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Expression of Aminopeptidase N on Human Choriocarcinoma Cells and Cell Growth Suppression by the Inhibition of Aminopeptidase N Activity

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We previously found that an aminopeptidase inhibitor, ubenimex (bestatin), had a growth-suppressive effect on choriocarcinoma cell lines *in vitro*. To clarify the mechanism of this action, we investigated the expression of aminopeptidase N (AP-N/CD13) on choriocarcinoma cells and other human tumor cells. Two choriocarcinoma cell lines, NaUCC-4 and BeWo, had higher AP-N activity than other cell lines (358.8 and 340.2 nmol/h/10^6 cells, respectively), as did the human myeloid leukemia cell line, HL-60 (373.8 nmol/h/10^6 cells). These choriocarcinoma and leukemia cell lines with abundunt AP-N activity showed much higher sensitivity to bestatin (IC50 = 0.5, 2.1 and 1.0 μg/ml, respectively) than the other cell lines. By immunoblotting and immunocytochemical staining, AP-N was detected as an approximately 165-kDa protein and localized on the cell membrane in choriocarcinoma cells. We also examined the effects of two other aminopeptidase inhibitors and three anti-CD13 monoclonal antibodies (MAbs) (WM15, MCS2 and MY7) on the growth of NaUCC-4 cells. Cell growth was markedly suppressed by the AP-N inhibitor actinonin as well as bestatin, but not by the AP-B inhibitor arphamenine. Of the three MAbs, only WM15, which is able to inhibit AP-N activity, suppressed cell growth in a dose-dependent manner. These results indicate that AP-N inhibitors show a growth-suppressive effect, presumably through inhibition of the enzymatic activity of AP-N on tumor cells, and suggest that AP-N may play important roles in the growth of certain tumors, such as choriocarcinoma and leukemia.

Key words: Choriocarcinoma — Aminopeptidase N — Ubenimex (Bestatin) — Tumor growth

Aminopeptidase N (AP-N, EC 3.4.11.2) is a glycoprotein localized in brush border membranes of the small intestine, renal proximal tubules, synaptic membranes of the central nervous system, and on the surface of monocytes and granulocytes. Recently the human myeloid plasma membrane glycoprotein CD13 has been proved to be identical to aminopeptidase N,[1)] and it is expressed in human myeloid leukemic cell lines[2)] and malignant mesenchymal tumors.[3)] However, the physiological roles of this enzyme in normal and malignant cells are poorly understood.[4)]

AP-N plays functional roles in human ovarian folliculogenesis or successful implantation.[5,6)] In malignancy, Saiki *et al.*[7)] and Menrad *et al.*[8)] have found AP-N to play important roles in the invasion of metastatic tumors *in vitro*. Thus, the inhibition of enzymes such as AP-N could be a new approach to suppress the growth or metastasis of cancer.

In our laboratory, it was previously found that AP-N was present abundantly on human normal trophoblasts in term placenta and may be involved in the physiology of pregnancy by metabolizing and degrading bioactive peptide hormones.[9–11)] We also reported that ubenimex (hereafter referred to as bestatin), an inhibitor of AP-N, showed a direct growth-suppressive effect on choriocarcinoma cells *in vitro*, suggesting the potential clinical usefulness of aminopeptidase inhibitors against certain cancer cells.[12)] The above two findings prompted us to investigate the expression of AP-N on choriocarcinoma cells, which are neoplastic transformants from normal placental trophoblasts, and to examine whether the inhibition of AP-N activity by enzyme inhibitors or antibodies against AP-N is correlated with growth suppression of choriocarcinoma cells.

MATERIALS AND METHODS

Cells and cell culture Two human choriocarcinoma cell lines were used. NaUCC-4, derived from pulmonary metastasis of choriocarcinoma, was established at our laboratory and BeWo established by Pattillo and Gey.[13)] HL-60, a human promyelocytic leukemia cell line[14)] was also used. All lines were maintained in RPMI 1640 medium (Nissui Pharmaceutical Co. Ltd., Tokyo) supplemented with 10% fetal calf serum (FCS, Cell Culture Laboratories, Cleveland, OH) at 37°C in a humidified 5% CO_2 atmosphere. Eight other human tumor cell lines differing in histological origin (listed in Table I) were

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cultured in RPMI 1640 or Eagle's minimal essential medium (Nissui) with 10% FCS.

