

Molecular Intercommunication between the Complement and Coagulation Systems

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The complement system as well as the coagulation system has fundamental clinical implications in the context of life-threatening tissue injury and inflammation. Associations between both cascades have been proposed, but the precise molecular mechanisms remain unknown. The current study reports multiple links for various factors of the coagulation and fibrinolysis cascades with the central complement components C3 and C5 *in vitro* and *ex vivo*. Thrombin, human coagulation factors (F) XIa, Xa, and IXa, and plasmin were all found to effectively cleave C3 and C5. Mass spectrometric analyses identified the cleavage products as C3a and C5a, displaying identical molecular weights as the native anaphylatoxins C3a and C5a. Cleavage products also exhibited robust chemoattraction of human mast cells and neutrophils, respectively. Enzymatic activity for C3 cleavage by the investigated clotting and fibrinolysis factors is defined in the following order: FXa > plasmin > thrombin > FIXa > FXIa > control. Furthermore, FXa-induced cleavage of C3 was significantly suppressed in the presence of the selective FXa inhibitors fondaparinux and enoxaparin in a concentration-dependent manner. Addition of FXa to human serum or plasma activated complement *ex vivo*, represented by the generation of C3a, C5a, and the terminal complement complex, and decreased complement hemolytic serum activity that defines exact serum concentration that results in complement-mediated lysis of 50% of sensitized sheep erythrocytes. Furthermore, in plasma from patients with multiple injuries ($n = 12$), a very early appearance and correlation of coagulation (thrombin-antithrombin complexes) and the complement activation product C5a was found. The present data suggest that coagulation/fibrinolysis proteases may act as natural C3 and C5 convertases, generating biologically active anaphylatoxins, linking both cascades via multiple direct interactions in terms of a complex serine protease system. *The Journal of Immunology*, 2010, 185: 000–000.

Traditionally, the complement and coagulation systems are described as separate cascades. As descendants of a common ancestral pathway, both proteolytic cascades are composed of serine proteases with common structural characteristics, such as highly conserved catalytic sites of serine, histidine, and aspartate (1, 2). Furthermore, both systems belong to a complex inflammatory network (3) and exhibit some similar charac-

teristics regarding the specialized functions of their activators and inhibitors. In particular, the clotting factor [human coagulation factor (F)] XIIa can activate the complement factor C1r and thereby initiate the classical pathway of complement activation. In turn, the C1 esterase inhibitor suppresses not only all three established complement pathways (classical, lectin, and alternative), but also the intrinsic coagulation cascade (kallikrein, FXIIa) (4, 5). Recently, it has been shown that thrombin is capable of generating the complement activation product C5a in the absence of C3 (6). In another study, Clark et al. (7) suggested that thrombin and plasmin may contribute to nontraditional complement activation during liver regeneration even in the absence of C4 and during inhibition of factor B. Thrombin may also act as a physiological agonist of the protein kinase C-dependent regulation of the complement decay-accelerating factor and thereby may provide a negative-feedback loop helping to prevent thrombosis during inflammation (8). In the setting of systemic inflammation, activation of the coagulation cascade is accompanied by a profound activation of the complement system, resulting in the generation of the anaphylatoxins C3a and C5a (9, 10). According to a previous report, C5a induces tissue factor (TF) activity in human endothelial cells (11) and may therefore be involved in the activation of the extrinsic coagulation pathway. Furthermore, C5a has been shown to stimulate the expression of TF on neutrophils via the C5aR, which was associated with a higher procoagulant activity (12). Additional evidence of procoagulant effects by complement components has been provided by a recent report demonstrating *in vitro* that mannan-binding lectin-associated serine protease 2 of the lectin pathway was capable of promoting fibrinogen turnover by cleaving prothrombin into thrombin (13).

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Abbreviations used in this paper: aPC, activated protein C; CH50, complement hemolytic serum activity that defines exact serum concentration that results in complement-mediated lysis of 50% of sensitized sheep erythrocytes; DPBS, Dulbecco's PBS; F, human coagulation factor; MAC, membrane attack complex; MBL, mannan-binding lectin; MS, mass spectrometry; PK, prekallikrein; TAT, thrombin-antithrombin complex; TCC, terminal complement complex; TF, tissue factor.

