sup> and activates macrophages.<sup>13</sup> Since bestatin does not exhibit antitumor activity in BALB/C



**Figure 2.** Relationship between number of formed colonies and added amounts of bestatin.

**Table 1.** Analysis of colony component cells by monoclonal antibodies (anti-CD3, 4, 8)

| Sample | Peripheral blood |     |                           |                    |                         |                         |                         | Number of colonies/<br>5 × 10 <sup>5</sup> cells | Colony component cells  |                         |                         |
|--------|------------------|-----|---------------------------|--------------------|-------------------------|-------------------------|-------------------------|--|-------------------------|-------------------------|-------------------------|
|        | Age              | Sex | WBC<br>(mm <sup>3</sup> ) | Lymphocytes<br>(%) | CD3 <sup>+</sup><br>(%) | CD4 <sup>+</sup><br>(%) | CD8 <sup>+</sup><br>(%) |  | CD3 <sup>+</sup><br>(%) | CD4 <sup>+</sup><br>(%) | CD8 <sup>+</sup><br>(%) |
| 1      | 46               | M   | 4800                      | 28.5               | 70                      | 42                      | 19                      | 112  | 100                     | 60.1                    | 19.2                    |
| 2      | 18               | F   | 8600                      | 46                 | ND                      | ND                      | ND                      | 210  | 100                     | 52.5                    | 25.0                    |
| 3      | 26               | F   | 3200                      | 52                 | 59                      | 50                      | 26                      | 80   | 100                     | 46.3                    | 12.1                    |
| 4      | 32               | F   | 5100                      | 20                 | 60                      | 32                      | 11                      | 168  | 99.1                    | 60.4                    | 22.2                    |
| 5      | 25               | F   | 7100                      | 19                 | ND                      | ND                      | ND                      | 396  | 98.2                    | 50.1                    | 30.5                    |
| 6      | 40               | M   | 10100                     | 29                 | ND                      | ND                      | ND                      | 79   | 100                     | 40.0                    | 23.3                    |
| 7      | 21               | M   | 4700                      | 40.5               | 79                      | 40                      | 17                      | 225  | 100                     | 39.0                    | 29.0                    |
| 8      | 29               | F   | 6300                      | 18                 | ND                      | ND                      | ND                      | 321  | 100                     | 71.7                    | 8.6                     |
| Mean   | 29.6             |     | 6237                      | 31.6               | 67                      | 41                      | 18.3                    | 199  | 99.6                    | 52.5                    | 21.2                    |
| ± SD   | 9.5              |     | 2240                      | 12.2               | 8.2                     | 6.4                     | 6                       | 106.9  | 0.7                     | 10.4                    | 7.2                     |

ND, not done.

nu/nu mice lacking T-cells,<sup>13</sup> it is speculated that bestatin exerts antitumor activity indirectly mediated by T-cells.

Bestatin increases bone marrow cells as well as neutrophils and platelets.<sup>14</sup>

In addition, bestatin increases colony forming units in culture (CFU-C) in the presence of colony stimulating factor (CSF),<sup>8</sup> but this effect disappears after removal of T-cells.<sup>10</sup> Bestatin also normalizes T-cell subsets of cancer patients,<sup>15</sup> increases natural killer cells<sup>15</sup> and promotes human B-cell colony formation.<sup>16</sup>

It is postulated that bestatin increases production of antibodies and potentiates delayed-type hypersensitivity reaction and immune response as a result of promotion of production of cytokines such as interleukin-1, 2 (IL-1, 2), interferon and CSF.<sup>17-19</sup>

Clinically, bestatin prolonged remission duration and survival time in patients with acute leukemia.<sup>20</sup>

From the results of cytotoxicity tests using monoclonal antibodies and complements,<sup>21</sup> steroid hormone and mitomycin C,<sup>11</sup> the colony forming cells appear to correspond to early to common thymocytes.<sup>22</sup>

For the differentiation and proliferation of colony forming cells, the presence of IL-2 is essential. The present study revealed that bestatin does not increase the number of colonies. Thus, it is unlikely that bestatin promotes production of IL-2.

However, in the presence of bestatin, CD4<sup>+</sup>·CD8<sup>+</sup> cells appeared in the colony component cells. These findings suggest that bestatin acts on precursor T-cells to favor the differentiation and proliferation of CD4<sup>+</sup> cells.

**Table 2.** Effects of bestatin (1 µg/ml) on surface markers of T-cell colony component cells

| Sample | Age   | Sex | WBC<br>(mm <sup>3</sup> ) | Lymphocytes<br>(%) | Number of<br>colonies | CD3 <sup>+</sup> | CD4 <sup>+</sup>   | CD8 <sup>+</sup>   | CD4 <sup>+</sup> ·8 <sup>+</sup> |
|--------|-------|-----|---------------------------|--------------------|-----------------------|------------------|--------------------|--------------------|----------------------------------|
| 1      | 18    | F   | 8800                      | 46.5               | 180                   | 98.2             | 53.2               | 13.3               | 6.0                              |
| 2      | 22    | F   | 7100                      | 26                 | 260                   | 100              | 63.1               | 15.6               | 10.2                             |
| 3      | 31    | F   | 4800                      | 18                 | 152                   | 95.6             | 59.3               | 20.1               | 3.6                              |
| 4      | 20    | M   | 9000                      | 50.5               | 188                   | 99.2             | 70.5               | 10.5               | 7.9                              |
| 5      | 38    | F   | 5300                      | 30                 | 236                   | 98.6             | 64.3               | 12.2               | 10.6                             |
| 6      | 24    | M   | 4100                      | 19                 | 292                   | 100              | 68.8               | 14.8               | 8.8                              |
| 7      | 29    | M   | 4600                      | 28                 | 226                   | 100              | 52.4               | 9.6                | 8.7                              |
| 8      | 40    | F   | 7700                      | 43                 | 226                   | 96.7             | 69.3               | 11.5               | 5.4                              |
| Mean   | 27.8  |     | 6425                      | 32.6               | 220                   | 98.5             | 62.6               | 13.5               | 7.7                              |
| ± SD   | ± 8.2 |     | ± 1837                    | ± 11.6             | ± 42.4                | ± 1.5            | ± 6.6 <sup>a</sup> | ± 3.2 <sup>b</sup> | ± 2.3                            |

<sup>a</sup> *P* < 0.05.<sup>b</sup> *P* < 0.01.

## Conclusion

The effects of bestatin on the differentiation and proliferation of human precursor T-cells was studied using a T-cell colony formation technique.

The results indicated that bestatin induces CD4<sup>+</sup> cells from human precursor T-cells.

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