Aminopeptidase inhibitors Bestatin, an inhibitor of AP-N, leucine aminopeptidase (LAP, EC 3.4.11.1) and aminopeptidase B (AP-B, EC 3.4.11.6), was kindly provided by Nippon Kayaku Co. Ltd., Tokyo. Actinonin, an inhibitor of AP-N and LAP, was obtained from Peptide Institute Inc., Osaka. Arphamenine, an inhibitor of AP-B, was from Peptide Institute Inc. All inhibitors were dissolved in distilled water and filtered through a 0.22 μm millipore filter, then diluted to an appropriate concentration with culture medium before use.

Antibodies Three mouse monoclonal antibodies (MAbs) specific to CD13/AP-N were used: WM15 purchased from Silenus Laboratories (Hawthorn, Australia), MY7 from Coulter Immunology (Hialeah, FL) and MCS2 from Nichirei Co. (Tokyo). Normal mouse IgG1 (Zymed Laboratories Inc., Los Angeles, CA) served as the negative control. Biotinylated goat anti-mouse immunoglobulin (Dako A/S, Glostrup, Denmark) was used as the second antibody for immunoblotting or immunocytochemistry.

Assay for enzymatic activity of AP-N Cell-surface AP-N activity in each cell line was detected spectrophotometrically as reported by Amoscato *et al.*[15] In brief, after incubating 5×10^5 cells in the culture medium in a 60×15 mm culture dish (Falcon 3002, Becton Dickinson Labware, Lincoln Park, NJ) for 48 h at 37°C, intact cells were used. After aspirating off the medium and washing with phosphate-buffered saline (PBS), prewarmed 1 m*M* alanine-*p*-nitroanilide (Peptide Institute, Inc.) was added to each dish as a substrate. Each dish was incubated at 37°C for 20 min, then the reaction was terminated by the addition of three volumes of ice-cold PBS. The solution was collected and after centrifugation for 5 min at 4°C, the optical density of the supernatant at 405 nm was measured with a spectrophotometer (Shimadzu Co., Kyoto). Enzymatic activity was determined from a standard curve and expressed as nmol/h/10^6 cells. All assays were conducted in triplicate and repeated three times.

Immunocytochemical staining The avidin-biotin immunoperoxidase technique was used for immunostaining. The cells were cultured on a 16-well chamber slide (Lab-Tek, Nunc Inc., Naperville, IL) for 48 h at 37°C in the culture medium. After washing with PBS, the cells were fixed with ice-cold 95% ethanol at 4°C for 5 min. Endogenous peroxidase activity was blocked by incubation for 10 min in 3% H_2O_2 and nonspecific immunoglobulin binding was blocked by incubation for 10 min with 5% normal goat serum. The primary antibody was added at a dilution of 1:40, followed by incubation for 1 h in a moist chamber at room temperature. The cells were rinsed five times with PBS and incubated for 30 min with biotinylated goat anti-mouse immunoglobulin (1:300 dilution). After five washes with PBS, the cells were incubated for 30 min with avidin-biotin-peroxidase complex (Dako A/S) and after five more washes, they were treated with 0.02% 3,3′-diaminobenzidine tetrahydrochloride as a chromogen (Katayama Chemical, Osaka) in PBS containing 0.01% H_2O_2 and 10 m*M* sodium azide for 1 to 2 min. After being rinsed with PBS, the cells were mounted with Canada balsam (Katayama Chemical). For negative controls, the primary antibody was replaced with normal mouse IgG1 at an appropriate dilution. Staining intensity was estimated by the use of an Olympus light microscope (Olympus Optical Co. Ltd., Tokyo).

