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Generation of interleukin-8 by plasmin from AVLPR-interleukin-8, the human fibroblast-derived neutrophil chemotactic factor

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Plasmin mainly cleaved the Arg¹-Ser² bond of Arg-Val-Leu-Pro-Arg-interleukin-8 (AVLPR-IL-8) produced by human dermal fibroblasts, which resulted in the conversion of AVLPR-IL-8 to IL-8 and the inactive pentapeptide, though a minor cleavage of AVLPR-IL-8 by plasmin at Lys⁵-Glu⁶ bond occurred.

Interleukin 8; Fibroblast; Plasmin; Neutrophil chemotactic factor

1. INTRODUCTION

In recent years it has become clear that a novel neutrophil chemotactic factor, IL-8, is secreted by several types of cells including monocytes, fibroblasts and endothelial cells in response to IL-1 and tumor necrosis factor [1–6]. The IL-8 is a peptide consisting of 72 amino acid residues, and its amino acid sequence is identical to the deduced amino acid sequence of a fragment of the 3-10C cDNA sequence [7]. Recently it has been demonstrated that human fibroblasts stimulated with cytokines mainly produce AVLPR-IL-8 (FDNCF) which is the NH_2-terminal extended 77-residue variant of IL-8 [8–10]. We report here that plasmin, an important proteinase in the regulation of inflammation, mainly cleaves FDNCF to IL-8 and the inactive AVLPR pentapeptide.

2. MATERIALS AND METHODS

2.1. *Purification of FDNCF*

Human dermal fibroblasts (SF-TY cell line) were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 25 mM Hepes, penicillin (0.1 mg/ml) and streptomycin (0.1 mg/ml) until they reached confluence. The cells were cultured in serum-free culture medium supplemented with 10^{-10} M recombinant human IL-1β and 0.1% bovine serum albumin. After culture for 2 days, cell-free conditioned medium was obtained by centrifugation at 1600 × *g* for 20 min. The chemoattractant in the conditioned medium was purified essentially as described previously [11]; the sample was chromatographed sequentially on CM-Sephadex, Sephadex G-75 and RP-HPLC.

Abbreviations: AVLPR-IL-8, Ala-Val-Leu-Pro-Arg-interleukin-8; FDNCF, fibroblast-derived neutrophil chemotactic factor; RP-HPLC, reverse-phase high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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2.2. *Treatment of FDNCF with plasmin*

FDNCF (40 μg) in a final volume of 0.2 ml of 50 mM Tris-HCl buffer (pH 8.0) was incubated with 0.8 μg of plasmin (purified from human plasma; Sigma, MO, USA) at 37°C for 30 min, and the reaction was stopped by the addition of leupeptin (final 1 mM). The reaction mixture was concentrated by a Speed-Vac centrifuge, dissolved in 6 M guanidine solution and loaded onto a C-18 reverse-phase column (0.45 × 15 cm; ODS-120T, Tosoh Co., Tokyo, Japan). Chemotactic factors were eluted with a linear concentration gradient of acetonitrile from 0% to 50.4% in 0.05% trifluoroacetic acid at a flow rate of 0.8 ml/min.

Neutrophil chemotaxis of plasmin-treated FDNCF was performed in vitro by using rat and human neutrophils as described previously [12]. As an index of chemotaxis, the number of neutrophils migrated into the lower chamber was expressed as percentage (migration rate) of that of neutrophils applied in the upper chamber. SDS-PAGE was carried out on a slab gel of a discontinuous 9.6%/16.5% acrylamide in tricine buffer system [13].

2.3. *NH_2-terminal amino acid sequencing analysis*

NH_2-terminal amino acid sequence determination was performed by automated Edman degradation on a gas-phase protein sequencer (model 470A, Applied Biosystems, CA, USA) equipped with a PTH analyzer (model 120A HPLC system).

