Inactivation of interleukin-8 by aminopeptidase N (CD13)

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Abstract: Aminopeptidase (APN) was found to degrade interleukin-8 (IL-8) and inactivate its chemotactic activity. The chemotactic activity of IL-8 was decreased by APN or neutrophil plasma membranes dose- and time-dependently. The chemotactic activity was not inactivated in the presence of bestatin or WM15 monoclonal antibody. The expression of IL-8 was measured by flow cytometry. On lipopolysaccharide (LPS) stimulation, IL-8 expression increased for 60 min and then decreased markedly. In contrast, on treatment with LPS and bestatin, the expression of IL-8 increased continuously for at least 120 min. These results suggest that the expression and release of IL-8 from phagocytic cells are regulated by the proteolytic effect of APN on IL-8. *J. Leukoc. Biol.* 57: 129-134; 1995.

Key Words: *interleukin-8 • aminopeptidase N • CD13 • bestatin • LPS*

INTRODUCTION

Interleukin-8 (IL-8) is a recently characterized cytokine that functions as a neutrophil chemotactic/activating factor. IL-8 was first shown to be produced by LPS-stimulated monocytes [1] and is now known to be expressed by a wide variety of cells including neutrophils and fibroblasts [2, 3]. IL-8 may be responsible for attracting neutrophils to sites of inflammation, and information is required on its production in various pathological states.

The cell surface glycoprotein CD13 was originally recognized as a marker for subsets of normal and malignant hematopoietic cells of the myeloid lineage [4, 5]. This glycoprotein is now thought to be identical to a major metalloprotease, aminopeptidase N (EC 3.4.11.2) (APN), from the predicted amino acid sequence of a biologically active cloned cDNA [6]. CD13/APN has also been found on the surface of many other cells and tissues beside those of the hematopoietic system, including fibroblasts and cells of the intestinal epithelium and renal tubular epithelium, and on synaptic membranes of the central nervous system [7, 8]. Although measurement of APN as leucine aminopeptidase activity is used clinically to detect liver diseases, the actual function(s) of APN is still controversial.

Here we report that IL-8 is degraded and inactivated by APN and suggest that APN may be a modulator of IL-8 on the cell surface.

MATERIALS AND METHODS

Materials

Recombinant human IL-8 (monocyte-derived, 72-amino-acid form) was a gift from Prof. Matsushima, Kanazawa University. Aminopeptidase N (porcine kidney) was obtained from Sigma (St. Louis, MO). This aminopeptidase N is the preparation derived from microsomal fractions of porcine kidney. By SDS-PAGE, three different polypeptide chains (molecular masses 140, 97, and 49 kDa) were recognized. The polypeptide chains of 97 and 49 kDa were produced from the 140-kDa form by a purification step. Its specific activity is 13.4 U/mg protein (one unit hydrolyzes 1.0 μM of leucine-*p*-nitroanilide per minute at pH 7.2 at 37°C). Endoproteinase (EC 3.4.21.40 mouse submaxillary grand), fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin G (IgG; sheep), FITC-conjugated anti-mouse IgG (rabbit), L-leucyl-*p*-nitroanilide, and bestatin were purchased from Sigma. Monoclonal mouse anti-human CD13 (WM47) was obtained from DAKO (Denmark) and anti-human WM15 monoclonal antibody from Nihon Kayaku Co. We determine whether the antibodies affect the enzymatic activity of the aminopeptidase N. WM47 antibody resulted in no effect on aminopeptidase activity but WM15 neutralized aminopeptidase activity. Polyclonal rabbit anti-human IL-8 from Upstate Biotechnology (New York). Modified Boyden chambers (tissue culture treated Transwell, 5.0 μm) were purchased from Costar (Cambridge, MA).

