A monoclonal antibody specific to the granulocyte-derived elastase-fragment D species of human fibrinogen and fibrin: its application to the measurement of granulocyte-derived elastase digests in plasma

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When granulocytes are stimulated under certain clinical conditions, elastase is released therefrom and digests fibrinogen independendy of the plasmin system, which may also be mobilized simultaneously. Thus, discrimination of these 2 systems becomes urgent for the diagnosis and treatment of the underlying diseases. Using as immunogen a 97-kd granulocyte-elastase digest of human fibrinogen, we raised an antibody IF-123 that specifically recognizes elastase digests of human fibrinogen. The 97-kd elastase fragment resembles plasmic fragment D1, and the epitope of this antibody is located on the Ao (196-204) residue segment. This segment appears to be masked in fibrinogen but exposed when the Ao Leu 204-Ile 205 peptide bond is cleaved by elastase. Cathepsin G concomitantly released from granulocytes failed to expose the epitope. By an enzyme immunoassay using IF-123 as the capture antibody, the elastase digests of fibrinogen can be measured in plasma samples without interference by abundantly coexisting fibrinogen. Indeed, we found that the elastase digests were mostly elevated in patients with inflammation or malignant tumors, but remained in normal range in patients with a benign gastrointestinal tract disease such as duodenal ulcer and polyps in the gallbladder or the colon. Like the plasmic D-dimer, the elastase digests predominately consisted of the DD/E complex and DD/E-containing high-molecular weight derivatives apparently corresponding to the phase-3 plasmic digests of cross-linked fibrin. (Blood. 2000;95:1721-1728)

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Introduction

Granulocyte-derived elastase (GE) is localized in azurophilic and specific granules of granulocytes and is released extracellularly in response to various stimuli such as endotoxin and cytokines.1 Released GE may degrade the components of the extracellular matrix, such as elastin and a variety of proteoglycans, and also plasma proteins including fibrinogen.2,3 GE has thus been implicated in the pathogenesis of a wide variety of diseases.

Granulocyte-derived elastase is regulated predominantly by α1-proteinase inhibitor (α1-PI) in vivo, although it is inhibited by several other inhibitors including α2-macroglobulin in vitro.4,5 The granulocyte-associated elastase, however, has been shown to be more resistant against α1-PI than free elastase.6,7 Like plasmin produced in blood of patients with certain diseases such as disseminated intravascular coagulation, GE may be released into the blood circulation and may digest fibrinogen and fibrin clots before being neutralized by α1-PI. Several lines of evidence have indicated that besides the major fibrinolytic protease plasmin, GE plays a role in fibrinogen degradation in vitro8-10 as well as in vivo.11-13 Of particular interest is that both plasmic and GE systems are often mobilized simultaneously, and discrimination of their fibrinogen degradation products becomes necessary to grasp the clinical conditions and to appropriately monitor their treatment. Based on these pieces of information, we have attempted to raise monoclonal antibodies specific to the GE digests of fibrinogen and to characterize and measure the GE digests in plasma from patients with a variety of diseases.

Materials and methods

Chemicals and reagents

All chemicals and reagents were purchased from commercial sources and used without purification unless otherwise specified.

Preparation of GE digests of human fibrinogen corresponding to plasmic fragment D1