3. RESULTS AND DISCUSSION

Human dermal fibroblasts stimulated with 10^{-10} M IL-1 mainly produced FDNCF, which is the NH_2-terminal extended 77-residue variant of IL-8 (AVLPR-IL-8). The homogeneity of the purified FDNCF was confirmed by SDS-PAGE, its NH_2-terminal amino acid sequence and its COOH-terminal amino acid determination. We found that the purified FDNCF attracts human neutrophils, but rat neutrophils are not attracted by the FDNCF at concentrations less than 10^{-8} M. On the other hand, 10^{-8} M

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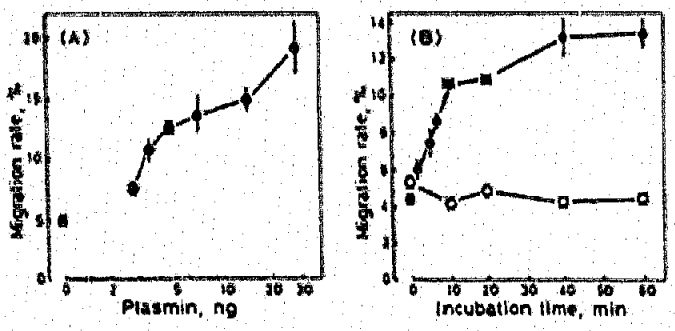

Fig. 1. (A) Rat neutrophil chemotactic activity of plasmin-treated FDNCF. FDNCF (1.4 μg) was incubated with the indicated amounts of plasmin at 37°C for 30 min. The reaction was stopped by addition of leupeptin (final concentration 1 mM). (B) Time course for rat neutrophil chemotactic activity of plasmin-treated FDNCF. (●) FDNCF (1.4 μg) was incubated with plasmin (30 ng) at 37°C for the indicated time and the reaction was stopped by addition of leupeptin (final concentration 1 mM), while as a control (○) plasmin was inactivated with leupeptin and then incubated with FDNCF. Rat neutrophil chemotactic activity of the reaction mixtures was measured at 3×10^{-8} M of the plasmin-treated FDNCF. Each point represents the mean 6 determinations. Horizontal bars indicate the SE.

IL-8 attracts both rat and human neutrophils. The results indicate that rat neutrophils are attracted by IL-8, but not by FDNCF at a concentration of 10^{-8} M. In the present studies, therefore, we used rat neutrophils at about 10^{-8} M concentration of attractants in chemotaxis assay to determine the conversion of FDNCF to IL-8 by plasmin.

When FDNCF was incubated with various amounts of plasmin, the reaction mixture dose-dependently increased the chemotactic activity for rat neutrophils (Fig. 1A). The chemotactic activity of the reaction mixture rapidly increased and reached a maximum within 40 min when FDNCF (1.4 μg) was incubated with plasmin (30 ng) at 37°C (Fig. 1B). The results suggest that IL-8 is rapidly formed from FDNCF by plasmin.

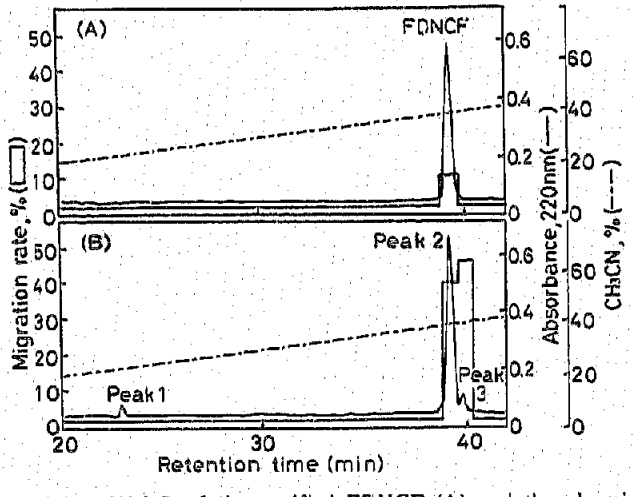

Fig. 2. RP-HPLC of the purified FDNCF (A) and the plasmin-treated FDNCF (B). Chemotactic activity of each fraction was assayed at 3×10^{-8} M in duplicate. Experimental conditions are described in section 2.

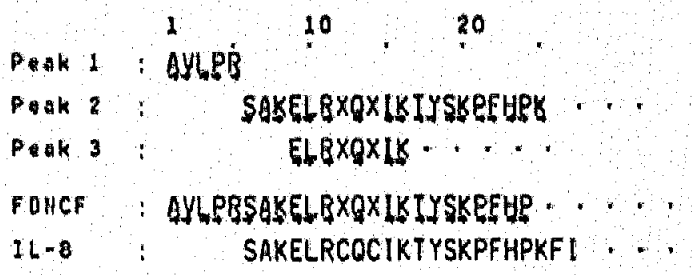

Fig. 3. NH_2-terminal amino acid sequences of peaks 1, 2 and 3 isolated from the plasmin-treated FDNCF by RP-HPLC (Fig. 2B). The amino acid residues identified by protein sequencer are marked by arrows under the residues. Partial sequences of IL-8 and the FDNCF purified from conditioned medium of SF-TY cell culture are shown.

