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Inhibition of Tumor Cell Invasion by Ubenimex (Bestatin) *in vitro*

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We have investigated the effect of the immunomodulator ubenimex (bestatin) on tumor cell invasion of reconstituted basement membrane (Matrigel). The invasion of B16-BL6 melanoma cells and Lewis lung carcinoma (3LL) cells into Matrigel-coated filters was inhibited by the presence of bestatin in a concentration-dependent manner. The pretreatment of either tumor cells or Matrigel with bestatin, however, had little effect on the invasion of tumor cells. Since bestatin was found to inhibit aminopeptidase in addition to its immunomodulating activities, the inhibition of tumor invasion by bestatin is likely to be associated with the action as an enzyme inhibitor. Other aminopeptidase inhibitors, arphamenine B and amastatin A, could also inhibit tumor cell invasion into Matrigel. Bestatin inhibited hydrolyzing activities towards substrates of aminopeptidases in B16-BL6 melanoma cells. However, bestatin did not have any effect on the haptotactic migration and adhesion of tumor cells to the substrates. These results indicated that bestatin may inhibit tumor cell invasion through a mechanism involving its inhibitory action on aminopeptidases in tumor cells.

Key word: Ubenimex (Bestatin) — Tumor metastasis — Invasion — Haptotactic migration — Adhesion

The binding of tumor cells to adhesion molecules, as well as their migration within extracellular matrices, plays an important role in the metastatic spread of malignant neoplasms.[1-4] Extracellular matrices in the vicinity of an invading neoplasm undergo significant degradation and remodeling during the invasion of malignant tumor cells. Two glycoproteins present in the extracellular matrices which play major roles in the process of tumor metastasis are fibronectin and laminin, which are involved in stimulating the adhesion[1,3] migration[5,6] and invasion of tumor cells. During the invasive cascade, proteolytic and other degradative enzymes have also been suggested to pass through connective tissue barriers consisting of adhesive proteins such as fibronectin, laminin and collagens.[1-4,7]

Ubenimex, designated bestatin, is a dipeptide discovered in the culture filtrate of *Streptomyces olivoreticuli*[8] and is a competitive inhibitor of aminopeptidase B and leucine aminopeptidase.[9-11] The immunomodulatory properties of bestatin have been examined in preclinical and clinical studies.[12-17] Bestatin has been shown to augment the humoral and cellular immune responses in experimental animals,[18-21] and can activate murine macrophages to become cytotoxic against tumor cells *in vivo* and *in vitro*, possibly through the T-independent mechanism.[13,21] Talmadge *et al.*[22] have reported significant therapeutic activity of bestatin against pre-existing experimental and spontaneous metastases when it was administered at high doses per animal. We also observed that bestatin significantly inhibited lung colonization of Lewis lung carcinoma when it was administered p.o. for 10 consecutive days after intravenous inoculation of the tumor (unpublished data). Further, the treatment of mice with bestatin reduces the frequency of spontaneous or methylcholanthrene-induced tumors.[15]

In the present study, we focused our attention on the enzyme-inhibitory properties of bestatin rather than the immunomodulatory activity, and also examined the effect of bestatin on the adhesion, motility (migration) and invasion of metastatic tumor cells *in vitro*.

MATERIALS AND METHODS

Cells Highly metastatic B16-BL6 melanoma cells, obtained by an *in vitro* selection procedure for invasion, were kindly provided by Dr. I. J. Fidler, M. D. Anderson Cancer Center, Houston, Texas. B16-BL6 cells and Lewis lung carcinoma (3 LL) cells, derived from C57BL/6 mice, were maintained as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 7.5% fetal bovine serum (FBS), vitamin solution, sodium pyruvate, nonessential amino acid and L-glutamine.