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In 1986, Sims et al. (14) showed that the terminal complement complex (TCC; C5b-9) can catalyze prothrombin cleavage to thrombin even in the absence of FV and thereby specifically increase platelet prothrombinase activity. In contrast, C5a has been described as having fibrinolytic effects by upregulating the plasminogen activator inhibitor I expression in the human mast cell line HMC-1 (15).

Thus, it is now becoming clear that both cascades may interact on a much larger scale than previously anticipated (16, 17). However, many of the underlying molecular mechanisms remain poorly understood. As indicated above, several factors of the coagulation/fibrinolysis cascade and components of the complement system display similar serine protease properties. In the current study, we hypothesized that various serine protease components of the clotting/fibrinolytic cascade directly cleave complement proteins, challenging the classic dogma that the two systems are separate cascades, and propose a model of a complex serine protease network.

Materials and Methods

Reagents

Unless stated otherwise, reagents were purchased from Sigma-Aldrich (Taufkirchen, Germany). Purified human C3 and C5 were obtained from Quidel (San Diego, CA). Human coagulation factors (F) VII, VIIa, IXa, Xa, Xia, and (activated) protein C were purchased from Calbiochem (Darmstadt, Germany). rFX, rFXI, and rTF were acquired from R&D Systems (Wiesbaden, Germany). Sodium enoxaparin and sodium fondaparinux were obtained from Sanofi-Aventis (Frankfurt, Germany).

In vitro cleavage of C3 and C5

In vitro experiments were performed by incubating native C3 (100 µg/ml) or C5 (100 µg/ml) in Dulbecco's PBS (DPBS) in the absence or presence of various coagulation factors [FXa, rFX, FXIa, rFXI, FIXa, FVIIa, FVII, FVIII, TF, protein C, activated protein C, thrombin (FIIa), plasmin and plasminogen] at 37°C in a dose- and time-dependent manner, followed by ELISA and Western blot analyses. Experiments were repeated in the absence or presence of selective FXa inhibitors (sodium enoxaparin and sodium fondaparinux). Human serum and plasma was incubated for 90 min at 37°C with FXa (ranging from 0–100 µg/ml) and assessed for C3a and C5a production as well as the assembly of TCC using Western blot and ELISA analysis.

Western blot analysis for C3a and C5a

Samples and controls were separated by SDS-PAGE under reducing conditions and transferred onto a polyvinylidene fluoride membrane (Schleicher & Schuell, Keene, NH). The blots were incubated overnight at 4°C with 1:1000 polyclonal rabbit anti-human C3a IgG (Calbiochem) or 1:1000 rabbit anti-human C5a IgG (Calbiochem). After washing, membranes were incubated for 1 h using alkaline phosphatase-conjugated goat anti-rabbit IgG (1:5000; Jackson ImmunoResearch Laboratories, West Grove, PA). For development, alkaline phosphatase substrate color development buffer (Bio-Rad, Hercules, CA) was used.

ELISA analysis of C3a, C5a, and TCC (C5b-9)

For quantitative analysis of C3a, C5a, and TCC (C5b-9), commercially available ELISA kits (Quidel and DRG Diagnostics, Marburg, Germany) were used according to the manufacturers' instructions.

Chemotaxis assay

HMC-1 (human mast cell line) and neutrophil chemotaxis assays were performed as previously described (18). Briefly, HMC-1 cells were fluorescein-labeled with 2', 7'-bis (2-carboxyethyl)-5-(and 6)-carboxy-fluorescein acetoxymethyl ester (Molecular Probes, Eugene, OR) and a chemotaxis assay was performed using a 5-µm porosity filter (NeuroProbe, Gaithersburg, MD). Labeled HMC-1 cells (5×10^6 cells/ml) were loaded into the upper chamber of a 96-well device (NeuroProbe) with C3 in the absence or presence of increasing concentrations of coagulation/fibrinolysis factors in the lower chamber. Glycosylated human C3a (100 ng/ml) served as a positive control.

