Leukemic Cell-Surface CD13/Aminopeptidase N and Resistance to Apoptosis Mediated by Endothelial Cells

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Background: Attachment of leukemic cells to vascular endothe- 
al cells induces the vascular endothelial cells to release endothelial cell-derived interleukin 8 (endothelial IL-8), which then induces leukemic cells to undergo apoptosis. NB4, a human promyelocytic leukemic cell line that expresses high levels of cell-surface CD13/aminopeptidase N, does not undergo endothelial IL-8-induced apoptosis. Consequently, we investigated the relationship between cell-surface aminopeptidase activity and endothelial IL-8 induction of apoptosis in various leukemic cell lines. Methods: CD13/aminopeptidase N activity and IL-8-induced apoptosis were examined in leukemic cell lines. Endothelial IL-8-induced apoptosis was examined further in NB4 cells, K562 cells (human chronic myelogenous leukemic cells expressing low levels of CD13/aminopeptidase N), CD13/aminopeptidase N-transfected K562 (K562/CD13) cells that overexpress aminopeptidase, and mock-transfected K562 cells (vector only). These cells were also cocultured with a vascular endothe- 
al cell layer to investigate the association between aminopeptidase activity and apoptosis in this system. All statistical tests were two-sided. Results: Endothelial IL-8 induced apoptosis in K562 cells but not in K562/CD13 cells. A combination of an aminopeptidase inhibitor (such as bestatin) and endothelial IL-8 induced apoptosis in NB4 cells and K562/CD13 cells (2.88-fold difference [95% confidence interval {CI} = 1.82-fold to 3.94-fold], P = .004 for bestatin-treated NB4 cells and 4.31-fold difference [95% CI = 3.52-fold to 5.10-fold], P < .001 for bestatin-treated K562/CD13 cells). When aminopeptidase activity in NB4 cells was modulated by aminopeptidase inhibitors, a statistically significant correlation was found between aminopeptidase activity and the proportion of apoptotic cells induced by endothelial IL-8 (r = −.837, P < .001 by Pearson’s correlation coefficient; r = −.697, P = .013 by Spearman’s correlation analysis by ranks). K562/CD13 cells cocultured with vascular endothelial cells did not undergo apoptosis, but the addition of bestatin resulted in the induction of apoptosis in K562/CD13 cells (2.70-fold difference [95% CI = 1.77-fold to 3.63-fold], P < .001). Bestatin treatment increased the level of IL-8 mRNA in and the amount of IL-8 secreted by vascular endothelial cells. Conclusions: High levels of cell-surface CD13/aminopeptidase N appear to allow leukemic cells to resist endothelial IL-8-induced apoptosis. The combination of endothelial IL-8 and bestatin induce leukemic cells expressing high levels of CD13/aminopeptidase N to undergo apoptosis. Bestatin may be useful for treating patients with leukemia. [J Natl Cancer Inst 2002;94:1020–8]

We have previously purified apoptosis-inducing factors from differentiated HL-60 cells (1–3). One of these factors is identical to endothelial cell-derived interleukin 8 (endothelial IL-8). Recombinant human endothelial IL-8 can induce apoptosis in many leukemic cell lines, but monocyte-derived IL-8 cannot. By testing various synthetic IL-8 peptides, we determined that a pentapeptide corresponding to the amino-terminal region of endothelial IL-8 is essential for its apoptosis-inducing activity (3). We also have reported that the direct interaction of a vascular endothelial cell monolayer and leukemic cells induces the release of endothelial IL-8 from endothelial cells and that the attached leukemic cells undergo apoptosis (1). Although endothelial IL-8 could induce apoptosis in many leukemic cell lines, endothelial IL-8 induces apoptosis in NB4 cells weakly or not at all (2), perhaps because NB4 cells, derived from a patient with acute promyelocytic leukemia, express high levels of a cell-surface aminopeptidase, CD13/aminopeptidase N (4).

CD13/aminopeptidase N was originally recognized as a marker for subsets of normal and malignant myeloid cells (5). The sequence of cDNAs for this marker revealed that it was a ubiquitously occurring Zn2+-dependent metalloprotease (6) that cleaves the amino-terminal neutral amino acid from proteins. Until recently, little was known about the biologic role of this enzyme, but evidence is accumulating that cell-surface pepti- 
dases play an important role in the control of growth and dif- 
ferentiation of many cellular systems (7–10). Ectoenzymes, in- 
cluding CD13/aminopeptidase N, cleave bioactive peptides on the cell surface to activate or inactivate them and to regulate their availability to adjacent cells (11–13). The purpose of our study was to evaluate the relationship between cell-surface ami- 
nopeptidase activity in seven leukemic cell lines and the ability of endothelial IL-8 to induce apoptosis in these cells.