Immunoblot analysis of AP-N For immunoblotting, cellular protein lysates were prepared from subconfluent cells in lysis buffer composed of 150 m*M* NaCl, 20 m*M* Tris (pH 7.5), 5 m*M* EDTA, 1% Nonidet P-40 containing 1 m*M* phenylmethylsulfonyl fluoride and 2 μg/ml aprotinin at 4°C for 30 min. Lysates were cleared by centrifugation at 15,000*g* for 30 min and stored at −80°C. Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Western blotting was carried out according to Towbin *et al.*[16] with minor modifications. Briefly, 30 μg of protein extract was separated by sodium dodecyl sulfate (SDS)/7.5% polyacrylamide gel electrophoresis (PAGE), transferred to mitrocellulose membrane and immunoblotted with the mouse MAb, WM15 (1:40 dilution). The biotinylated secondary antibody was used at 1:500 dilution. Immunoreactive proteins were stained with avidin-biotin-peroxidase complex (Dako). SDS-PAGE molecular weight standards (Bio-Rad) served as molecular mass markers. In negative control experiments, the primary antibody was replaced with normal mouse IgG1.

Assay for cell growth suppression The succinate dehydrogenase inhibition test (SDI test) using MTT was conducted with some modifications as previously described.[12] In brief, a single cell suspension obtained following treatment with 0.25% trypsin-EDTA was incubated at 37°C for 96 h or 144 h in a 24-well culture plate (Corning Glass Works, Corning, NY) in the presence or absence of reagents. Then 0.1 *M* sodium succinate and 0.4% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO) were added to each well. After further incubation at 37°C for 3 h, formazan formed from MTT was extracted by adding dimethylsulfoxide and mixing for 10 min. Immediately, the absorbance was measured at 550 nm using a spectrophotometer (Easy Reader, SLT-Labinstruments, Austria). All experiments were performed in quadruplicate and repeated three times. The reagents used were bestatin, actinonin, arphamenine, normal mouse IgG1 and three kinds of anti-CD13 MAbs. The percent survival relative to the control specimen (without enzyme-

inhibitor or antibody) was calculated as follows; [(absorbance in test well − absorbance in background well)/(absorbance in control well − absorbance in background well)] × 100(%). All data were expressed as means ± SD. Significance of differences was determined by using Student's *t* test.

RESULTS

AP-N activity on choriocarcinoma cells Two human choriocarcinoma cell lines, NaUCC-4 and BeWo, were examined for enzymatic activity of AP-N on the cell membrane. Both cell lines showed higher AP-N activity than other solid tumor cell lines. The degree of AP-N activity on choriocarcinoma cell lines was essentially the same as that of HL-60, a promyelocytic leukemia cell line known to be CD13-positive, and approximately 2 to 3 times higher than those of three ovarian cancer cell lines and 5 times those of three gastro-intestinal cancer cell lines (Table I). AP-N activity on NaUCC-4 cells was reduced to less than 50% by preincubation for 2 h with 10 μg/ml of bestatin, an inhibitor of AP-N (data not shown).

Expression of AP-N on choriocarcinoma cells by immunoblotting and immunocytochemistry To confirm the expression of AP-N on choriocarcinoma cells, immunoblotting was conducted using CD13-specific MAb WM15. In NaUCC-4 and BeWo, AP-N was detected as an approximately 165-kDa protein, as it also was in the leukemic cell line, HL-60 (Fig. 1). In the uterine cervical cancer cell line SiHa, a weaker band of AP-N was found, possibly due to difference in the degree of AP-N activity, as shown in Table I.

Immunocytochemical staining with the same MAb WM15 was conducted by the avidin-biotin-peroxidase complex method to determine the localization of AP-N on choriocarcinoma cells. AP-N was abundantly detected on the membrane of NaUCC-4 cells (Fig. 2). HL-60, a positive control for immunostaining, showed intense membrane staining by WM15 (data not shown).