Preparations of neutrophils, monocytes, and plasma membranes

Normal human mononuclear cells and neutrophils were isolated by Ficoll-Hypaque density gradient centrifugation of heparin-anticoagulated peripheral blood from normal adult volunteers, as previously described [9]. Cell viabilities were >98% as judged by trypan blue exclusion. Contaminating erythrocytes were lysed by brief exposure (5 min) of the cells to lysis buffer (160 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Cells were stored at room temperature in Hanks' buffer. For some experiments, the cells were cultured overnight in RPMI 1640 medium supplemented with L-glutamine, 10% fetal calf serum and antibiotics to allow monocytes to adhere to the plastic. The nonadherent cells enriched in lymphocytes and adherent cells enriched in monocytes were harvested.

Plasma membranes were purified from neutrophils by a modification of the procedure previously described [10]. Washed cells in Hanks' buffer were homogenized by 10 strokes of a HISCOTON homogenizer and centrifuged at

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Abbreviations: APN, aminopeptidase N; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; IL-8, interleukin-8; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TNF-α, tumor necrosis factor α.

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Received March 3, 1994; accepted August 25, 1994.

40,000*g* for 30 min at 4°C. The pellet was then suspended in 0.25 M sucrose solution and layered on 1 M sucrose solution in phosphate-buffered saline (PBS) containing 0.2 mM $MgCl_2$ and 1% bovine serum albumin (BSA) in a ratio of 3:8. The resulting gradient was centrifuged at 100,000*g* for 1 h at 4°C. The pelleted membranes were solubilized by homogenization in 100 mM Hepes buffer, pH 7.6, containing Hanks' buffer. Protein was measured by the method of Lowry et al. with BSA as a standard. The purity of the plasma membrane fraction was assessed by assays of lactate dehydrogenase, elastase, and APN as markers of the cytosol, lysosomes, and plasma membranes, respectively. No lactate dehydrogenase or elastase activity was detectable, but the APN activity was increased 20-fold. The APN activity was determined as 5.4 U/mg protein, so it was 60% lower than that of pure porcine APN. APN-rich neutrophil plasma membranes are referred to as plasma membranes in this paper.

SDS-PAGE of IL-8

SDS-PAGE was performed by the method of Laemmli [11]. Aliquots of IL-8 (2 μg/10 μl) in Tris-buffered saline containing 1 mM $ZnCl_2$ were preincubated with 100 ng/ml and 10 μg/ml APN for 30 min and 4 h, respectively, at 37°C and then subjected to 20% SDS-PAGE under nonreducing conditions. Similar aliquots of IL-8 (2 μg/100 μl) in the buffer were preincubated with 200 μg/ml neutrophil plasma membranes for 4 h at 37°C and then subjected to 20% SDS-PAGE under nonreducing conditions. The gels were stained with Coomassie Blue.

Identification of the proteolytic cleavage site in IL-8 by sequence analysis

Samples of 20 μg of IL-8 were digested with 1 μg of porcine aminopeptidase N or 200 μl of PBS for 20 h at 23°C. The digested samples and a control sample were dialyzed against 3% acetic acid, and aliquots of each sample were sequenced by Edman degradation in a prototype spinning-cup sequenator [12]. The amino acid derivatives were identified by isocratic high-performance liquid chromatographic analysis.

Chemotactic assay

Neutrophil chemotaxis was assayed by a modification of the Boyden method [13]. Briefly a small culture chamber (upper chamber) of which the bottom dish is made of microporous membrane is inserted into a 6.5-mm-diameter well (lower chamber). The pore size is 5.0 μm and the membrane is made of polycarbonate. Normal human neutrophils (10^6) were placed in the small chamber. IL-8 solution (100 ng/ml) was preincubated with APN (10–200 ng/ml), plasma membranes (10–200 ng/ml), or porcine endoproteinase (200 ng/ml) as control at 37°C for 0–80 min and then aliquots (600 μl) were placed in the lower chamber. Bestatin (100 μg/ml) or WM15 monoclonal antibody (50 μg/ml), which neutralizes APN activity [14], was also preincubated at 37°C for 1 h with some samples of plasma membranes. Incubations were performed at 37°C for 45 min and then the filters (5 mm pore size) were carefully removed, fixed, and stained. All material from the upper surface of the filter was carefully removed by scraping with a cotton-tipped roll, and chemotactic cells adhering to the lower surface of the filter were counted under a microscope (×400). Values are expressed as numbers of cells in randomly chosen areas on the surface of the filter. Each experiment was carried out in triplicate; cells on three areas of each filter were counted.