A commercial human fibrinogen product (FIB-I: Enzyme Research Laboratories, South Bend, IN, about 2.6% [w/v] in 20 mmol/L citric acid-HCl/glycine, pH 7.4) was diluted to 1.0% [w/v] with 50 mmol/L Tris-HCl, pH 7.5, containing 0.15 mol/L NaCl (TBS) and 1.0 mmol/L disodium EDTA and applied onto a lysine-Sepharose 4B column (Pharmacia Biotech, Tokyo, Japan) to remove plasminogen and plasmin, if any. The passage-through fractions were pooled and treated with 2.0 mol/L urea to denature factor XIII, a trace contaminant. The fibrinogen fraction (20 mL) was digested with 2.0 mg human GE (purified from the human purulent sputum, and free of cathepsin G, myeloperoxidase, and reagents containing active proteases such as human trypsin, chymotrypsin, and elastase) in 200 mL TBS and brought to 10 mg/mL with the same buffer. The fibrinogen fraction was then dialyzed against TBS and brought to 10 mg/mL with the same buffer. The fibrinogen fraction (20 mL) was digested with 2.0 mg human GE (purified from the human purulent sputum, and free of cathepsin G, myeloperoxidase, and reagents containing active proteases such as human trypsin, chymotrypsin, and elastase) in 200 mL TBS and brought to 10 mg/mL with the same buffer. The fibrinogen fraction was then dialyzed against TBS and brought to 10 mg/mL with the same buffer. The fibrinogen fraction (20 mL) was digested with 2.0 mg human GE (purified from the human purulent sputum, and free of cathepsin G, myeloperoxidase, and reagents containing active proteases such as human trypsin, chymotrypsin, and elastase) in 200 mL TBS and brought to 10 mg/mL with the same buffer. The fibrinogen fraction was then dialyzed against TBS and brought to 10 mg/mL with the same buffer. The fibrinogen fraction (20 mL) was digested with 2.0 mg human GE (purified from the human purulent sputum, and free of cathepsin G, myeloperoxidase, and reagents containing active proteases such as human trypsin, chymotrypsin, and elastase) in 200 mL TBS and brought to 10 mg/mL with the same buffer. The fibrinogen fraction was then dialyzed against TBS and brought to 10 mg/mL with the same buffer. The fibrinogen fraction (20 mL) was digested with 2.0 mg human GE (purified from the human purulent sputum, and free of cathepsin G, myeloperoxidase, and reagents containing active proteases such as human trypsin, chymotrypsin, and elastase) in 200 mL TBS and brought to 10 mg/mL with the same buffer.
Preparation of GE digests of cross-linked fibrin

The human fibrinogen fraction was brought to 10 mg/mL in TBS containing 5.0 mmol/L NaCl, 1 mg/mL benzamidine HCl, and 1.0 mmol/L trans-4-aminoethyl-l-1-cyclohexanecarboxylic acid (t-AMCHA) for 2 hours at 37°C. This enzyme was also found to be free of trypsin-type and chymotrypsin-type enzymes (data not shown). The ratio of enzyme/substrate (wt/wt) was 1:100. To terminate the digestion, diisopropylfluorophosphate (DFP) was added to the reaction mixture at 1.0 mmol/L. The digests were applied onto a Sephacryl S-300 HR column (5.0 × 90 cm; Pharmacia Biotech), and the fractions containing a 97-kd fragment corresponding to plasmic fragment D1 were collected and pooled. We tentatively designated this 97-kd fragment as GE-D.

Preparation of GE digests of cross-linked fibrin

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Preparation of plasmic degradation products

Plasmic degradation products of human fibrinogen were prepared essentially as described elsewhere. The degradation products were found to contain lysozyme, Elastin Products, Owensville, MO) in the presence of 5.0 mmol/L NaCl, 1.0 mmol/L benzamidine HCl, and 1.0 mmol/L trans-4-aminoethyl-l-1-cyclohexanecarboxylic acid (t-AMCHA) for 2 hours at 37°C. This enzyme was also found to be free of trypsin-type and chymotrypsin-type enzymes (data not shown). The ratio of enzyme/substrate (wt/wt) was 1:100. To terminate the digestion, diisopropylfluorophosphate (DFP) was added to the reaction mixture at 1.0 mmol/L. The digests were applied onto a Sephacryl S-300 HR column (5.0 × 90 cm; Pharmacia Biotech), and the fractions containing a 97-kd fragment corresponding to plasmic fragment D1 were collected and pooled. We tentatively designated this 97-kd fragment as GE-D.