Materials and Methods

Reagents

Recombinant human endothelial IL-8 was purchased from Genzyme (Cambridge, MA). Bestatin was provided by Nippon

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Kayaku Co. Ltd. (Tokyo, Japan). All other peptidase inhibitors were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Lines and Cell Culture

Seven leukemic cell lines were used to investigate the apoptosis-inducing activity of endothelial IL-8 in diverse cell lines. A human chronic myelogenous leukemia cell line, K562; a human promyelocytic leukemia cell line, NB4; a human myelogenous leukemia cell line, HL-60; a human monocytic leukemia cell line, U937; a human acute myelogenous leukemia cell line, KG-1, were obtained from the American Type Culture Collection (ATCC; Manassas, VA). All cell lines were maintained in Dulbecco’s modified Eagle medium/nutrient mixture F-12 Ham (Sigma Chemical Co.) supplemented with 10% serum-free medium, GIT® (Wako Pure Chemicals, Osaka, Japan) (15). For coculture experiments, human umbilical vascular endothelial cells (HUVECs) were purchased from BioWhittaker (Walkersville, MD) and were maintained in CS-C® medium (Cell Systems, Kirkland, WA).

Assay for Aminopeptidase N Activity

Cell surface aminopeptidase activity was measured in triplicate samples in triplicate experiments. After incubation with various aminopeptidase inhibitors or monoclonal antibody for 48 hours, leukemic cells were suspended in phosphate-buffered saline (PBS, pH 7.4) at 1 x 10^5 cells per mL, and then 100 μL of cell suspension was mixed with an equal volume of 200 μM Ala-(7-amino-4-methylcoumarin) (Peptide Institute, Osaka, Japan) acetic acid in a 96-well microtiter plate and incubated at 37 °C for 60 minutes. Reactions were stopped by adding 50 μL of 100 mM EDTA. The quantity of 7-amino-4-methylcoumarin generated was measured with a fluorometric plate reader (excitation wavelength = 355 nm; emission wavelength = 460 nm; Fluoroskan Ascent, Helsinki, Finland). The aminopeptidase activity was calculated from the fluorescence of a 7-amino-4-methylcoumarin standard (16).

Immunofluorescence Staining and Flow Cytometry

Flow cytometry was used to detect the expression of CD13/aminopeptidase N. Cells were washed with PBS and incubated for 30 minutes at 4 °C with fluorescein isothiocyanate (FITC)-labeled anti-CD13 monoclonal antibody (Leu-M7; BD Biosciences, Franklin Lakes, NJ) (17) and analyzed by flow cytometry with a FACSscan using CellQuest software (BD Biosciences).

Cell Proliferation Assay

We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) assay to quantitate cell proliferation. K562 cells were seeded at 1 x 10^5 cells per mL onto a confluent HUVEC layer in CS-C® medium (Cell Systems). After 3 days, K562 cells were collected, and their capacity to reduce MTT was determined as described (18). Ten microliters of MTT at 5 mg/mL of PBS was added to 100 μL of cell suspension, and the preparation was incubated at 37 °C for 4 hours. Cells that reduced MTT to its formazan form were dissolved in 0.04 N HCl in 2-propanol, and absorbance was measured at 595 nm with a microplate reader (Bio-Rad, Hercules, CA). MTT formazan reflects cell proliferation by using the succinate–tetrazolium (components of the respiratory chain in metabolically active cells) reductase system.

