Inhibition of Tumor Cell Invasion and Matrix Degradation by Aminopeptidase Inhibitors

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We investigated the effects of several types of aminopeptidase inhibitors on tumor cell-associated aminopeptidase activity and invasion. The aminopeptidase expressed by the human metastatic HT1080 fibrosarcoma cells was effectively suppressed by actinonin A, bestatin, leuhistin and matlystatin A, which are capable of inhibiting the purified aminopeptidase N, but not by arphamenine B specific for aminopeptidase B. The aminopeptidase N inhibitors inhibited HT1080 cells from degrading the subendothelial matrix and from invading into Matrigel in parallel with their aminopeptidase inhibitory activities. Matlystatin A, with multiple inhibitory activity against both aminopeptidase N and matrix metalloproteinases (MMP), was the most effective inhibitor of invasion. However, leuhistin and bestatin, without MMP inhibitory activity, also exhibited significant inhibition of invasion. The results suggest that aminopeptidase N plays a crucial role in the degradation and invasion of extracellular matrices by fibrosarcoma cells and that aminopeptidase inhibitors may be useful for preventing the spread of malignant tumors.

Key words fibrosarcoma; invasion; aminopeptidase N; aminopeptidase inhibitor; gelatinase

During the metastatic cascade, a tumor cell penetrates several connective tissue extracellular matrices and basement membranes, which consist of unique macromolecules such as collagens, glycoproteins and proteoglycans.[1-5] Proteolytic enzymes are believed to play an essential role in the metastatic process, particularly in those steps which involve the ability of tumor cells to traverse the basement membrane and collagenous stroma.[6-8] Several classes of enzymes have been implicated, including matrix metalloproteinases (MMPs), serine proteinases, cysteine proteinases and aminopeptidases. These enzymes are secreted not only by a variety of metastatic tumor cells but also by normal cells under certain conditions. Understanding the role of these enzymes may help in the development of antimetastatic therapies.

Extracellular MMPs are Zn^{2+}-dependent proteases that are produced by eukaryotic cells. These proteases can initiate the degradation of extracellular matrix (ECM) macromolecules, such as collagen and proteoglycans.[9,10] It is assumed that malignant cells can exploit these enzymes to promote invasion and metastasis, whereas normal physiological processes such as morphogenesis[11] and tissue repair[12] are dependent on careful spatial regulation of the activity.

Aminopeptidase N is also a Zn^{2+}-dependent exoprotease that binds to membranes through N terminal segments as an ecto enzyme. This enzyme has been postulated to have multiple functions, including hydrolytic inactivation of regulatory peptides, such as enkephalins. The aminopeptidase N is identical to the cell surface antigen CD13, which is expressed not only on myeloid cells but also on many other types of cells.[13] We have reported that anti-aminopeptidase N/CD13 monoclonal antibody, capable of inhibiting aminopeptidase activity, can suppress the degradation and invasion of ECM by tumor cells.[14] These findings have suggested the involvement of aminopeptidase N/CD13 in the molecular mechanisms of tumor cell invasion.

Bestatin, a potent inhibitor of aminopeptidase N, aminopeptidase B and leucine aminopeptidases, has been shown to augment humoral and cellular responses in experimental animals. Tsuruo *et al.*[15] first demonstrated the inhibition by bestatin of P388 leukemia metastasis in mice. Talmage *et al.*[16] reported that the administration of high doses of bestatin resulted in a significant inhibition of experimental and spontaneous metastasis of melanoma in mice. We found that bestatin inhibited the invasion and degradation of type IV collagen by tumor cells *in vitro*.[17,18]

In the present study, several inhibitors with different specificities for aminopeptidases were examined for inhibitory activity against matrix degradation and invasion by fibrosarcoma cells. Our aims were to confirm the critical role of aminopeptidase N in tumor invasion and to seek a possibility of the use of aminopeptidase inhibitors for preventing the spread of metastatic tumors.

MATERIALS AND METHODS

Cells Metastatic human fibrosarcoma HT-1080 cells and human embryonal lung fibroblast WI-38 cells were obtained from American Type Culture Collection (Rockville, MD). THP-1 leukemia cells were provided by Riken Cell Bank (Wako, Japan). Rat lung endothelial cells, clone-4 (RLE-4), were established as described previously.[19] These cells were maintained as a monolayer culture in RPMI-1640 containing 10% fetal bovine serum (FBS).