Effects of aminopeptidase inhibitors on choriocarcinoma cell growth The effects of three aminopeptidase inhibitors on the growth of choriocarcinoma cells *in vitro* were studied. Dose-response curves for bestatin, arphamenine

Table I. Aminopeptidase N Activity of Human Tumor Cell Lines

Cell lines	Origin	AP-N activity (nmol/h/10^6 cells)
NaUCC-4	choriocarcinoma	358.8
BeWo	choriocarcinoma	340.2
NOS3	ovarian cancer	189.6
NOS4	ovarian cancer	107.4
SKOV	ovarian cancer	142.8
SiHa	cervical cancer	122.4
NUGC-3	gastric cancer	79.2
NUGC-4	gastric cancer	76.8
SW1083	colorectal cancer	66.6
A-431	skin cancer	62.4
HL-60	leukemia	373.8

Cells were cultured for 48 h, and 1 m*M* alanine-*p*-nitroanilide was added, followed by incubation at 37°C for 20 min. AP-N activity was detected spectrophotometrically by monitoring the increase in optical density at 405 nm. Assay was repeated three times. The results of a representative experiment are shown.

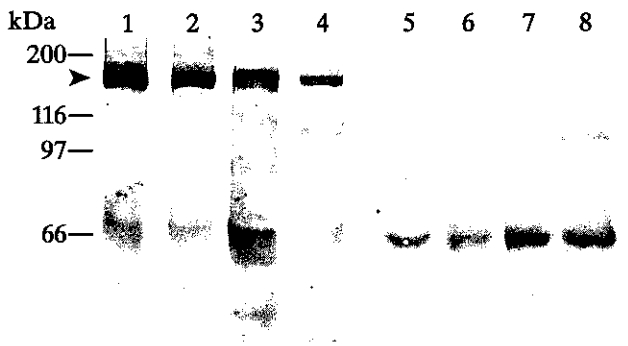

Fig. 1. Immunoblot analysis of AP-N using CD13-specific MAb WM15. Proteins (30 μg) extracted from NaUCC-4 (lanes 1, 5), BeWo (lanes 2, 6), HL-60 (lanes 3, 7) and SiHa (lanes 4, 8) cells were separated on SDS/7.5% polyacrylamide gel and electroblotted onto a nitrocellulose membrane. AP-N was detected by staining with WM15 (lanes 1, 2, 3, 4), but not with normal mouse IgG1 (lanes 5, 6, 7, 8). Arrowhead, AP-N band of around 165 kDa.

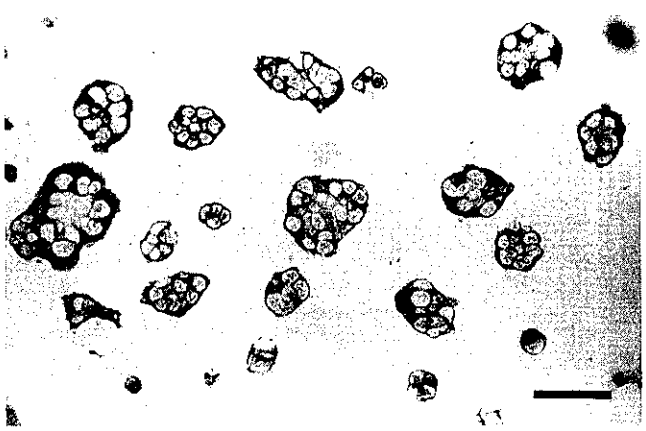

Fig. 2. Immunocytochemical localization of AP-N in choriocarcinoma NaUCC-4 cells with CD13-specific MAb WM15. Intense membrane staining was observed. The scale bar represents 120 μm.

and actinonin on NaUCC-4 cells assayed by SDI test using MTT are shown in Fig. 3. Bestatin and actinonin, which inhibit AP-N, exerted a dose-dependent suppressive effect on the cell growth of NaUCC-4 at concentrations from 0.1 to 30 μg/ml. Arphamenine, a strong inhibitor of AP-B alone, had no such effect.

Growth-suppressive effects of bestatin on various human tumor cell lines were also investigated by SDI test using MTT. Dose-response curves were obtained for eleven human tumor cell lines used in the enzymatic assay. The sensitivity of each cell line to bestatin was evaluated in terms of the 50%-inhibitory concentration (IC50) calculated from the dose-response curve (Fig. 4). NaUCC-4 and BeWo, with higher AP-N enzymatic activity, showed much lower IC50, as did HL-60, indicating higher sensitivity to bestatin. In contrast, cell lines with relatively low AP-N activity, such as NOS4, NUGC-3, NUGC-4, SW1083 and A-431, showed less sensitivity to bestatin.