Flow cytometric analysis

LPS stimulation of cells

Samples of neutrophils and monocytes (1×10^6) were incubated with or without LPS for 3 h (neutrophils) or 24 h (monocytes) in a CO_2 incubator. Then the cells were incubated with excess CD13-specific monoclonal antibody (WM47) or IL-8 polyclonal antibody at 4°C for 30 min. They were then washed twice with cold Hanks' buffer and incubated for 30 min at 4°C with phycoerythrin-conjugated affinity-purified sheep immunoglobulin G (IgG) to mouse IgG for CD13 or FITC-conjugated affinity-purified swine IgG to rabbit IgG for IL-8. After two more washings with cold Hanks' buffer, the cells were resuspended in the buffer at 5×10^5 cells/ml in Hanks' buffer and two-color fluorescence was analyzed in an EPICS R Profile Analyzer. The fluorescence intensities (mean channel) of CD13- and IL-8–positive cells were calculated by subtraction of the background fluorescence intensity of cells stained with an isotype-matched control IgG of a nonimmuned mouse.

Bestatin treatment

Samples of neutrophils or monocytes (1×10^6 cells) were incubated with or without 10 mg/ml of bestatin (aminopeptidase inhibitor) at 37°C in a CO_2 incubator for 20, 40, 60, and 90 min (neutrophils) or 4 and 24 h (monocytes). Then they were incubated with an excess amount of IL-8 polyclonal antibody at 4°C for 30 min and stained by the same procedure as described above.

RESULTS

SDS-PAGE and NH_2-terminal analysis

The proteolytic action of APN on IL-8 was verified by SDS-PAGE (**Fig. 1**). IL-8 was degraded to a form with a lower

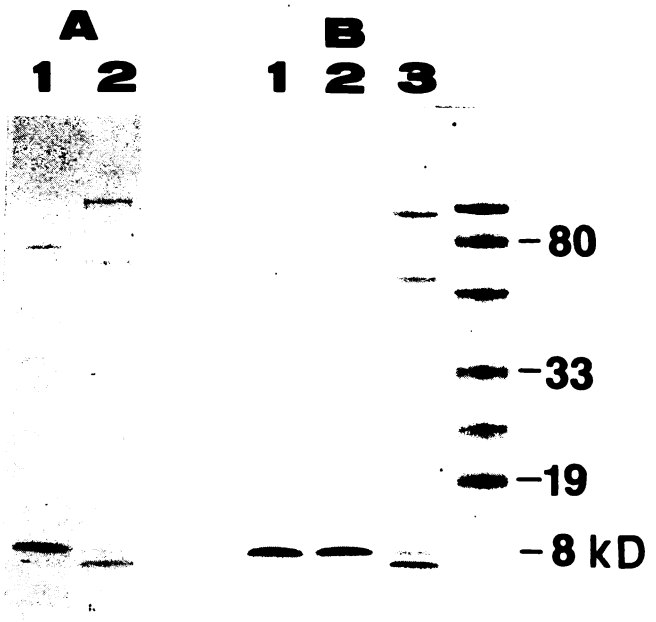

Fig. 1. SDS-PAGE of IL-8 treated with aminopeptidase N. (A) Incubation time 4 h. A1, untreated IL-8; A2, IL-8 with 10 μg/ml aminopeptidase N (porcine). (B) Incubation time 30 min. B1, untreated IL-8; B2, IL-8 with 100 ng/ml aminopeptidase N; B3, IL-8 with 10 μg/ml aminopeptidase N. Degraded IL-8 of 6 kDa is observed in lanes A2, B2 (weak), and B3. Bands of marker proteins are shown on the right.