Chemical Reagents Ubenimex[20] (bestatin) was prepared by Nippon Kayaku Co., Ltd.; arphamenine B,[21] leuhistin,[22] and actinonin[23] were obtained from the Institute of Microbial Chemistry, Tokyo. Matlystatin A[24] was kindly provided by Dr. Tanzawa, Sankyo Co., Ltd., Tokyo, Japan.

Aminopeptidase Assay Aminopeptidase activity was assayed by measuring the 7-amino-4-methylcoumarin (AMC) liberated from L-alanine 4-methyl-coumaryl-7-amide (Ala-MCA, Peptide Institute, Osaka, Japan). A mixture containing 0.1 mM Ala-MCA and aminopeptidase

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N (Boehringer-Mannheim, Germany) or tumor cells (5×10^3) in 200 μl of Hank's buffer solution was placed in each well of a 96-well microplate and incubated at 37 °C. The incubated sample solution was mixed with 50 μl of 0.1 M EDTA to terminate the reaction, and was then subjected to fluorometric determination of AMC (7-amino-4-methylcoumarin) using a Baxter Fluorescence Concentration Analyzer (Baxter, Mandolin, IL) with excitation at 365 nm and emission at 450 nm. The aminopeptidase activity was calculated from the amount of AMC formed during the incubation period.

Gelatinase Assay Gelatinases A and B were purified from the conditioned media of WI-38 cells and TPA-stimulated THP-1 cells, respectively. The supernatants were applied on a column of gelatin-Sepharose (Sigma, St. Louis, MO). The column was washed with 10 mM Tris–HCl, pH 7.5, containing 1 M NaCl. The enzymes were eluted with 10 mM Tris–HCl, pH 7.5, containing 10% DMSO. The gelatinase fractions were dialyzed against 50 mM Tris–HCl, pH 7.5, containing 150 mM NaCl and 1 mM $CaCl_2$, and were then subjected to gel filtration chromatography using a Sephacryl-S 200 column equilibrated with 50 mM Tris–HCl, pH 7.5, containing 150 mM NaCl. Aliquots of 50 μl of the fractions were analyzed by gelatin zymography as previously described.[25] Gelatinolytic activity was also determined by degradation assay using *N*-[^{3}H]acetylated gelatin with a specific radioactivity of 1004000 dpm/mg. *N*-[^{3}H]acetylated gelatin was prepared with [^{3}H]acetic anhydride (New England Nuclear, Boston, MA) according to the methods of Mookhtiar *et al.*[26] The purified gelatinases were activated by incubation with 1 mM APMA at 37 °C for 30 min and then incubated in the presence or absence of aminopeptidase inhibitors for 15 min at 37 °C in 100 μl of 50 mM Tris–HCl, 1 mM $CaCl_2$, 150 mM NaCl, 0.4% Briji 35, pH7.5. A 100 μl aliquot of substrate solution containing 5 μg of [^{3}H]gelatin (5020 dpm) was preincubated at 70 °C for 5 min and then added to the assay mixture containing activated gelatinases. After a 2 h incubation period at 37 °C, the assay solution was mixed with 100 μl of 50% ice-cold trichloroacetic acid (TCA) and incubated at 4 °C for 30 min. TCA-insoluble proteins were precipitated by centrifugation for 10 min at 10000 × ***g***, and the radioactivity in the supernatant containing acid-soluble [^{3}H]gelatin peptides was measured by a liquid scintillation counter.

Invasion Assay The invasion activity of tumor cells was assayed using Transwell cell-culture chambers according to the method previously reported.[17] Briefly, the lower surface of polyvinylpyrrolidone-free polycarbonate filters with an 8.0 μm pore size (Nucleopore, Pleasanton, CA) was pre-coated with 0.1 mg/ml of fibronectin or laminin in phosphate-buffered saline (PBS) (5 μg/filter). Matrigel was diluted to 1 mg/ml with cold PBS, applied to the upper surfaces of the filters (20 μg/filter), and dried at room temperature under a hood. The filters were designated Matrigel/fibronectin- or Matrigel/laminin-coated filters. The coated filters were washed extensively in PBS and then dried immediately before use. Tumor cells were harvested with 1 mM EDTA in PBS, washed three times with serum-free minimum essential medium (EMEM), and resuspended to a final concentration of 10^6 cells/ml in EMEM with 0.1% bovine serum albumin. Cell suspensions (100 μl) with or without aminopeptidase inhibitors were added to the upper compartment, and incubated for a certain period 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 surfaces of the filter were wiped out with cotton swabs. The cells which appeared on the lower surface were manually counted under a microscope at a magnification of 400 ×. Each assay was performed in triplicate.