Preparation of plasmic degradation products

Plasmic degradation products of human fibrinogen were prepared essentially as described. The degradation products were found to contain fragments X, Y, D1A, D1, and E3. When necessary, fragment D1 and phase-3 digests of cross-linked fibrin were prepared as described elsewhere.22 The amounts of bound antibodies were calculated from microtiter plates coated with either GE-D or GE-XDP as described previously.22 The amounts of bound antibodies were calculated from calibration curves constructed with known amounts of the bound reactive antibodies. The dissociation constant was calculated as described previously.23

Immunoblotting

Reactivity of IF-123 to various antigens was analyzed by immunoblotting essentially as described elsewhere.24 The separation of α-chain remnants of GE-D bound to IF-123 and their digestion with lysyl endopeptidase

Preparation of GE digests of fibrinogen

We inoculated Balb/c mice with 50 µg of GE-D in complete Freund’s adjuvant followed by 50 µg of GE-D without the adjuvant as immunogen essentially by a hybridoma technique of Köhler and Milstein25 with minor modifications as described elsewhere.25 Selection of clones secreting monoclonal antibodies (mAbs) specific to the GE digests of fibrinogen, but not to the parent molecule fibrinogen or its plasmic digests was carried out by a direct-binding enzyme-linked immunosorbent assay (ELISA) using GE-D, plasmic fragment D1, and fibrinogen as antigens.

Binding of mAbs to antigens determined by a direct-binding ELISA

Binding between the antigens and mAbs was studied by a direct-binding ELISA as described previously.22 Briefly, wells of polystyrene microtitre plates (Immuron-II, Dynatech, Chantilly, VA) were coated overnight at 4°C with 50 µL of respective antigens at 5 µg/mL in 50 mmol/L Tris-HCl, pH 8.5. The antigen-coated wells were washed with 0.15 mol/L NaCl containing 0.05% (w/v) Tween-20 (NaCl-Tween) and incubated with 50 µL of the culture supernatant for 1 hour at 25°C. After decantation of the reaction mixture, the wells were washed with NaCl-Tween. Horseradish peroxidase (HRPO)-conjugated antimouse IgG rabbit antibody (DAKO, Glostrup, Denmark) diluted 200-fold with 50 mmol/L Tris-HCl, pH 8.0, containing 0.15 mol/L NaCl and 0.05% (w/v) Tween-20 was added to each well as the second antibody. The bound antibodies were determined using 50 mmol/L Tris-HCl, pH 7.5, containing 0.5 mmol/L 4-aminonitrophenyl, 10 mmol/L phenol, and 0.005% hydrogen peroxide as substrate, and the color produced was read at 492 nm on an MPR-A4i Microplate reader (Tosoh, Tokyo, Japan).

Inhibition by synthetic peptides of the binding of IF-123 to immobilized affinity-purified GE-D or a synthetic 13-residue peptide corresponding to Aα (192-204)

When the separated peptides were tested for binding with IF-123, only a single peptide, named peptide #19, was found to react with the antibody in a direct-binding ELISA. Its full sequence was determined by a protein sequencer, model 476A (Applied Biosystems, Foster City, CA), and assigned to the Aα (192-204) residue segment. Wells of polystyrene microtitre plates were coated with 50 µL of 4 mmol/L affinity-purified GE-D or 40 mmol/L synthetic peptide with a sequence of Aα (192-204), Asp-Leu-Leu-Phe-Ser-Arg-Asp-Arg-Gln-His-
Leu-Pro-Leu, tentatively named s-peptide 19, in 50 mmol/L Tris-HCl, pH 8.5, for 16 hours at 4°C. The antigen-coated wells were washed 3 times with NaCl-Tween and incubated with IF-123, which had been mixed with various concentrations of either one of the synthetic peptides (TANA Laboratories, Houston, TX) in TBS-Tween for 1 hour at 25°C. The bound antibody was detected as described above.

Preparation of protease(s) released from activated granulocytes

To release the enzymes stored in granules, the granulocytes (5.7 x 10^7 cells) isolated from 30 mL of heparinized venous blood obtained from healthy volunteers described elsewhere,25 were preincubated for 3 minutes at 37°C with 5 µg/mL of cytochalasin B (Sigma) in the presence of 1.3 mmol/L CaCl_2 and 1.0 mmol/L MgCl_2, and activated with 500 mmol/L N-formyl-Met-Leu-Phe (FMLP, Sigma) for 5 minutes at 37°C. After centrifugation, the supernatant was collected and passed through a cellulose acetate membrane filter (0.2 µm pore, Advantec, Tokyo, Japan). The concentrations of GE and cathepsin G in the supernatant were measured by spectrophotometric assays using MeO-Suc-Ala-Ala-Pro-Val-NA and Suc-Ala-Ala-Pro-Phe-NA (Sigma) as substrates, respectively.26 Concentrations of GE and cathepsin G were calculated from calibration curves constructed with known amounts of the commercially available purified GE and cathepsin G (purified from human purulent sputum; elastase, myeloperoxidase, lysozyme free; Elastin Products).