The reaction products of plasmin-treated FDNCF were isolated by RP-HPLC to confirm the formation of IL-8. The RP-HPLC revealed one major peak (peak 2) and two minor peaks (peaks 1 and 3); the area of peak 2 was about 90% of total peak areas on RP-HPLC (Fig. 2B). Peaks 2 and 3 had chemotactic activity for rat neutrophils, but chemotactic activity was not found in peak 1 (Fig. 2B).

The peaks 1–3 obtained from the RP-HPLC were concentrated and loaded onto a gas-phase protein sequencer. NH_2-terminal amino acid sequences of peaks 1–3 are summarized in Fig. 3. The sequence of peak 1 is AVLPR, and the subsequential amino acid was not detected (Fig. 3). In addition, amino acid analysis of peak 1 showed that the peptide of peak 1 is constituted by Ala, Val, Leu, Pro and Arg in equal number of moles (data not shown). The results indicate that peak 1 is the pentapeptide, AVLPR.

NH_2-terminal amino acid sequence of peak 2 was identical to that of IL-8 (Fig. 3), and other sequences

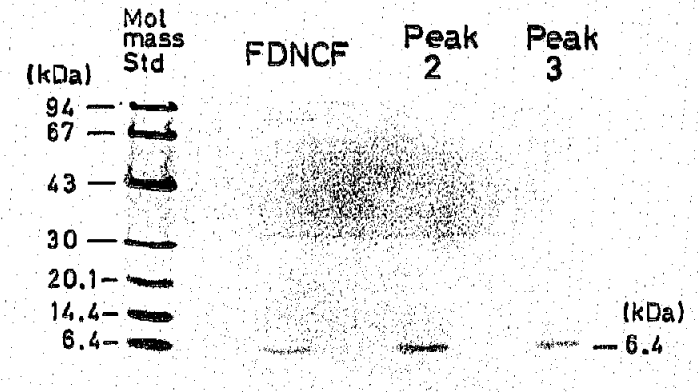

Fig. 4. SDS-PAGE of FDNCF, peak 2 and peak 3. Peaks 2 and 3 were isolated from the plasmin-treated FDNCF (Fig. 2B). The disulfide bonds of samples were reduced with 25% (v/v) 2-mercaptoethanol, and subjected to SDS-PAGE. The molecular mass standards (Std) were run simultaneously.

including FDNCF were not found in significant amounts by NH_2-terminal sequence analysis of peak 2, although FDNCF and IL-8 were eluted at the same retention time on RP-HPLC (Fig. 2A and B). The results suggest that FDNCF was almost completely converted to IL-8 by plasmin under the present conditions. The carboxypeptidase A treatment of peak 2 and FDNCF resulted in the release of the same amino acid, Ser which is identical to carboxy-terminal amino acid of IL-8 (data not shown). In addition, both the peak 2 and FDNCF revealed a similar molecular mass (about 6.4 kDa) on SDS-PAGE (Fig. 4). The results indicate that peak 2 is IL-8 with 72 amino acids. On the basis of NH_2-terminal amino acid sequence of peak 3 (Fig. 3) and the molecular mass of peak 3 which was similar to that of FDNCF (Fig. 4), it is probable that peak 3 is the NH_2-terminal truncated 69-amino acid variant of IL-8. The ratio of peak 2 (IL-8)/peak 3 was about 9:1 on the basis of the area of each peak on RP-HPLC (Fig. 2B), suggesting that plasmin mainly cleaves the Arg^5–Ser^6 bond, and the partial cleavage of Lys^8–Glu^9 bond may occur as a result of the secondary attack of plasmin. Plasmin did not cleave any other Arg–X and Lys–X bonds of FDNCF under the present experimental conditions. This stability of FDNCF except for NH_2-terminal portion may be due to the fact that IL-8 contains a triple stranded anti-parallel β-sheet structure and a long COOH-terminal helix [14,15].

It can be assumed that plasmin is generated from plasminogen by plasminogen activator which is secreted by the stimulated inflammatory cells including monocytes/macrophages and endothelial cells in the inflammatory region [16]. In the present study we have shown for the first time that plasmin is responsible for the limited proteolysis of FDNCF; plasmin can selectively cleave FDNCF to IL-8 and the inactive AVLPR pentapeptide, though a minor secondary reaction occurs.

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