molecular mass of 6 kDa by porcine APN, its degradation being almost complete on treatment with 100 ng/ml APN for 4 h. The faint bands of 68 kDa (in all lanes) indicate small amount of BSA for stabilization of IL-8 and the bands of APN were shown in lanes A2 and B3. Similar degradation was obtained with the neutrophil plasma membrane. The APN cleavage site in IL-8 was determined by NH_2-terminal amino acid sequence analysis of degraded IL-8 in comparison with untreated IL-8. The cleavage sites were found to be between Lys-15 and Pro-16 or between Ser-14 and Lys-15 (**Table 1**).

Chemotactic assay

We next examined the effect of APN on the chemotactic activity of IL-8. The chemotactic activity of IL-8 was rapidly lost on incubation of IL-8 with APN or plasma membranes. The inactivation was observed time dependently (**Fig. 2**). The activity of pure APN per 1 mg protein is 50% higher than that of plasma membranes but the degree of inactivation by APN and plasma membranes had a similar pattern. The chemotactic activity of IL-8 was not lost on incubation of IL-8 with endopeptidase (data not shown). When we mixed APN or plasma membrane with IL-8 at 37°C for 80 min, the inactivation of IL-8 was observed with increase of the concentration of APN or plasma membranes (**Fig. 3**). The rate of inactivation by pure APN was higher than by plasma membranes, especially at 50 and 100 ng/ml. This difference is thought to be due to APN activity of pure APN and plasma membranes, because APN activity of plasma membranes is approximately 50% lower than that of pure APN. When APN or plasma membranes were preincubated with bestatin and then incubated with IL-8 at 37°C for 1 h, the chemotactic activity of IL-8 was similar to that of IL-8 without treatment (Fig. 3). Moreover, in the presence of monoclonal antibody WM15, which neutralizes APN activity, the chemotactic activity of IL-8 was scarcely changed even by APN at 200 ng/ml. Both bestatin and WM15 antibody inhibited the inactivation of IL-8 by APN in plasma membranes. When we used 10 ng/ml IL-8, pure APN and plasma membranes also inhibited the chemotactic activity (**Fig. 4**). The chemotactic activity of pure APN and plasma membranes showed a very low level.

TABLE 1. *NH_2*-terminal Amino Acid Sequence Analysis of IL-8 Treated by APN[a]

	1st residue of IL-8 treated by APN (pmol)	2nd residue of IL-8 treated by APN (pmol)	3rd residue of IL-8 treated by APN (pmol)
D	4.07	2.55	0.65
N	1.47	1.20	1.38
S	1.37	0.25	0.66
Q	1.27	1.26	0.96
T	1.22	0.59	0.41
G	8.9	2.73	2.43
E	24.6	11.85	3.87
H	0.92	0.61	49.0
A	4.00	4.99	1.71
R	0.75	0.76	6.9
Y	0.67	0.44	0.88
C	6.64	0.96	0.1
P	99.2	69.4	9.5
M	1.04	0.54	0.45
V	0.96	0.90	0.91
F	0.08	71.8	44.3
I	4.46	3.36	0.40
K	85.7	12.1	3.74
L	1.27	17.2	2.18

[a]IL-8 sequence: SAKELRCNCIKYYSKPFH--. Samples of 20 μg of IL-8 were digested with 1 μg of porcine aminopeptidase N in 200 μl of PBS for 20 h at 23°C. NH_2-terminal amino acid was analyzed by an amino acid sequencer. NH_2-terminal sequences of IL-8 digested by APN were determined to be PFH--- or KPF---.