Chemical reagents Ubenimex (bestatin) was prepared by Nippon Kayaku Co. Ltd. Various enzyme inhibitors such as pepstatin, leupeptin, amastatin and arphamenine B were kindly provided by Dr. T. Aoyagi, Institute of Microbial Chemistry, Tokyo. Soybean trypsin inhibitor (SBTI) was obtained from P-L Biochemicals Inc. WI. 1,10-Phenanthroline was purchased from Wako Pure

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Chemical Industries, Ltd., Tokyo. L-Arginine-β-naphthylamide (L-Arg-β-NA) and L-leucine-β-naphthylamide (L-Leu-β-NA) were purchased from Sigma Chemical Co. Enzyme inhibitors were dissolved in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) before use. Purified mouse fibronectin was purchased from Seikagaku Kogyo Co. Ltd., Tokyo. Purified mouse laminin and Basement Membrane Matrigel (containing laminin, collagen type IV, heparan sulfate proteoglycan and entactin) were obtained from Collaborative Research Inc. MA. All the reagents and media in this study were endotoxin-free (approximately <1.0 ng/ml) as determined by a colorimetric assay (Pyrodick, Seikagaku Kogyo Co.).

Haptotactic migration assay Tumor cell migration along a gradient of substratum-bound fibronectin or laminin was assayed in a Transwell cell culture chamber (Costar No. 3422, Cambridge, MA; as shown in Fig. 1) according to the methods reported by McCarthy and Furcht[5] with some modifications. Polyvinylpyrroridone-free polycarbonate filters with 8.0 μm pore size (Nucleopore, Pleasanton, CA) were precoated with 5 μg of fibronectin or laminin in a volume of 50 μl on the lower surface, and dried overnight at room temperature. The coated filters were washed extensively in PBS and then dried immediately before use. Log-phase cell cultures of tumor cells were harvested with 1 m*M* EDTA in PBS, washed three times with serum-free MEM, and resuspended to a final concentration of 10^6/ml in MEM with 0.1% bovine serum albumin (BSA). Cell suspensions (100 μl) with or without agents were added to the upper compartment, and incubated for 4 h at 37°C in a 5% CO_2 atmosphere. The filters were fixed with methanol, and stained with hematoxylin and eosin. The cells on the upper surface of the filters were removed by wiping with a cotton swab. The cells that had migrated to various areas of the lower surface were manually counted under a microscope at a magnification of 400, and each assay was performed in triplicate.

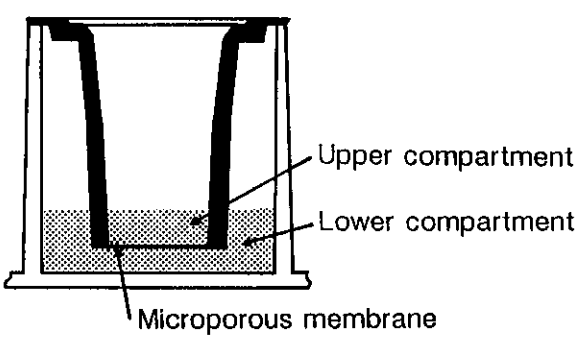

Fig. 1. Diagram of the Transwell cell culture chamber equipped with a microporous membrane (pore size, 8 μm; Costar No. 3422). The volumes of media added to the lower and upper compartments are 600 and 100 μl, respectively.

Invasion assay The invasive activity of tumor cells was assayed according to the method reported by Albini *et al.*[23] with some modifications. Briefly, the lower surface of the filters was precoated with fibronectin or laminin as described above. The Matrigel was diluted to 100 μg/ml with cold PBS, applied to the upper surface of the filters (5 μg/filter), and dried at room temperature under a hood. The filters thus prepared were designated Matrigel/laminin- or Matrigel/fibronectin-coated filters, respectively. The following procedures were the same as those of haptotactic migration assay.

Aminopeptidase (APase) assay APase activities were assayed by measuring β-naphthylamine (β-NA) liberated from amino-acid-β-NA (L-Leu-β-NA or L-Arg-β-NA) after incubation.[9] The mixture containing amino acid-β-NA and tumor cells (2×10^4) in a total volume of 1.0 ml was incubated for 2 h at 37°C. At every 30 min, an aliquot was taken and cooled to 0°C to terminate the reaction. The supernatant of the mixture at each time point was separated by centrifugation, heated for 5 min on a boiling water bath and subjected to fluorometric determination of β-naphthylamine with a Hitachi 650-10S fluorescence spectrophotometer. APase activity was expressed as pmol/min/2×10^4 cells calculated from the amount of β-naphthylamine formed by the added tumor cells.

Statistical analysis The significance of differences between groups was calculated by applying Student's two-tailed *t* test.