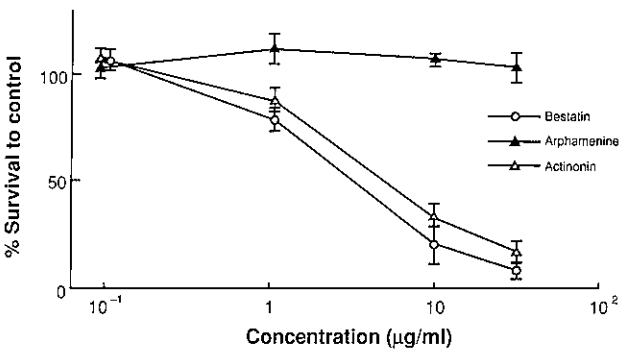

Fig. 3. Dose-response curves for three aminopeptidase inhibitors on NaUCC-4 cells. Cells were treated with each inhibitor at various concentrations as indicated for 96 h. Survival of the control, taken as 100%, was determined by SDI test. Each point and bar represent the mean and SD of three experiments.

Effects of CD13-specific MAb on choriocarcinoma cell growth The effects of three CD13-specific MAbs, WM15, MY7 and MCS2, on the growth of NaUCC-4 were studied. WM15 strongly inhibits AP-N activity, while the two other MAbs either lack or have minimal inhibitory activity against AP-N (they recognize different epitopes).[2, 17, 18] As shown in Fig. 5 mouse IgG1, MY7 and MCS2 at the concentration of 2.5 μg/ml had no effect on the growth of NaUCC-4 cells, whereas cell growth was significantly suppressed by WM15 at the same concentration ($P<0.05$). WM15 at 0.01 to 10 μg/ml showed a dose-dependent growth-suppressive effect on NaUCC-4 and HL-60 (Fig. 6).

DISCUSSION

Bestatin, an inhibitor of AP-N, or AP-N/CD13-specific MAb potently suppresses the invasion of cancer

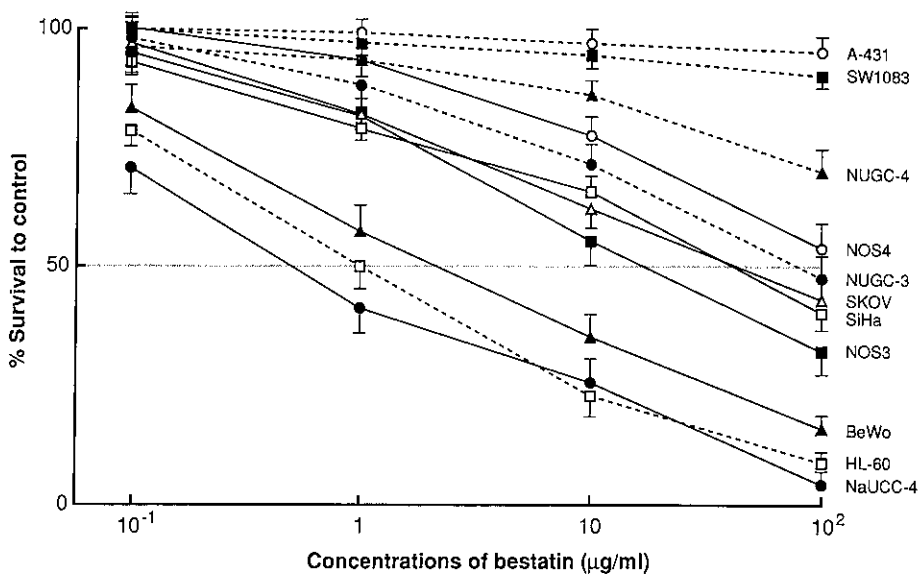

Fig. 4. Sensitivity of various human tumor cell lines to the AP-N inhibitor bestatin. Cells were treated with bestatin at various concentrations as indicated for 144 h. Survival of the control, taken as 100%, was determined by SDI test as in Fig. 3. Each point and bar represent the mean and SD of three experiments. IC50 values (μg/ml) for bestatin were as follows: NaUCC-4, 0.5; BeWo, 2.1; NOS3, 18.5; NOS4, 100<; SKOV, 45; SiHa, 41.3; NUGC-3, 71.7; NUGC-4, 100<; SW1083, 100<; A-431, 100<; HL-60, 1.0.