Characterization of GE digests by immunoprecipitation with IF-123–conjugated Sepharose 4B

Purified IgG fractions of IF-123 and JIF-23,27 an mAb that specifically recognizes the amino-terminal disulfide-linked conformation of the plasmic fragment D species (D1, D2, and D3), were individually coupled to CNBr-activated Sepharose 4B (Pharmacia Biotech) according to the manufacturer’s instruction, and the IgG-conjugated Sepharose 4B was suspended in an equal volume of 50 mmol/L Tris-HCl, pH 8.0, containing 0.15 mol/L NaCl and 0.05% sodium azide. One hundred microliters of either purified GE digests (10 µg of GE digests of fibrinogen and GE-XDP) or patient-derived plasma samples were incubated with 200 µL of the gel suspension and 1 mL of 20 mmol/L Tris-HCl, pH 7.6, containing 0.5 mol/L NaCl and 0.05%(w/v) Tween-20 (suspension buffer) for 16 hours at 4°C, and the mixture was centrifuged at 10 000g for 3 minutes at 25°C. The gels precipitated were washed and washed 5 times with the same buffer, each time by rotating the tubes for 20 minutes at 25°C, and then centrifuged at 10 000g for 3 minutes at 25°C. The precipitate was resuspended at 1.0 mL of 20 mmol/L Tris-HCl, pH 7.6, containing 0.15 mol/L NaCl and 2% SDS to solubilize the proteins bound to the antibody-coupled gels. Proteins in 0.2 mL of the supernatant after centrifugation at 10 000g for 5 minutes at 25°C were isolated by acetone precipitation and subjected to SDS-PAGE using 3.5% to 9.0% gradient gels under nonreducing conditions as described elsewhere.24 The proteins were visualized by silver staining.

Measurement of GE digests of fibrinogen and fibrin in clinical samples by a sandwich ELISA

Wells of polystyrene microtiter plates were coated overnight at 4°C with 20 µg/mL Fab(1)2 fragment of IF-123 in 50 mmol/L Tris-HCl, pH 8.5, and 50 fold-diluted plasma samples in 50 mmol/L Tris-HCl, pH 8.0, containing 0.05%(w/v) Tween-20 and 1 mol/L urea were allowed to react with the immobilized Fab(1)2 fragment of IF-123 for 1 hour at 25°C. After decantation of the reaction mixture, the wells were washed with NaCl-Tween, and HRPO-conjugated antihuman fibrinogen rabbit antibody (DAKO) was added to each well as the second antibody. The amounts of bound antibodies were determined by reading A_492 on an MPR-A4i Microplate reader using 4-aminoantipyrine-phenol-hydrogen peroxide as substrate. Proteins specifically bound to IF-123 were determined on a calibration curve constructed with pooled normal plasma spiked with known amounts of GE-XDP.

Statistical analysis

To compare the levels of GE digests in plasma between patients and the control, Welch’s t test was used. A P value of less than 0.05 was considered significant.

Results

Characterization of an mAb specific to GE digests of human fibrinogen and cross-linked fibrin

Among the mAbs thus prepared, there was an antibody that specifically reacted with GE-D but not with fibrinogen or its plasmic D1, when analyzed by a direct-binding ELISA. The reactivity of this antibody IF-123 was independent of calcium ions, although the fibrinogen D domain contains a high-affinity calcium binding site29,30 (data not shown). This antibody was classified into IgG2 with k-type light chains. When analyzed by immunoprecipitation followed by SDS-PAGE, GE-D and GE-XDP were adsorbed to IF-123 (Figure 1A, GE-D and GE-XDP in lanes 5 and 6, respectively). Fibrinogen, its plasmic digests, or plasmic XDP were not adsorbed (Figure 1B, lanes 4-6). The dissociation constants of IF-123 with GE-D and GE-XDP were 1.20 x 10^-9 mol/L and 1.23 x 10^-9 mol/L, respectively.