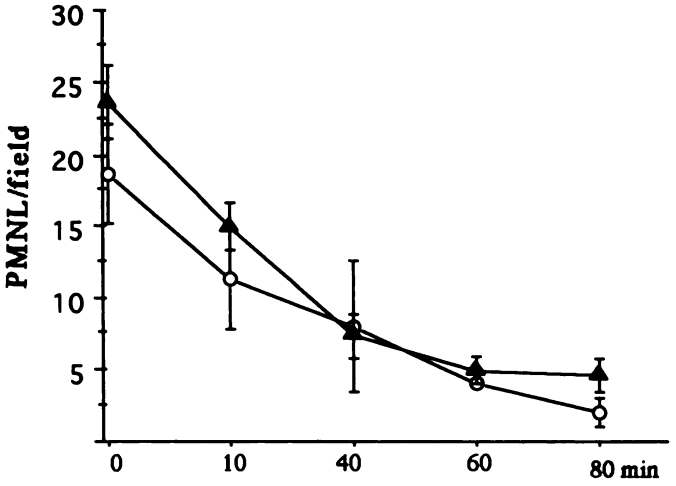

Fig. 2. Time-dependent inactivation of chemotactic activity of IL-8 by APN, APN-rich neutrophil plasma membrane. IL-8 (100 ng/ml) was preincubated with 200 ng/ml APN or APN-rich plasma membranes for the indicated times. A membrane invasion culture system was used. The ordinate shows numbers of polymorphonuclear leukocytes (×400) below the filter. Values are mean ± SD for three experiments. (○) APN; (▲) plasma membranes.

Flow cytometric analysis

To detect the effect of APN on IL-8 in living cells, we analyzed IL-8 expression on phagocytic cells. For this we used LPS as an IL-8 inducer and bestatin as a specific inhibitor of APN. The cell surface expressions of APN and IL-8 of cells with LPS showed an inverse relationship at 120 min (**Fig. 5**, top left). The maximum IL-8 expression by neutrophils was observed at 60 min; it was 50% higher than that

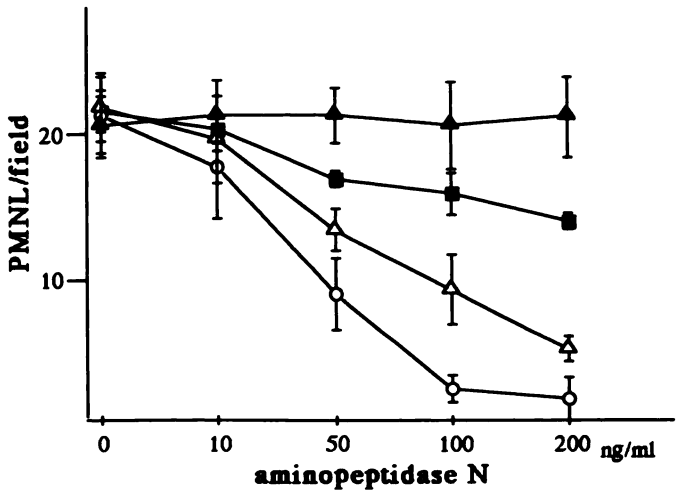

Fig. 3. Dose-dependent inactivation of chemotactic activity of IL-8 by APN or APN-rich neutrophil plasma membranes. IL-8 (100 ng/ml) was preincubated for 80 min at 37°C with different concentrations of APN (○), plasma membranes (△), plasma membranes + 0.1 mg/ml bestatin (▲), and plasma membranes + 50 μg/ml WM15 antibody (■). The ordinate shows numbers of polymorphonuclear leukocytes (×400) below the filter. Values are mean ± SD for three experiments.