Subendothelial Matrix Degradation Assay Rat lung endothelial cells (RLE-4) were grown in 24-well tissue culture plates (Costar 3422, Cambridge, MA) at 37°C using RPMI-1640 containing 10% FBS. Confluent cells were incubated for 48 h with 1 μCi/ml of [^{14}C]leucine (specific radioactivity 341 Ci/mmol, New England Nuclear, Boston, MA) in RPMI-1640 containing 5% FBS. The extracellular matrix was isolated according to the method of Kramer *et al.*[27] The isolated matrix was incubated with 1 ml of PBS containing 1% of BSA for 16 h. Tumor cells were suspended in 1 ml of a mixture of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient medium (DMEM/F12) plus 0.1% BSA with or without aminopeptidase inhibitors, and were then placed onto the isolated RLE matrix and incubated for 48 h at 37 °C in a CO_2 incubator. The culture supernatants were withdrawn and centrifuged at 18000 × ***g*** for 15 min. The radioactivity of digested materials in 500 μl of the supernatant was measured by liquid scintillation counting.

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

RESULTS

Effects of Aminopeptidase Inhibitors on Purified Aminopeptidase N and Tumor Cell-Associated Aminopeptidase Activity We have previously reported that bestatin inhibited aminopeptidase activity in murine and human metastatic tumor cells. Here, we examined the effects of several types of aminopeptidase inhibitors on HT1080 fibrosarcoma aminopeptidase and on the purified porcine intestinal aminopeptidase N. As shown in Table 1,

Table 1. Inhibition of Alanine Aminopeptidase Activity in Tumor Cells by Aminopeptidase Inhibitors

Inhibitors	IC_{50} (μg/ml)	
	Aminopeptidase N	HT-1080
Actinonin	0.1	0.25
Arphamenin B	40	>100
Bestatin	2.0	2.4
Leuhistin	0.25	0.1
Matlystatin A	0.08	0.8

Alanine aminopeptidase activities in human HT-1080 fibrosarcoma cells (5×10^3) were measured by incubation with 0.2 mM Ala-MCA in a total volume of 0.2 ml in the presence or absence of aminopeptidase inhibitors. Porcine intestinal aminopeptidase N (1 μU) was incubated with 0.2 mM of Ala-MCA in Hank's buffer, pH 7.5, in the presence or absence of aminopeptidase inhibitors.

leuhistin, actinonin, and matlystatin A demonstrated similar inhibitory activities and were more effective than bestatin. In contrast, arphamenine B, specific for aminopeptidase B, had little effect on those aminopeptidase activities.

Effect of Aminopeptidase Inhibitors on Gelatinases Extracellular matrix metalloproteinases are Zn^{2+}-dependent proteinases. These proteases can degrade ECM macromolecules, such as collagens and proteoglycans. Aminopeptidase N is also a Zn^{2+}-dependent exoprotease and shares homology with MMPs within the zinc binding motif. We examined the effects of aminopeptidase inhibitors on purified MMPs using [^{3}H]-labeled gelatin. Table 2 shows the IC_{50} values of aminopeptidase inhibitors against gelatinases A (MMP-2) and B (MMP-9). Matlystatin A and actinonin suppressed these MMP activities, whereas neither arphamenine B, bestatin, or leuhistin showed any inhibitory activity on these MMPs.

Effect of Aminopeptidase Inhibitors on Enzymatic Degradation of Subendothelial ECM by Tumor Cells Degradation of ECM during tumor invasion involves a number of enzymes, including collagenase and aminopeptidases. We examined the inhibitory effects of these aminopeptidase inhibitors on the degradation of extracellular matrix by HT-1080 fibrosarcoma cells. [^{14}C]-Leucine-labeled subendothelial matrices were isolated from RLE cultures in 1.5 cm wells of multiple tissue culture plates and seeded with HT-1080 cells. The amount of ^{14}C-active materials released into the media during a 24 h period incubation was measured. Leuhistin, bestatin, and matlystatin inhibited 50—72% of the degradation of ECM by HT1080 cells. Matlystatin inhibited the matrix degradation more effectively than leuhistin and bestatin. However, arphamenine B did not show inhibitory effect on the matrix degradation (Fig. 1). Under the conditions used, the aminopeptidase inhibitors, except actinonin, had no significant effect on tumor cell growth when the

Table 2. IC_{50} Values of Aminopeptidase Inhibitors for MMPs

	IC_{50} (μg/ml)	
	MMP 2	MMP 9
Actinonin	1.6	0.15
Arphamenine B	>100	>100
Bestatin	>100	>100
Leuhistin	>100	>100
Matlystatin A	0.9	0.16

MMP-2 (19.1 ng) or MMP-9 (240 ng) was activated with 1 mM APMA. [^{3}H]-Gelatin (5 μg) was mixed with the activated MMP solutions and incubated in 50 mM Tris–HCl, 10 mM $CaCl_2$, 0.1% Briji 35, and 0.4% Triton X-100, pH 7.5 at 37 °C for 3 h.