Epitope mapping for IF-123

By immunoblotting run under reducing conditions, neither fibrinogen nor plasmic D1 was stained (Figure 2B, lanes 1 and 2), whereas 2 α-remnant species of GE-D were stained with this antibody (Figure 2B, lane 3). The epitope was thus localized to an approximately 12-kd Aco-chain–derived segment specifically cleaved by GE. The 12-kd fragment purified (Figure 3A, Pe-α/GE-D) and their lysyl endopeptidase digests were fractionated by reverse-phase HPLC on a Cosmosil 5C18SP column (Figure 3B). Separated peak fractions were coated onto immobilplates and their reactivities with IF-123 were examined. Among them, a fraction denoted by peak #19 (Figure 3B) reacted with IF-123. By sequence analysis, we assigned this peptide to the Aco domain of GE-D and the lysyl endopeptidase hydrolyzes specifically the carboxyl side bond of lysyl residues,31 it is very likely that peptide #19 lacking the carboxyl-terminal Lys residue constitutes the carboxyl-terminal segment of the α-remnant of GE-D. To confirm that the epitope resides in...
peptide #19, we conducted inhibition assays by using ELISAs, where the affinity-purified GE-D and a synthetic peptide with the same sequence as peptide #19, s-peptide 19, were individually immobilized, and binding of IF-123 was tested in the presence of various synthetic analogs of peptide #19. Two synthetic peptides, s-peptide 19 and a peptide analog, Ser 196-Leu 204, lacking the first 4 amino acids of s-peptide 19 were able to inhibit the binding of IF-123 to the affinity-purified GE-D (Figure 4A) as well as to s-peptide 19 (Figure 4B) in the same manner. When the first 3 Ser-Arg-Asp residues of the (Ser 196-Leu 204) peptide had been removed, binding with IF-123 decreased nearly two magnitudes (Figure 4A and B). On the other hand, deletion of Leu 204 from or addition of Ile 205 or Ile 205-Lys 206 to the (Ser 196-Leu 204) peptide resulted in complete loss of binding with IF-123 (Figure 4A and B). The results together suggested that the Arg (Ser 196-Leu 204) residue segment functioned as the epitope for IF-123, and that its carboxyl-side residues were critical for full expression of the epitope, as schematically shown in Figure 5.

Expression of the epitope by proteases released from activated granulocytes

When granulocytes (10^7 cells/mL) had been stimulated with 500 nmol/L fMLP, they were estimated to release GE and cathepsin G

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**Figure 2. Immunoblot analyses.** Immunoblot analyses were run under reducing conditions for fibrinogen and its plasmic and GE-digests with an antihuman fibrinogen rabbit antibody (A) and IF-123 (B). Lane 1, fibrinogen; lane 2, plasmic fragment D1; and lane 3, GE-D.

**Figure 3. Reverse-phase HPLC.** Separation of the α-remnants of GE-D (A) and its lysyl endopeptidase-digests (B) by reverse-phase HPLC. Only peptide #19 was reactive to IF-123.

**Figure 4. Synthetic peptide inhibition.** Inhibition by synthetic peptides Aα (192-204), Aα (196-204), and their analogs of binding of IF-123 to immunoaffinity-purified GE-D (A) and the Aα (192-204) peptide corresponding to peptide #19 (B). Synthetic peptides correspond to Aα (192-204) (□), Aα (196-204) (○), Aα (199-204) (□), Aα (196-203) lacking Leu 204 (□), Aα (196-205) linked with Ile 205 (△) and Aα (196-206) linked with Ile 205-Lys 206 (▲).
at the concentration of 610 nmol/L and 56 nmol/L, respectively. These proteases were tested for digestion of fibrinogen with or without prior treatment with various protease inhibitors. When the supernatant of activated granulocytes had been treated with a mixture of Z-Gly-Leu-Phe-CH2Cl, Nα-tosyl-L-lysine chloromethylketone (TLCK), and Nα-tosyl-L-phenylalanine chloromethylketone (TPCK), inhibitors of cathepsin G, trypsin-type enzymes, and chymotrypsin-type enzymes, respectively, profiles of fibrinogen degradation and appearance of the reactivity with IF-123 (Figure 6B) were nearly identical with those for the nontreated supernatant (Figure 6A) as examined by SDS-PAGE and a sandwich ELISA. When the supernatant had been treated with a GE-specific inhibitor, MeO-Suc-Ala-Ala-Pro-Val-CH2Cl, fibrinogen was degraded gradually, but none of the degradation products reacted with IF-123 (Figure 6C).