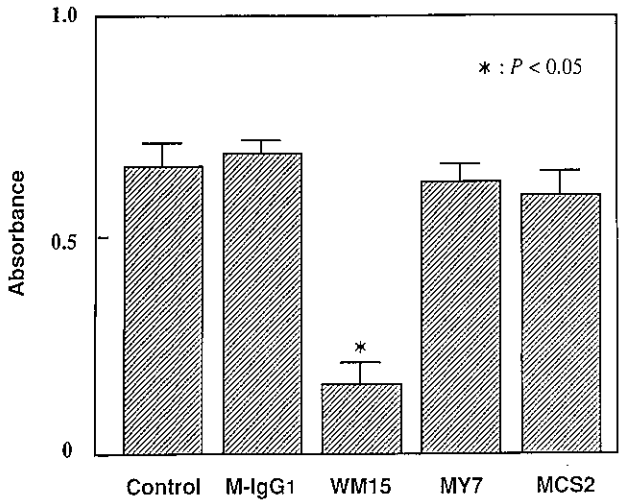

Fig. 5. Effects of CD13-specific MAbs on the proliferation of NaUCC-4 cells. Cells were treated with each antibody at 2.5 μg/ml for 96 h. Growth suppression was assayed by SDI test. Each column and bar represent the mean and SD of three experiments. * Significant difference compared to others by the Student's t test ($P<0.05$).

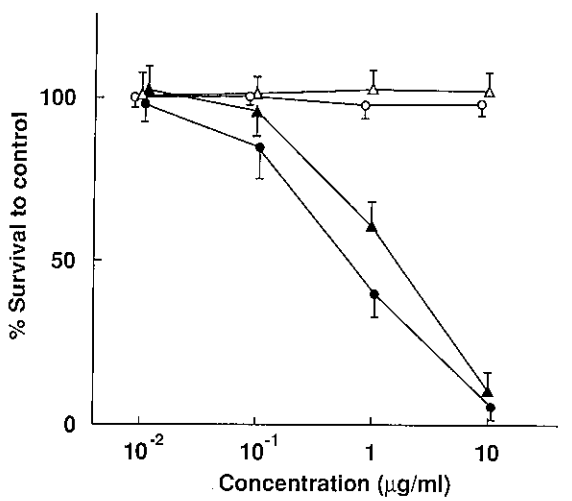

Fig. 6. Dose-response curves for CD13-specific MAb WM15. NaUCC-4 cells (△,▲) and HL-60 cells (○,●) were treated with WM15 (▲,●) or normal mouse IgG1 (△,○) at various concentrations as indicated for 96 h. Survival of the control, taken as 100%, was determined by SDI test as in Fig. 3. Each point and bar represent the mean and SD of three experiments.

cells *in vitro*.[7, 19, 20] We previously reported that bestatin markedly suppressed the growth of four choriocarcinoma cell lines *in vitro*.[12] We and Shibuya *et al.* also reported the suppressive effect of bestatin on proliferation of human leukemic cell lines.[21, 22] From these findings, the AP-N inhibitor would appear to have a suppressive effect not only on tumor-cell invasion but also on tumor-cell proliferation. Thus, in this study, we attempted to determine the relation between AP-N and tumor cell growth.