Apoptosis Assays

The first test for apoptosis was DNA fragmentation, as measured by the terminal deoxynucleotidyltransferase-mediated Fig. 1. Induction of apoptosis by endothelial cell-derived interleukin 8 (endothelial IL-8) and measurement of cell-surface aminopeptidase activity. K562, KG-1, HL-60, Jurkat, U937, THP-1, and NB4 cells (each at 1 x 10^5 cells per mL) were cultured with or without IL-8 (20 ng/mL) at 37 °C. Left, after 48 hours, apoptotic cells were detected by the TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP [deoxyuridine 5’-triphosphate] nick end labeling) assay. Data are expressed as the apoptotic proportion (mean and 95% confidence interval [assumption of normal distribution] for three experiments with triplicate samples). A statistically significant difference in the proportion of cells undergoing apoptotic cell death was observed when cells treated with endothelial IL-8 were compared with cells treated with monocyte-derived IL-8 (for all three experiments, P < .001 for K562, KG-1, HL-60, Jurkat, and THP-1 cells and P = .003 for U937 cells; χ^2 test). Right, the activity of aminopeptidase N was assessed by using a quenched fluorescent peptide. Data are the mean ± 95% confidence interval of the mean (error bars) from three experiments with triplicate samples.
dUTP (deoxyuridine 5'-triphosphate) nick end labeling (TUNEL) method. Reagents were obtained from a Boehringer Mannheim (Indianapolis, IN) kit and were used as described by the manufacturer (19). Briefly, $10^6$ cells were fixed in PBS containing 2% paraformaldehyde. After washing with PBS, cells were permeabilized on ice in a hypotonic solution containing 0.1% Triton X-100 and 0.1% sodium citrate for 2 minutes, washed twice in PBS, and resuspended in the TUNEL reaction mixture. After washing, the label incorporated at damaged DNA sites was visualized by fluorescence microscopy (Olympus, Tokyo, Japan). The percentage of apoptotic cells in cultures was determined by examining more than 200 cells per time point under a microscope.

The second test used for apoptosis was Annexin V staining and flow cytometry. Cells were washed twice with ice-cold PBS and then resuspended in 100 μL of binding buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, and 2.5 mM CaCl$_2$). Cells were incubated with 5 μL of FITC-conjugated Annexin V (Roche Diagnostics, Indianapolis, IN) for 15 minutes at room temperature in the dark, 400 μL of binding buffer was added, and cells were analyzed by flow cytometry with FACScan (BD Biosciences) with CellQuest software.

**Transfection of K562 Cells With a Full-Length Human CD13 Gene**

To further investigate the apoptosis-resistance mechanisms of CD13/aminopeptidase N, we transfected the cDNA of CD13/aminopeptidase N into K562 cells, which express low levels of CD13/aminopeptidase N. A. T. Look (Dana-Farber Cancer Institute, Harvard University, Boston, MA) provided the plasmid vector containing the full-length human CD13 gene (pZIPneoCD13). K562 cells were transfected with the pZIPneoCD13 vector (K562/CD13) or with an empty control pZIPneo vector (K562/mock) by electroporation. For stable transfectants, transfected K562 cells were selected with G418 (250 μg/mL).

**Northern Blot Analysis**

To assess the level of IL-8 mRNA expression, northern blotting was performed as we previously described (20). Briefly,
after coculture, total RNA from HUVECs and K562 cells was isolated with RNAzol B (Tel-Test, Inc., Friendswood, TX). Five micrograms of total RNA was fractionated by electrophoresis in formaldehyde/1.5% agarose gels, and bands were blotted onto a nitrocellulose membrane. RNA was cross-linked to membranes by UV irradiation (Stratalinker® UV Crosslinker, model 1800; Stratagene, La Jolla, CA). The membrane was hybridized first with a cDNA probe for human IL-8 and then with a cDNA for glyceraldehyde-3-phosphate dehydrogenase, radiolabeled by use of a Random-Prime Labelling kit (Amersham Biosciences, Piscataway, NJ). Hybridization was performed at 65 °C for 2 hours, followed by stringent washing in 2.0x standard saline citrate/0.1% sodium dodecyl sulfate (SDS) at 65 °C for two 15-minute periods, 1.0x standard saline citrate/0.1% SDS at 65 °C for two 15-minute periods, 0.5x standard saline citrate/0.1% SDS at 65 °C for two 15-minute periods, and finally in 0.1x standard saline citrate/0.1% SDS at 65 °C for two 15-minute periods. Hybridized membranes were exposed to Hyperfilm MP (Amersham Biosciences) at –80 °C.

**Statistical Analysis**

The proportion of apoptotic cells was calculated by dividing the number of TUNEL-positive cells by the total number of cells. Differences were tested for statistical significance with a χ2 test with 1 degree of freedom (df) by use of the statistical program StatView (SAS Institute Inc., Cary, NC). A two-sided Pearson’s correlation coefficient and Spearman’s correlation coefficient by ranks were calculated to determine whether there was a correlation between the aminopeptidase activity and the proportion of apoptotic cells. Quantification of IL-8 was carried out in triplicate; statistical analysis was performed with a two-sided Student’s t test. The level of statistical significance was set at P<.05. All statistical tests were two-sided.