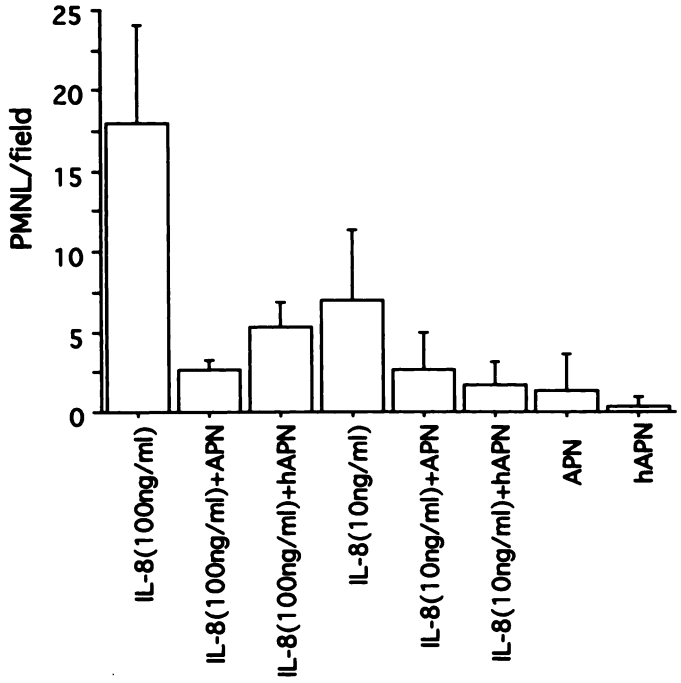

Fig. 4. Chemotactic activity of 100 ng/ml and 10 ng/ml IL-8 with or without APN (porcine aminopeptidase N) or plasma membranes (hAPN). APN and plasma membranes inhibited the chemotactic activity of both concentrations of IL-8.

of control cells and then decreased. APN expression increased after 60 min. The effects of bestatin on the levels of APN and IL-8 on the cell surface are shown in Figure 5 (bottom left). IL-8 expression increased after treatment with bestatin, reaching a high level at 60 and 120 min that was 30% higher than the level without bestatin. APN expression upon treatment with bestatin and LPS was not so increased compared with LPS only.

On monocytes, IL-8 expression was maximal 24 h after LPS stimulation, being 300% higher than that before LPS stimulation (Fig. 5, right). In contrast, APN expression was greatly decreased after 24 h of stimulation with LPS. IL-8 expression by monocytes increased significantly on treatment with LPS or with LPS and bestatin.

DISCUSSION

IL-8 is an unusually stable mediator of inflammation, and the mechanism of its regulation by enzymatic degradation or release from cells is not yet known. Suhail et al. [15] reported that a C5a-inactivating protease from serosal fluid degrades IL-8 and inactivates its chemotactic activity and they characterized this protease as a 55-kDa serine protease. IL-8 very rapidly combines with its receptor on cells and down-regulates expression of its own receptor associated with ligand internalization [16]. Thus intracellular or cell surface regulation of IL-8 also seems to be important. The DNA se-