What cancers express AP-N is not yet apparent though some leukemia cell lines express it abundantly.[2, 23] In the present study, AP-N activity in various human solid tumor cell lines was found to vary (Table I), and to our knowledge, this is the first report to demonstrate clearly, both enzymologically and immunologically, that choriocarcinoma cells express AP-N. By immunoblot analysis with anti-CD13 MAb WM15, AP-N was detected as an approximately 165-kDa protein in two choriocarcinoma cell lines (Fig. 1). This is consistent with the result of Bradstock *et al.*,[24] but there are several reports indicating different molecular weights of AP-N. Look *et al.* found AP-N (CD13) expressed on the human myeloid plasma membrane to be a 150-kDa glycoprotein,[1] whereas Menrad *et al.* have reported that human melanoma cells express AP-N as a 143-kDa protein.[8] O'Connell *et al.* have noted variable glycosylation of CD13/AP-N.[25] The differences in molecular weight found by immunoblotting may possibly be due to variation in glycosylation or oligosaccharide composition.

This study shows that choriocarcinoma cell growth is markedly suppressed by the AP-N inhibitor actinonin, as well as bestatin (an inhibitor of both AP-N and AP-B), but not by the AP-B inhibitor arphamenine (Fig. 3). Growth is also significantly suppressed by CD13-specific MAb WM15, but not by MY7 or MCS2 (Fig. 5) Of these three anti-CD13 MAbs, only WM15 inhibits the enzyme activity of AP-N, presumably because it recognizes the functionally active site of this enzyme. Thus growth-suppressive effects on choriocarcinoma cells may be correlated with the inhibition of AP-N activity.

It is of interest that choriocarcinoma and leukemia cell lines with higher AP-N activities showed higher sensitivity to bestatin (Table I and Fig. 4). Namely, the growth-suppressive effect was strong on cell lines with abundant AP-N activity, but weak on those with little activity, at least among the cell lines tested. These results suggest that AP-N may play specific roles in the growth of certain tumor cells, such as choriocarcinoma or leukemia.

How does AP-N physiologically function on the choriocarcinoma cell surface? AP-N is a zinc-metalloproteinase anchored in the plasma membrane and catalyzes the removal of N-terminal amino acids from peptides. Enzymological study indicates that it hydrolyzes various biologically active peptides.[11, 26, 27] Recently Ansorge *et al.* have suggested AP-N to be essential to the regulation of T cell growth and possibly involved in cytokine/

lymphokine mediated signaling between immune cells.[28] It is well known that various growth factors, cytokines and biologically active peptides operating under autocrine and/or paracrine mechanisms control the growth or differentiation of trophoblasts.[29-32] Similar mechanisms may be operative in the growth of choriocarcinoma cells which are generated by neoplastic transformation from normal trophoblasts.[33] We have noted the expression of epidermal growth factor (EGF) receptor on a choriocarcinoma cell line NaUCC-4 and an autocrine function for EGF.[34] Takahashi *et al.* reported that the AP-N inhibitor bestatin suppressed EGF-induced proliferation in rat hepatocytes.[35] These results indicate that one possible function of AP-N is to degrade and convert some peptides to the active form during the activation of certain growth factors or cytokines, or their receptors. Thus, AP-N may possibly contribute to the growth of choriocarcinoma cells. Further study is required to determine the functional roles of AP-N in choriocarcinoma cell growth.

The clinical potential of AP-N inhibitors seems interesting. Bestatin has already been used in the treatment of cancer, especially acute myeloid leukemia,[36] though formerly only as an immuno-enhancer or biological response modifier (BRM). We have shown that the suppressive effects of bestatin on the growth of hydatidiform molar trophoblastic cells, choriocarcinoma cells and leukemic cells *in vitro* are not exerted through immunomodulatory action.[12, 21, 37] We have also reported the efficacy of the combined use of bestatin with chemotherapeutic agents against trophoblastic tumors and ovarian cancers *in vitro* and *in vivo*.[38, 39] Thus, aminopeptidase inhibitors could become new drugs for cancer treatment, not as BRM, but as anti-cancer chemicals exerting direct suppressive action on the growth or metastasis of certain cancers.

In conclusion, choriocarcinoma cells express AP-N/CD13 on the cell surface membrane abundantly, and AP-N inhibitor and anti-CD13 MAb suppress the cell growth possibly through inhibitory action against AP-N activity. Further investigation of the functional role of AP-N in tumor growth is needed.

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