Effects on the epitope expression of plasmic digestion of GE-XDP and GE digestion of plasmic XDP

To see whether plasmic digestion of GE-XDP and GE digestion of plasmic XDP affect the structure required for the epitope expression, we digested GE-XDP with plasmin and plasmic XDP with GE, both at 1:1000 of the enzyme/substrate ratio (wt/wt), and examined the reactivity of the digests to IF-123 by the immunoprecipitation method. Although GE-XDP was converted by plasmin to the phase-4 digests (Figure 7A, lanes 1-4), the epitope for IF-123 was retained in the digests containing the fragment D components (Figure 7A, lanes 5-8). Digestion of plasmic XDP with GE also yielded the phase-4 digests (Figure 7B, lanes 1-4), and the new epitope was expressed at later stages, where plasmic DD and DY had been further degraded to smaller fragments DD and DY (Figure 7B, lanes 7 and 8, indicated by arrowheads).

Measurement of GE digests in plasma by a sandwich ELISA

By a sandwich ELISA, we were able to measure GE-D and GE-XDP spiked in plasma up to 80 µg/mL without any interference.
by fibrinogen (Figure 8, open and closed circles, respectively). When plasma samples, 282 in total, derived from patients with a variety of diseases were subjected to measurement of plasmic fragment D species and GE-digests by ELISAs using JIF-23 and IF-123, respectively, there was a moderate correlation, \( r = 0.652 \), between these 2 fibrin(ogen) digests (Figure 9). However, they were found to be independent entities, as shown by 3 representative examples: (a) GE-digests were markedly elevated but plasmic digests were low; (b) both GE and plasmic digests were elevated; and (c) GE digests were low but plasmic digests were elevated. By immunoprecipitation analysis, multiple protein fractions were abundantly precipitated with IF-123 from examples a and b (Figure 10A, lanes 2 and 3), but only a little from example c (Figure 10A, lane 4). On the contrary, only small amounts of proteins were precipitated with JIF-23 from example a (Figure 10B, lane 2), whereas considerable amounts of multiple proteins were precipitated from examples b and c (Figure 10B, lanes 3 and 4).

When the GE digests were measured in plasmas derived from various diseases, including acute promyelocytic leukemia (APL), sepsis, systemic lupus erythematosus (SLE), and benign gastrointestinal tract diseases, they were highly elevated in APL and sepsis, and moderately elevated in SLE (Figure 11). No significant increase was observed in benign gastrointestinal tract diseases. The GE digests in 23 normal healthy men and women between 22 and 48 years of age ranged from 0 to 0.70 \( \mu \text{g/mL} \) (mean \( \pm \text{SD:} \) 0.30 \( \pm \) 0.17 \( \mu \text{g/mL} \)).