**RESULTS**

**Cell-Surface Aminopeptidase Activity and Sensitivity to Endothelial IL-8-Induced Apoptosis**

We previously reported that endothelial IL-8 induced apoptosis in many human leukemic cell lines (2). In fact, in our hands, the promyelocytic leukemia cell line NB4, which expresses high levels of CD13/aminopeptidase N, was the only line that appeared resistant to endothelial IL-8-induced apoptosis (4). To investigate the relationship between cell-surface aminopeptidase activity and susceptibility to endothelial IL-8-induced apoptosis, we assessed cell-surface aminopeptidase activity and determined the proportion of apoptotic cells after incubation with endothelial IL-8 or monocyte-derived IL-8 as a control. All cell lines that were examined, except for NB4, showed statistically significantly greater amounts of apoptosis with endothelial IL-8 than with monocyte-derived IL-8 (for all three experiments, P<.001 for K562, KG-1, HL-60, Jurkat, and THP-1 cells; P = .003 for U937 cells). NB4 cells, however, had the highest aminopeptidase activity and did not show statistically significantly more apoptosis with endothelial IL-8 than with monocyte-derived IL-8. THP-1 and U937 cells that responded to the apoptosis-inducing activity of endothelial IL-8 had intermediate levels of aminopeptidase activity (Fig. 1).

To verify that the aminopeptidase activity of NB4 cells was responsible for their resistance to endothelial IL-8-induced apoptosis, we examined the apoptosis-inducing activity of recombinant endothelial IL-8 in the presence or absence of the CD13-neutralizing monoclonal antibody WM-15 or of an aminopeptidase inhibitor, such as bestatin, amastatin, or actinonin. When the neutralizing antibody or an aminopeptidase inhibitor was added, cell-surface aminopeptidase activity of NB4 cells was reduced in a dose-dependent manner (Fig. 2, A).

The proportion of apoptotic NB4 cells cultured with endothelial IL-8 (20 ng/mL) was statistically significantly increased when CD13/aminopeptidase N was inhibited with an aminopeptidase inhibitor at 1 μg/mL or with WM-15 at 10 μg/mL (P = .004 for bestatin; P = .043 for arphamenine B; P<.001 for amastatin; P = .005 for actinonin; and P = .001 for WM-15; Fig. 2, B). We performed a correlation analysis to clarify the relationship between aminopeptidase activity and sensitivity to apoptosis. As shown in Fig. 2, C, a statistically significant correlation was found between aminopeptidase activity and the proportion of apoptotic cells induced by endothelial IL-8 (r = –.837, P<.001 by Pearson’s correlation efficient; r = –.697, P = .013 by Spearman’s correlation analysis by ranks). The morphology of Wright-Giemsa-stained cells indicated the presence of apoptotic cells with aggregated chromatin or nuclear and cytoplasmic condensation in cultures treated with endothelial IL-8 or with the aminopeptidase inhibitor bestatin (Fig. 3).

**Apoptosis-Inducing Activity of Endothelial IL-8 in CD13/K562 Cells**

Several types of peptidases are expressed on the surface of both normal and malignant hematopoietic cells (21–24). To determine whether CD13/aminopeptidase N by itself can modulate the apoptosis-inducing activity of endothelial IL-8, we transfected K562 cells that express low levels of CD13/aminopeptidase N with a vector expressing CD13/aminopeptidase N, and we confirmed that the CD13/aminopeptidase N was expressed on the cell surface by immunofluorescence. To determine the relationship between cell-surface aminopeptidase activity and sensitivity to endothelial IL-8-induced apoptosis, we assessed cell-surface aminopeptidase activity and determined the proportion of apoptotic cells after incubation with endothelial IL-8 or monocyte-derived IL-8 as a control. All cell lines that were examined, except for NB4, showed statistically significantly greater amounts of apoptosis with endothelial IL-8 than with monocyte-derived IL-8 (for all three experiments, P<.001 for K562, KG-1, HL-60, Jurkat, and THP-1 cells; P = .003 for U937 cells). NB4 cells, however, had the highest aminopeptidase activity and did not show statistically significantly more apoptosis with endothelial IL-8 than with monocyte-derived IL-8. THP-1 and U937 cells that responded to the apoptosis-inducing activity of endothelial IL-8 had intermediate levels of aminopeptidase activity (Fig. 1).