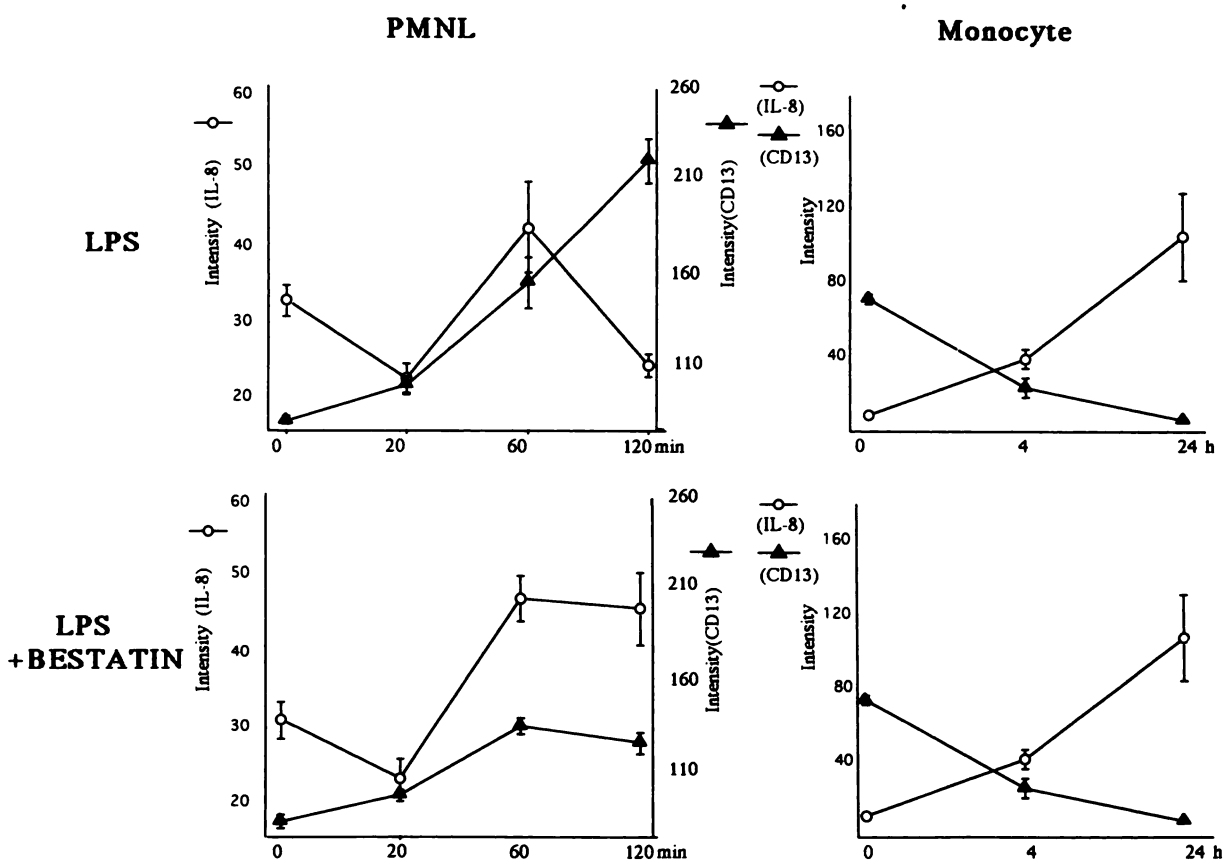

Fig. 5. Flow cytometric analysis of neutrophil (polymorphonuclear leukocyte) and monocyte expressions of CD13 (▲) and IL-8 (O) after incubation with LPS (10 μg/ml) or LPS and bestatin (1 mg/ml). (Left) Neutrophils: (right) monocytes; (top) LPS stimulation; (bottom) LPS + bestatin stimulation. IL-8 expression by PMNL was still high at 120 min on incubation with LPS and bestatin, but not on incubation with LPS only, whereas that by monocytes was high after 24 h on incubation with either LPS or LPS + bestatin. Intensity on the ordinate shows mean fluorescence channel.

quence of APN was found to be identical to that of surface antigen CD13 [6] expressed on phagocytic cells, but the precise function(s) of APN on phagocytic cells has not been reported. We found that APN cleaves and inactivates IL-8 in vitro. APN degraded IL-8 of 8 kDa to a molecule of 6 kDa. The NH_2-terminal amino acid of the degradation product with APN was Pro-16 or Lys-15. Baldwin and colleagues [17, 18] proposed that residues 4 to 9 of the amino terminus and the β-bend, which includes His-33, of IL-8 may be important for receptor binding. They found that residues 29–36 form the β-bend, which is attached covalently to the amino-terminal strand through a disulfide bridge between residues 7 and 34. This and our results indicate that the structure of the receptor binding site could be changed by this degradation, so that the degraded molecule does not have receptor binding capacity. The APN degradation of IL-8 is thought to be a interesting phenomenon, because APN mostly cleaves dipeptides or oligopeptides. The molecular weight of IL-8 is 8K and it has 72 amino acids. IL-8 is a markedly large polypeptide for APN to degrade. Therefore, the cleavage of IL-8 by APN is thought to be novel.