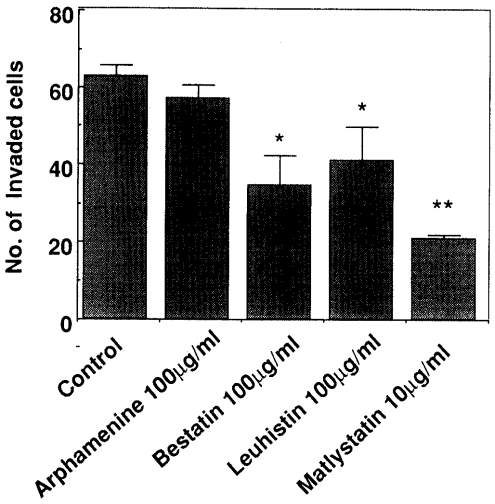

Fig. 1. Effects of Aminopeptidase Inhibitors on Matrix Degradation by HT-1080 Cells

HT-1080 cells (1×10^5) suspended in DMEM/F12 containing 0.1% BSA were seeded on the subendothelial matrix produced by RLE cells, metabolically labeled with [^{14}C] leucine, and incubated at 37 °C for 24 h. The radioactivity in the supernatant was measured by a liquid scintillation counter. $*p>0.05$, $**p>0.01$.

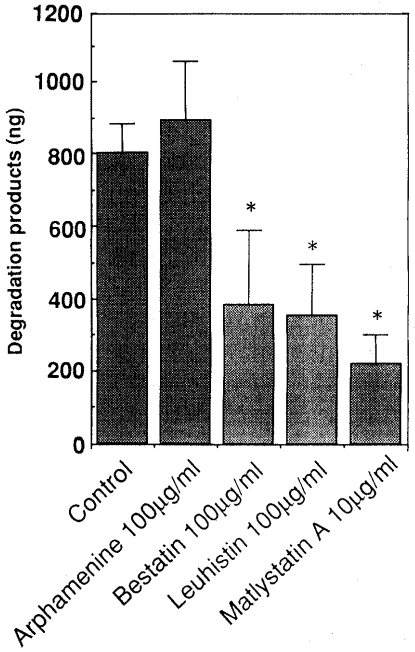

Fig. 2. Invasion Inhibition by Aminopeptidase Inhibitors

HT-1080 cells (1×10^5/100 μl) suspended in DMEM/F12 containing 0.1% BSA were seeded with or without aminopeptidase inhibitors into the upper compartment of a Transwell chamber. Filters in the chamber were precoated with laminin (5 μg) on the lower surface and with Matrigel (20 μg) on the upper surface. The cells that invaded Matrigel and appeared on the lower filter surface were counted after a 6 h incubation period. $*p<0.05$.

Table 3. Effects of Aminopeptidase Inhibitors on Tumor Cell Invasion through Reconstituted Basement Membrane

Inhibitors	μg/ml	Number of tumor cells which invaded Matrigel	% of control
Bestatin	0	34.6± 2.6	100
	1	37.3± 2.5	110
	10	36.7± 5.1	108
	100	16.7± 2.5*	49.1
Leuhistin	0	43.0± 2.6	100
	1	44.6± 7.2	104
	10	21.6± 6.7*	50.1
	100	23.3± 6.7*	54.2
Matlystatin A	0	52.3±11.8	100
	0.1	47.3± 4.0	90.4
	1	25.6± 3.2*	49.1
	10	19.0± 7.2*	36.3

HT-1080 cells (5×10^4/100 μl) in DMEM/F12 containing 0.1% BSA were seeded with or without aminopeptidase inhibitors into the upper compartment of a Transwell chamber. Filters in the chamber were pre-coated with laminin (5 μg) on the lower surface and with Matrigel (20 μg protein) on the upper surface. The invaded cells on the lower surface were counted after incubation for 6 h. ($*p<0.05$)

cell growth was analyzed by MTT assays after a 72 h period incubation (data not shown). As actinonin was found to be highly cytotoxic to HT-1080 cells, it was not tested in further experiments with live cells.