RESULTS

Effect of bestatin on tumor cell invasion Invasion is a complex process involving cell adhesion, motility, and the secretion of different classes of enzymes. Since bestatin is a potent competitive inhibitor of aminopeptidase B and leucine aminopeptidase, in addition to being an immunomodulator,[9,10] we first examined the effect of bestatin on the invasion of two different tumor cells, B16-BL6 and 3LL cells, into reconstituted basement membrane components Matrigel (Table I). B16-BL6 or 3LL cells were incubated for 8 h at 37°C with bestatin at concentrations ranging from 0.5 to 100 μg/ml in the upper compartment of the Transwell chamber. The invasion of both tumor cells into the Matrigel/laminin-coated filters was inhibited by bestatin in a concentration-dependent fashion (Expt. 1 of Table I). Similarly bestatin was shown to inhibit the invasion of B16-BL6 cells into Matrigel/fibronectin-coated filters (Expt. 2 of Table I). We also observed that bestatin at concentrations ranging from 0.01 to 500 μg/ml did not block the tumor cell adhesion to Matrigel-coated substrates, which

Table I. Effect of Bestatin on the Tumor Cell Invasion into Matrigel/Laminin- or Matrigel/Fibronectin-coated Filters

Treatment	Concentration (μg/ml)	No. of invaded cells (mean±SD)	
		B16-BL6	3LL
Expt. 1			
None	—	52±5	99±9
Bestatin	0.5	52±7	77±7
	1	38±10	68±12
	5	22±9*	61±14*
	10	15±8*	53±11*
	50	17±4*	46±10*
	100	15±5*	32±5*
Expt. 2			
None	—	60±4	124±16
Bestatin	1	39±6*	90±12
	10	20±4*	85±6*
	100	17±5*	67±6*

B16-BL6 melanoma cells (2×10^5) or 3LL carcinoma cells (2×10^5) in 0.1% BSA-medium were seeded with or without bestatin into the upper compartment of the Transwell chamber. Filters in the chamber were precoated with either 5 μg of laminin (Expt. 1) or 5 μg of fibronectin (Expt. 2) on the lower surface, and with Matrigel (5 μg) on the upper surface. After an 8-h incubation, the invaded cells on the lower surface were counted.
*, $P<0.001$.

Table II. Effect of Bestatin on the Invasion of B16-BL6 Melanoma Cells into Matrigel/Laminin-coated Filter

Addition or treatment		No. of invaded cells (mean±SD)
None	—	39±3
Bestatin	1 μg/ml	17±1*
	10	15±4*
	100	8±2*
Pretreatment of tumor cells with bestatin, 100 μg/ml		27±3
Pretreatment of Matrigel with bestatin, 100 μg/ml		35±3

B16-BL6 melanoma cells (2×10^5) in 0.1% BSA-medium were seeded with or without bestatin into the upper compartment of the Transwell chamber. Filters in the chamber were precoated with laminin (5 μg) on the lower surface and with Matrigel (5 μg) on the upper surface. After an 8-h incubation, the invaded cells on the lower surface were counted.
*, $P<0.001$.

is considered to be an initial step in the invasive process (data not shown).

Influence of treatment of tumor cells or Matrigel with bestatin on tumor cell invasion We next examined whether the pretreatment of tumor cells or Matrigel with bestatin caused the inhibition of tumor cell invasion or not (Table II). Tumor cells or Matrigel/laminin-coated filters were incubated for 1 h at 37°C with 100 μg/ml bestatin, and washed 3 times with PBS. This pretreatment of either tumor cells or Matrigel with bestatin did not affect the invasive activity into the Matrigel/laminin-coated filters. However, tumor cell invasion into Matrigel/laminin was inhibited by presence of bestatin (1 to 100 μg/ml) in the upper compartment of the Transwell chamber as had already been shown above. We also observed that bestatin has no inhibitory effect on the incorporation of ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine into the tumor cells after a 24 h coincubation. These results suggest that bestatin may inhibit the enzymatic destruction of basement membrane barriers by tumor-associated enzymes during tumor invasion.