We demonstrated loss of chemotactic activity of IL-8 degraded by APN or plasma membranes. Loss of the chemotactic activity of IL-8 was extensive at 80 min on treatment with APN or plasma membranes and was dependent on the concentration of APN or plasma membranes. Although APN activity of plasma membranes is 50% lower than that of pure APN, inactivation of chemotactic activity had a similar pattern (Fig. 2). This suggests that other factors such as phospholipid and unknown membrane proteins may involve IL-8 inactivation. However, when plasma membrane was preincubated with bestatin or monoclonal antibody WM15, it did not inactivate the chemotactic activity (Fig. 3). These data indicate that APN plays a central role in inactivating IL-8 on plasma membranes. It is known that neutral peptidase on the neutrophil cell membrane cleaves and inactivates the chemotactic peptide f-Met-Leu-Phe [19]. APN may control the chemotactic activity of IL-8 on the surface of neutrophils as neutral peptidase does that of f-Met-Leu-Phe.

Yeager et al. [20] found that APN is a major receptor for coronavirus. Fridkin et al. [21] reported that APN liberates the NH_2-terminal of tuftsin, transforming the synthetic tetrapeptide into an inactive tripeptide that competitively inhibits tuftsin binding to its receptors. However, these substances are formed in pathological conditions, so we studied how APN affects IL-8 on the cell surface. To detect the precise function of APN on the cell surface, we examined phagocytic cells by flow cytometric analysis. Expression of IL-8 by LPS-stimulated neutrophils increased for 60 min and then decreased. In contrast, their APN expression continued to increase for 120 min. Once IL-8 is produced and on LPS stimulation, it binds to its receptor and is internalized. This cycle must be terminated at some stage to restore the normal condition of the cells. Thus APN on the cell surface may be increased to degrade IL-8 and suppress its overproduction. Our results suggest that APN (CD13) inhibits autocrine stimulation of neutrophils by IL-8.

Bauvois et al. [22] investigated the relationships of membrane-bound peptidase with tumor necrosis factor (TNF), IL-1, and interferon-γ. They found that TNF-α is a selective substrate for dipeptidyl aminopeptidase (CD26) and tripeptidyl endopeptidase, whereas APN did not appear to participate in the degradation of any cytokine tested [22]. APN and CD26 are involved in cytokine-mediated T cell growth [23]. Dipeptidyl aminopeptidase (CD26) is suspected to attack X-Pro bonds in NH_2-terminal sequences such as those of IL-1, IL-2, IL-6, granulocyte colony-stimulating factor, TNF-β, and granulocyte-macrophage colony-stimulating factor [24]. The amino acid cleavage by APN is very slow at the X-Pro sequence or proline [24] APN does not cleave such cytokines because they contain proline in sequences of the NH_2-terminal region. APN is thought to be a specific protease for the cytokine IL-8. C5a is also an important chemotactic factor in inflammation sites. It is known that C5a-inactivating enzyme contains serosal fluid [15]. We did not examine C5a-inactivating protease in our assay system. As APN and C5a-inactivating protease may have similar characteristics, further comparative study is needed.

Taken together, our findings suggest that APN regulates IL-8 functions at many sites, such as on the cell surface, in the extracellular matrix, and in the serosal fluid. Further studies should provide precise details of the system regulating inflammation.

ACKNOWLEDGMENTS

We thank Prof. Kouji Matushima, Kanazawa University, for a gift of recombinant IL-8. We also thank Dr. Michio Fujie and Elizabeth Ichihara for helpful suggestions. This study was supported in part by Grants-in-Aid for Specific Research (no. 06454469) from the Japanese Ministry of Education.

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