Effect of Various Aminopeptidase Inhibitors on Tumor Cell Invasion We examined the effects of five aminopeptidase inhibitors on HT1080 cell invasion. As shown in Fig. 2, bestatin, leuhistin and matlystatin A effectively inhibited the invasion of HT-1080 cells into Matrigel/laminin-coated filters. Arphamenine B did not exhibit a significant effect on the invasion at the concentration used. These results suggest that the invasion inhibition by these inhibitors is most likely associated with the inhibition of aminopeptidase N. Invasion inhibition by bestatin was observed at concentrations higher than 100 μg/ml, and leuhistin was effective at 10 μg/ml (Table 3). Matlystatin A demonstrated a significant inhibition of invasion at a concentration as low as 1 μg/ml.

DISCUSSION

We have previously reported that bestatin and the anti-CD13 antibody markedly inhibited tumor cells from invading Matrigel and degrading type IV collagen.[14] We further tested various aminopeptidase inhibitors for invasion inhibition to confirm the significant role of aminopeptidase N in metastatic invasion and to search for inhibitors with a good potential for therapeutic applications. In the present study we examined five aminopeptidase inhibitors: actinonin, arphamenine B, bestatin, leuhistin and matlystatin A.

In our previous study, the high expression of aminopeptidase N/CD13 was observed on the cell surface of HT1080 fibrosarcoma by flow cytometry with FITC-labeled anti-CD13 antibodies.[14] Therefore, we first examined the effects of these aminopeptidase inhibitors on the aminopeptidase activity of HT1080 cells using Ala-MCA, which is a preferable substrate to aminopeptidase N. The results were compared with those on purified porcine intestinal aminopeptidase N. Bestatin, leuhistin, actinonin, and matlystatin A showed a similar degree of potent inhibitory activity on both the purified porcine aminopeptidase N and HT1080 cell-associated aminopeptidase. However, arphamenine B, which is known to be an aminopeptidase B specific inhibitor, showed little effect on those aminopeptidases. Thus, the major aminopeptidase expressed by HT1080 cells is likely to be aminopeptidase N.

Bestatin, leuhistin, and matlystatin A exhibited inhibitory activities on the basement membrane invasion by HT1080 fibrosarcoma cells. In contrast, arphamenine B did not decrease the number of cells penetrating through the reconstituted basement membrane. Therefore, the inhibitory effect of the aminopeptidase inhibitors on HT1080 cell invasion is likely to be correlated with the inhibition of aminopeptidase N activity.

A substantial correlation between MMP activity and the metastatic potential of a variety of human and animal tumor cell lines has been reported.[10] Aminopeptidase N is a Zn^{2+}-dependent protease and shares homology with MMPs within the Zn^{2+} binding motif. Thus, we also examined the effects of the aminopeptidase inhibitors on purified MMP-2 and MMP-9 (gelatinases A and B). Matlystatin A and actinonin strongly inhibited both MMPs, whereas the other inhibitors did not show any effect on either MMP-2 or MMP-9 at the concentrations used. Basement membrane invasion and subendothelial matrix degradation by HT1080 cells were inhibited by aminopeptidase inhibitors without showing any MMP inhibitory activity. Therefore, aminopeptidase inhibition itself appeared to be an important event in the inhibition mechanisms of invasive degradation. However, both basement membrane invasion and subendothelial matrix degradation were most effectively suppressed by matlystatin A, suggesting a significant involvement of MMPs in these phenomena. Incidentally, actinonin, a potent inhibitor of both aminopeptidase N and MMPs, was highly toxic to HT1080 cells and could not be applied to further *in vitro* assays with live cells.

ECM metalloproteinases, as well as aminopeptidase N, are Zn^{2+}-dependent proteinases and share homology in the catalytic domains containing a Zn^{2+} binding site. Matlystatin A and actinonin demonstrated potent inhibition against not only MMPs but also aminopeptidase N. As matlystatin A was the most effective invasion inhibitor among those tested, a dual enzyme inhibitor may be a better candidate for therapeutic applications.

In conclusion, we demonstrated that aminopeptidase N inhibitors inhibited the invasion and degradation of ECMs by metastatic fibrosarcoma cells through a mechanism based on the inhibition of tumor cell-associated aminopeptidase N activity. Potent inhibitors of aminopeptidase N, such as leuhistin, as well as multiple inhibitors against both aminopeptidase N and MMPs, such as matlystatin A, may be possible candidates for invasion prevention therapy.

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