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**Fig. 3.** Induction of apoptosis in NB4 cells by a combination of bestatin and endothelial cell-derived interleukin-8 (endothelial IL-8). NB4 cells (1×10⁷ cells per mL) were cultured alone (a), with endothelial IL-8 at 20 ng/mL (b), with bestatin at 1 μg/mL (c), or with both endothelial IL-8 at 20 ng/mL and bestatin at 1 μg/mL (d) at 37 °C for 48 hours. After centrifugation in a cytocentrifuge, cells were stained with Wright-Giemsa stain. Arrowheads = apoptotic cells.
peptidase N with a CD13 cDNA and established stable transfectants that overexpress CD13/aminopeptidase N (termed K562/CD13 cells) (Fig. 4, A). K562/CD13 cells and NB4 cells have similarly high levels of aminopeptidase activity (Fig. 4, B). To examine the susceptibility of K562/CD13 cells to endothelial IL-8-induced apoptosis, recombinant endothelial IL-8 was added to the culture medium (final IL-8 concentration = 20 ng/mL), and 48 hours later, the number of apoptotic cells was assessed with the TUNEL assay. Compared with untreated cultures, almost no change in the number of K562/CD13 cells undergoing apoptosis was detected. In the presence of endothelial IL-8 at the same concentration, however, statistically significantly more K562/mock transfectants (K562 cells transfected with an empty vector) underwent apoptosis than untreated K562/mock transfectants (for all, \( P < 0.001 \)). We next investigated the effect of inhibiting aminopeptidase activity by adding endothelial IL-8 (20 ng/mL) with various concentrations of bestatin to cultures of K562/CD13 cells and NB4 cells. Addition of bestatin at 1 \( \mu \)g/mL effectively suppressed the aminopeptidase activity of K562/CD13 cells and NB4 cells and allowed endothelial IL-8 to induce apoptosis to the same level detected in K562/mock transfectants (2.88-fold difference [95% CI = 1.82-fold to 3.94-fold], \( P = 0.004 \), for bestatin-treated NB4 cells and 4.31-fold difference [95% CI = 3.52-fold to 5.10-fold], \( P < 0.001 \), for bestatin-treated K562/CD13 cells; Fig. 4, C and D). Thus, the aminopeptidase activity of CD13/aminopeptidase N appears to be involved in the resistance of K562/CD13 cells and NB4 cells to endothelial IL-8-mediated apoptosis.

**Fig. 4.** Effect of bestatin on aminopeptidase activity of CD13-transfected K562 cells (K562/CD13 cells) expressing high levels of CD13/aminopeptidase N and on the apoptosis-inducing activity of endothelial cell-derived interleukin 8 (endothelial IL-8). K562 cells were transfected with pZIPneoCD13 (K562/CD13 cells) or empty pZIPneo (K562/mock cells) by electroporation and then selected with G418. A) Expression of surface CD13 was detected with fluorescein isothiocyanate-conjugated mouse immunoglobulin G1 (IgG1) anti-human CD13 antibody (shaded peak). Nonspecific fluorescence was estimated with an isotype-matched control antibody (open peak). One representative pair of clones of six pairs (K562/CD13 and K562/mock cells) is shown. B) Aminopeptidase activity of K562, NB4, K562/CD13, and K562/mock cells was assessed. Data are the mean ± 95% confidence interval of three experiments with triplicate samples. C) Aminopeptidase activity was assessed in the presence of bestatin at 0–10000 ng/mL. Data are the mean ± 95% confidence interval of three experiments with triplicate samples. D) CD13/K562 cells and K562/mock cells were cultured with or without recombinant endothelial IL-8 (20 ng/mL) at 37 °C in the presence of bestatin at 0–10000 ng/mL. After 48 hours, apoptotic cells were detected by TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP [deoxyuridine 5′-triphosphate] nick end labeling) assay. Data are the apoptotic proportion and 95% confidence interval of a representative experiment of three experiments, all with triplicate samples. All experiments had similar results. Two-sided \( x^2 \) tests were performed to determine statistical significance of effects between treated and untreated cultures (* \( P < 0.001 \)).
Coculture of Leukemic Cells and Vascular Endothelial Cells