Effect of bestatin and various enzyme inhibitors on tumor cell invasion We next examined the effects of various kinds of enzyme inhibitors; pepstatin (acid-protease inhibitor), leupeptin (serine- and cysteine-protease inhibitor), SBTI (serine-protease inhibitor) and 1,10-phenanthroline (metallo-protease inhibitor). As shown in Table III, bestatin and 1,10-phenanthroline significantly inhibited the invasion of B16-BL6 and 3LL cells into the Matrigel/laminin-coated filters. The invasion of 3LL cells was also inhibited by SBTI. Other enzyme inhibitors, pepstatin and leupeptin, did not inhibit the invasion of either of the tumor cell lines at the concentrations used. However, the inhibition may depend on the concentration of inhibitors or the kinds of tumor cells. These inhibitor concentrations were chosen because they afforded optimal enzyme inhibition in our hands and were not toxic to the B16-BL6 cells in this short-term assay. However, we observed that 1,10-phenanthroline decreased the cell proliferation *in vitro* after a 24 h incubation (data not shown). On the other hand, bestatin and the other enzyme inhibitors used in this study did not have any effect on the haptotactic migration of tumor cells of either line to filters precoated on the lower surface with 5 μg of laminin. The results suggested that the inhibition of invasion might be associated with the specific enzyme-inhibitory properties of bestatin. Therefore, we selected other aminopeptidase inhibitors as well as bestatin and tested them for activity in the tumor invasion assay. Arphamenine B is an inhibitor of aminopeptidase B, and amastatin A is an inhibitor of aminopeptidase A and leucine aminopeptidase.[10] Table IV shows that these aminopeptidase inhibitors significantly inhibited tumor cell invasion into the Matrigel/laminin-coated filters.

Table III. Effect of Bestatin on the Haptotactic Migration and Invasion of B16-BL6 Melanoma and Lewis Lung Carcinoma (3LL) Cells

Tumor	Treatment	Concentration (μg/ml)	No. of cells (mean±SD)	
			Invasion	Haptotaxis
B16-BL6 melanoma	None	—	57±7	80±8
	Pepstatin	1	44±5	75±4
	Leupeptin	10	59±4	66±6
	SBTI	50	45±6	77±5
	1,10-Phenanthroline	50	20±2*	74±2
	Bestatin	10	33±2*	69±5
Lewis lung carcinoma (3LL)	None	—	56±5	67±6
	Pepstatin	1	45±5	67±8
	Leupeptin	10	60±2	65±6
	SBTI	50	32±4*	66±7
	1,10-Phenanthroline	50	20±3*	68±7
	Bestatin	10	29±5*	68±6

B16-BL6 cells (2×10^5) or 3LL cells (2×10^5) in 0.1% BSA medium were seeded with or without enzyme inhibitor into the upper compartment of the Transwell chamber. Filters in the chamber were pre-coated with laminin (5 μg) on the lower surface for haptotactic migration assay, and in addition, were coated with Matrigel (5 μg) on the upper surface for invasion assay. The migrated or invaded cells on the lower surface were counted after a 4-h incubation for haptotaxis, or an 8-h incubation for invasion. *, $P<0.001$.

Table IV. Effect of Aminopeptidase Inhibitors on the Invasion of B16-BL6 Melanoma into Matrigel/Laminin-coated Filter

Inhibitor		Concentration (μg/ml)	No. of invaded cells (mean±SD)	
			I	II
None			121±7	110±9
Bestatin		100	81±4**	72±14*
Amastatin	A	100	76±10**	72±12*
Arphamenine	B	100	95±10*	86±11*

B16-BL6 melanoma cells (2×10^5) in 0.1% BSA medium were seeded with or without aminopeptidase inhibitor into the upper compartment of the Transwell chamber. Filters were precoated with laminin (5 μg) on the lower surface and with Matrigel (5 μg) on the upper surface. After an 8-h incubation, the invaded cells on the lower surface were counted.
*, $P<0.01$; **, $P<0.001$.