**Discussion**

Granulocytes are known to release intrinsic proteolytic enzymes including elastase and cathepsin G in a variety of pathologic conditions.\(^3\) In fact, they may degrade the tissue-constituent including elastase and cathepsin G in a variety of pathologic diseases. Granulocytes are known to release intrinsic proteolytic enzymes such as elastin and a variety of proteoglycans, and also plasma proteins including fibrinogen and fibrin.\(^4\)-\(^7\),\(^13\) Although a variety of proteases may be released, cathepsin G, the other major granulocyte-derived protease, or trypsin-type and chymotrypsin-type proteases if any, were not to affect the epitope expression in the GE digests of fibrinogen and fibrin (see Figure 6). Degradation of fibrinogen and fibrin by GE was distinct from degradation of those by plasmin,\(^7\)-\(^13\) and discrimination of their fibrin(ogen) degradation products have been attempted by SDS-PAGE\(^10\) and immunochemical techniques.\(^9\),\(^13\) In this study, we raised an mAb IF-123 that recognized the \( \alpha \)-remnants of GE-D, but not fibrinogen nor its plasmic fragment D. By HPLC analysis of GE-D, and inhibition studies by ELISAs using a variety of peptide analogs, the epitope was localized within the \( \alpha \) carboxyl-terminal 204-204 residue segment (Figures 4 and 5). Although removal of the amino-terminal 3 residues of this peptide decreased the reactivity to IF-123 significantly by 2 magnitudes, deletion of the carboxyl-terminal Leu 204, and addition of Ile 205 and Ile 205-Lys 206 resulted in complete loss of the reactivity. Thus, for eliciting the epitope, a full sequence of the \( \alpha \) carboxyl-terminal 196-204 residues and its local conformation seems to be needed, and its carboxy-terminal portion appears to be critical. In a crystal structure study on tryptic fragment D of human fibrinogen,\(^33\) the \( \alpha \) 196-197 residues, exactly corresponding to the amino-terminal dipeptide of the epitope (Figure 5), were not appropriately positioned, however. Furthermore, the hydrophobicity at the carboxy-terminal residue of this segment seems to contribute to the local conformation needed for the epitope as observed by partial inhibition of the reaction to IF-123 by peptide analogs with either a Val or a Phe residue at their carboxy-terminal (profiles not shown). The \( \alpha \) Lys 206 is a potential P1-site for plasmic cleavage of the \( \alpha \) chain to form the fragment D species,\(^32\) and the carboxy-terminal 404 residues, \( \alpha \) Met 207-Val 610, are proposed to compose the flexible polar protuberance.\(^34\) Therefore, the epitope for IF-123 seems to be situated very close to the carboxy-terminal globular D domain, but is still masked even though the carboxy-terminal flexible protuberance is cleaved by plasmin at the \( \alpha \) Lys 206-Met 207 peptide bond. Only when the \( \alpha \) 204-205 peptide linkage of the fibrinogen \( \alpha \) chain is cleaved directly by GE, or when the carboxy-terminal 2 Ile 205-Lys 206...
residues of plasmic fragment D are removed by GE, the epitopic segment becomes accessible to IF-123 (Figure 4A and B and Figure 5). As shown in an experiment, in which the plasmic phase-3 digests were further degraded by GE to smaller phase-4 digests, the reactivity to IF-123 was clearly demonstrated in at least 2 bands (Figure 7B, lanes 7 and 8, indicated by arrowheads). Although not shown in results, sequence analysis of the first 3 cycles of Pe-α/GE-D revealed the presence of 4 amino-termini to be assigned to Ala Ser 100, Asn 102, Tyr 108, and Ser 112. Thus, the 2 bands that reacted with IF-123 on immunoblotting are likely to have these amino acids at their amino-termini. Conversely, when the GE-XDP was further digested with plasmin, the epitope was retained in all the D- and DD-containing segments (see Figure 7A). These data altogether indicate that the epitope is buried in fibrinogen and plasmic fragments D and XDP, and is exposed when the αα Leu 204-Ile 205 peptide bond is cleaved by GE either directly from fibrinogen and fibrin, or from plasmic fragments D and XDP, which may have been produced prior to digestion by GE. Therefore, GE digests and plasmic digests were certainly distinct entities, even though they may be produced simultaneously under many disease conditions (Figure 9). Furthermore, GE digests of cross-linked fibrin resembled the plasmic phase-3 digests consisting of DD/E and much higher molecular weight fragments (Figure 10). Therefore, discrimination of these 2 species of fibrinogen/fibrin degradation products will allow us to grasp the patient’s condition more accurately and to respond more quickly and appropriately than monitoring by plasmic fragments alone in terms of fibrinogen/fibrin degradation products.

Although the diseases tested are still limited at this stage of this investigation, we found that the level of GE-D species, that is, GE-D and GE-XDP, were increased significantly in APL and sepsis, and moderately in SLE. On the other hand, the level of GE-D species was in the normal range in patients with a benign gastrointestinal tract disease. To comprehend clinical implications of the plasma level of GE-D species in relation to diseases, and also to evaluate the proposed assay system using IF-123, we must await further investigation.

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References