We have previously reported that when leukemic cells attach to vascular endothelial cells, vascular endothelial cells release endothelial IL-8, which induces the attached leukemic cells to undergo apoptosis (1). Consequently, we studied K562/CD13 and K562/mock transfectants in this system. Compared with control parental K562 cells cultured alone, the growth of K562/mock cells cultured on a HUVEC layer was reduced by 75.3% (95% CI 72.1% to 78.5%), and the growth of K562/CD13 cells was reduced by 95.0% (95% CI 91.8% to 93.2%) (data not shown). We next tested the aminopeptidase inhibitor bestatin in the coculture system. After 48 hours of coculture with HUVECs, the proportion of apoptotic K562/CD13 cells was statistically significantly lower than that of parental K562 cells and K562/mock transfectants (P = .002 versus parental K562, and P = .004 versus K562/mock transfectants). After the addition of bestatin at 1 μg/mL, the number of apoptotic K562/CD13 cells was statistically significantly higher (2.70-fold difference [95% CI 1.77-fold to 3.63-fold]; for all, P<.001), but the number of apoptotic K562/mock transfectants was not (Fig. 5, A). Similar data were obtained from TUNEL assays (Fig. 5, A) and Annexin V staining (Fig. 5, B). Conditioned medium from K562/CD13 cells or K562/mock transfectants cocultured with vascular endothelial cells had higher concentrations of IL-8, and after the addition of bestatin to these cultures, the concentration of IL-8 was further increased. Higher concentrations of IL-8 were also detected after the addition of bestatin to HUVEC cultures alone but not to K562 cultures (Fig. 6, A). Addition of bestatin to the HUVEC culture medium induced the expression of IL-8 mRNA in HUVECs (Fig. 6, B).

Thus, leukemic cells that express high levels of CD13/aminopeptidase N may escape apoptosis induced by endothelial IL-8 secreted by endothelial cells that have interacted with leukemic cells. Bestatin may increase the sensitivity to apoptosis by inhibiting the cell-surface aminopeptidase activities of leukemic cells and/or, perhaps, by inducing vascular endothelial cells to secrete endothelial IL-8.

DISCUSSION

High levels of aminopeptidase activity appear to allow various leukemic cells to resist endothelial IL-8-induced apoptosis. NB4 cells and K562/CD13 cells are resistant to endothelial IL-8-induced apoptosis. Addition of WM-15, a CD13-neutralizing monoclonal antibody, or various aminopeptidase inhibitors, to NB4 cells or K562/CD13 cells inhibited the cell-surface aminopeptidase activity, and the subsequent addition of recombinant endothelial IL-8 induced apoptosis in these cells. Sensitization of these cells to apoptosis apparently involved inhibition of the aminopeptidase activity, because the number of apoptotic cells induced by endothelial IL-8 correlated inversely with the aminopeptidase activity. Sekine et al. (25) reported that bestatin induced apoptosis in leukemic cells, that bestatin at less than 1 μg/mL did not induce NB4 cells or K562 cells to undergo apoptosis, and that a more than additive effect with endothelial IL-8 was not noted. However, it remains to be clarified why cell

Fig. 5. Effect of bestatin on vascular endothelial cell-induced apoptosis of CD13-transfected K562 cells (K562/CD13 cells) expressing high levels of CD13/aminopeptidase N. A) Untransfected K562 cells, K562/CD13 cells, and K562/mock cells were cultured with or without a human umbilical vascular endothelial cell (HUVEC) monolayer at 37 °C in the presence or absence of bestatin at 1 μg/mL. After 48 hours, cells were collected, and apoptotic cells were detected by the TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP [deoxyuridine 5'-triphosphate] nick end labeling) assay. Data are expressed as the apoptotic proportion (mean and 95% confidence interval of three experiments with triplicate samples). Two-sided χ² tests were performed to determine statistical significance. B) Annexin V staining was performed to confirm that apoptosis occurred when cells were cocultured with HUVECs. Control cells were cultured with the HUVEC monolayer. K562/CD13 cells were collected after a 48-hour coculture, incubated with fluorescein isothiocyanate-conjugated Annexin V, and analyzed by flow cytometry. Left, untreated control. Right, treated with bestatin at 1 μg/mL.
lines such as THP-1 or U937 that express relatively high levels of CD13 undergo endothelial IL-8-induced apoptosis to the same extent as cell lines, such as K562 or Jurkat, which express low levels of CD13. A threshold level of aminopeptidase activity may protect cells from endothelial IL-8-induced apoptosis. Indeed, inhibition of aminopeptidase activity in NB4 cells to the level detected in THP-1 or U937 cells (0.3–0.4 nmol per mL per min) increased the number of NB4 cells undergoing apoptosis. Alternatively, another mechanism may exist that can overcome or mimic the high levels of CD13/aminopeptidase N.