Table V. Inhibition of Aminopeptidase Activities in B16-BL6 Cells by Bestatin and 1,10-Phenanthroline

Inhibitors	Concentration (μg/ml)	Aminopeptidase activity (pmol/min/2×10^4 cells)	
		L-Leu-β-NA	L-Arg-β-NA
None	—	14.7	25.5
Bestatin	0.1	14.8	16.6 (35%)
	1	11.9 (19%)	6.0 (76%)
	10	9.6 (35%)	1.4 (95%)
	100	4.0 (73%)	0.4 (98%)
1,10-Phenanthroline	10	8.7 (46%)	24.7 (3%)
	100	4.7 (68%)	0.3 (99%)

The incubation mixture for assay of the activities contained 2×10^4 B16-BL6 cells and 0.2 m*M* L-Leu-β-NA or L-Arg-β-NA in a total volume of 1.0 ml. The aminopeptidase activity was expressed as pmol/min/2×10^4 cells calculated from the amount of β-NA. Values in parentheses represent percent inhibition compared with the control (in the absence of inhibitor).

Effect of bestatin on aminopeptidase activities in tumor cells Bestatin was found to be a competitive inhibitor not only of the isolated microsomal and cytosolic leucine aminopeptidase but also of the aminopeptidases present in membrane preparations and on the cell surface of L5178Y cells.[10] The results shown in Table IV demonstrated that the three aminopeptidase inhibitors (including bestatin) could similarly inhibit the tumor cell invasion *in vitro*. Therefore, we examined whether or not bestatin could inhibit the aminopeptidase activities in B16-BL6 tumor cells by measuring β-NA liberated from amino acid-β-NA (Table V). The aminopeptidase activities in the absence of bestatin were 14.7 pmol/min/2×10^4 cells (L-Leu-β-NA) and 25.5 pmol/min/2×10^4 cells (L-Arg-β-NA). Bestatin at concentrations ranging from 0.1 to 100 μg/ml inhibited the L-Leu-β-NA- and L-Arg-β-NA-hydrolyzing activities of B16-BL6 cells in a concentration-dependent manner, as did 1,10-phenanthroline. We also found that the amino acid-β-NA-hydrolyzing activities of 3LL cells were inhibited by bestatin *in vitro* (data not shown).

DISCUSSION

The pharmacokinetics and biotransformation of ubenimex (bestatin) have been examined in detail.[24, 25] In particular, the antitumor activity of bestatin has been shown to be mediated by immunomodulatory activities *in vivo*.[14, 18] Bestatin was also found to possess inhibitory activities against aminopeptidases which are associated with outer membranes of most mammalian cells, including spleen cells, macrophages and tumor cells.[11] The aminopeptidase activity was shown to increase in association with the activation and differentiation of macrophages *in vivo*.[26, 27]

Tumor invasion into extracellular matrices and basement membranes is a crucial step in the complex multistage process which leads to the formation of metastasis. It seems likely that the penetration of tumor cells into basement membranes involves distinct events including attachment of tumor cells, secretion by the tumor cells of enzymes that cause the degradation of the adjacent basement membrane, and migration of the cells into the target tissue. Proteolytic enzymes including lysosomal hydrolases,[28–30] collagenase,[31–34] and in some cases plasminogen activator[35] are elevated in invasive cells and in tumors with metastatic potential. The modulation of cell surface enzymatic activities on tumor cells by the inhibitors may affect some steps in the invasive cascade. We therefore investigated the effect of bestatin on the tumor cell invasion into reconstituted basement membrane (Matrigel) *in vitro*. Bestatin inhibited the invasion of B16-BL6 and 3LL cells into Matrigel/laminin- or Matrigel/fibronectin-coated filters in a concentration-dependent manner (Table I). The preincubation of tumor cells or Matrigel substrate with bestatin did not affect the invasive character of the tumor cells (Table II). Bestatin did not have any effect on the growth of tumor cells, attachment of tumor cells to Matrigel substrate (data not shown) or the motility (haptotactic migration) of tumor cells to laminin-coated substrate without Matrigel (Table III). Other enzyme inhibitors, 1,10-phenanthroline, arphamenine B and amastatin A, which are considered to be inhibitors of metallo-peptidases, could inhibit the tumor cell invasion as well as bestatin (Table IV). The inhibitory effect of bestatin on tumor cell invasion seems to be partially attributable to the inhibition of aminopeptidase in tumor cells. The amino acid-β-NA hydrolyzing activities with tumor cells were significantly inhibited by bestatin in a concentration-dependent manner (Table V).

In conclusion, we have demonstrated that bestatin, which has antitumor and immunomodulatory activities, potently inhibits tumor cell invasion, possibly by inhibiting enzyme(s) required for the degradation of extracellular matrices and basement membrane.

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