Recent studies indicated that CD13/aminopeptidase N might play an important role in the control of growth and differentiation of hematopoietic cells [for review, see (26)]. CD13/aminopeptidase N is expressed on proliferating progenitors of granulocyte-macrophage colony-forming units (21) and on malignant acute myeloblastic cells and lymphoblastic leukemia cells (23,24). The expression of CD13/aminopeptidase N is associated with the proliferation of activated T lymphocytes and dendritic cell/macrophage progenitors (9,27). Inhibition of CD13 expression or aminopeptidase N activity reduces the proliferation of various types of cells (7,9,10,26,28). Many articles have reported that CD13/aminopeptidase N is involved in hematopoietic proliferation or differentiation, but the underlying mechanisms remain elusive. Our data indicate that the aminopeptidase activity of CD13/aminopeptidase N may contribute to the proliferation of hematopoietic cells by protecting them from proapoptotic peptide factors such as endothelial IL-8.

Vascular endothelial cells contribute to antitumor host defense by expressing nitric oxide synthase in response to interferon-γ and tumor necrosis factor-α. Nitric oxide synthase then produces nitric oxide, which kills leukemic cells (29). Moreover, as we demonstrated previously, vascular endothelial cells release endothelial IL-8 after they directly interact with leukemic cells, and IL-8 induces the attached leukemic cells to undergo apoptosis (1). Vascular endothelial cells secreting IL-8—like other antitumor cells, such as macrophages and natural killer cells—have the antitumor property of inducing apoptosis in attached leukemia cells. In this study, we found that K562/CD13 cells escaped apoptosis induced by contact with vascular endothelial cells. Addition of bestatin restored this sensitivity. Thus, leukemic cells that express high levels of CD13/aminopeptidase N should have a survival advantage in the vascular system.

The role of angiogenesis in tumor vascularization and tumor metastasis has been discussed previously (30–32). Tumor cells
secrete metallocproteinase to destroy matrix proteins and to damage endothelial cells, and then the tumor cells invade the extra-vascular space (33,34). Bhagwat et al. (35) reported that CD13/aminopeptidase N in endothelial cells is activated by angiogenic signals and is essential for capillary tube formation. They demonstrated that CD13/aminopeptidase N inhibitors effectively abrogate the ability of the cells to organize capillary formation. Another group described a novel monoclonal antibody that inhibits cell motility and in vitro invasion of tumor cells (36). The epitope for this antibody was identical to a CD13/aminopeptidase N epitope. This group also demonstrated that the expression of CD13/aminopeptidase N is a useful indicator of a poor prognosis for lymph node-positive patients with colon cancer (36). In this study, we observed that bestatin increases the expression of IL-8 mRNA in endothelial cells. After attachment of leukemic cells, endothelial cells secrete more IL-8 in the presence of bestatin than in its absence. Inhibition of CD13/aminopeptidase N thus appears to reinforce the antitumor property of vascular endothelial cells by preventing tumor vascularization and by inducing the secretion of antitumor factors, such as endothelial IL-8.

Bestatin has been used clinically for years as an immunotherapy drug. The combination of bestatin and maintenance chemotherapy was reported to prolong the survival of patients with acute nonlymphocytic leukemia (37). Recently, randomized phase III studies of bestatin as postoperative adjuvant treatment in patients with stage I squamous cell lung cancer were carried out, and statistically significant clinical improvement in overall survival and disease-free survival was ascertained (38). Some researchers suggested that bestatin has T-cell-mediated antitumor activity (39), but the antitumor mechanisms of bestatin were not fully clarified. Our present study offers additional evidence to explain the mechanisms contributing to good clinical outcomes in adjuvant treatments using bestatin. We have also demonstrated that bestatin enhanced STI-571-induced apoptosis in K562 cells (data not shown).

Various types of tumor cells can protect themselves from the induction of apoptosis. Our data strongly indicate that the high level of CD13/aminopeptidase N expression on the surface of some leukemic cells may be important for their survival. Aminopeptidase N inhibitors such as bestatin may be useful in treating leukemia because they make cells more sensitive to the induction of apoptosis.

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NOTES

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