Human neutrophils were isolated from whole blood of healthy human volunteers (approved by the Independent Ethics Committee of the University of Ulm, Ulm, Germany, No. 44/06, following written informed

consent of all individuals). Whole blood was drawn from the antecubital vein into syringes containing the anticoagulant citrate dextrose (1:10; Baxter Health Care, Deerfield, IL). Neutrophils were isolated using Ficoll-Paque gradient centrifugation (Pharmacia Biotech, Stockholm, Sweden) followed by a dextran sedimentation step. After hypotonic lysis of residual RBCs, neutrophils were resuspended in HBSS and fluorescein labeled with 2', 7'-bis (2-carboxyethyl)-5-(and 6)-carboxy-fluorescein acetoxymethyl ester (Molecular Probes) for 30 min at 37°C. Labeled neutrophils (5×10^6 cells/ml) were loaded into the upper chamber of a 96-well device (NeuroProbe) and separated by a polycarbonate filter with a porosity of 3 µm (NeuroProbe). The lower chambers were loaded with recombinant human C5a (100 ng/ml, positive control) or C5 in the absence or presence of increasing concentrations of coagulation/fibrinolysis factors. Postincubation at 37°C for 30 min, the number of cells that had migrated through the polycarbonate membrane was determined by cytofluorometry (Cytofluor II, Per Septive Biosystems, Framingham, MA).

Mass spectrometry analysis by MALDI-TOF

Deglycosylation by PNGase F. PNGase F was purchased from New England BioLabs (Ipswich, MA). For glycan cleavage, the procedure followed the product's guide. Protein samples were reduced with 5 mM DTT for 45 min at 56°C. Afterwards, 1 µl enzyme PNGase F was added to the samples, and the solutions were incubated at 37°C overnight. After desalting and enrichment by C18 Ziptips (Millipore, Billerica, MA), the deglycosylated proteins were ready for mass spectrometry (MS) analysis.

MALDI-TOF MS analysis. Protein samples were analyzed by MALDI-TOF. Spectra were acquired on a MALDI-Micro MX (Waters, Milford, MA) in linear mode. The instrument is equipped with an N2 UV laser emitting at 337 nm, a pulsed ion extraction source, an electrostatic reflectron of 2.3 m effective path length, a 2 GHz 8-bit transient analog to digital converter with real time peak display and fast dual microchannel plate detectors. All sample solutions were mixed in a 1:2 volume ratio with a matrix solution (10 mg/ml sinapinic acid in 4:6 acetonitrile/0.1% trifluoroacetic acid). Then, 0.8 µl each sample matrix mixture was spotted on the target plate and allowed to dry under moderate vacuum for 1 min. Thirty single-shot mass spectra were summed to give a composite spectrum. All data were reprocessed using Waters MassLynx software (Waters). The mass scale was calibrated externally using a defined peptide mixture (insulin, cytochrome-c, hemoglobin, myoglobin, and trypsinogen).

Complement hemolytic serum activity

Hemolytic activity of human serum in the absence and presence of FXa (100 µg/ml) was assessed as previously described (19, 20). Briefly, sheep erythrocytes (Oxoid, Wesel, Germany) were sensitized with hemolysin (Colorado Serum Company, Denver, CO) and exposed to dilutions of serum samples in TBS (pH 7.35, 37°C, 60 min). The complement reaction was stopped by the addition of ice-cold TBS followed by a centrifugation step (700 × g, 5 min). Absorption values of the supernatant fluids were determined by spectrophotometry at 541 nm. The complement hemolytic serum activity (CH50) defines the exact serum concentration that results in complement-mediated lysis of 50% of sensitized sheep erythrocytes.

Measurement of thrombin–antithrombin complexes and C5a early after multiple injury in humans

Twelve patients after multiple injury (10 males, 2 females, median age: 38 y, range: 19–74 y) with a median injury severity score of 48 (range: 25–60) as defined by the Consensus Criteria were enrolled in the study in accordance to the Independent Local Ethics Committee of the University of Ulm (approval number 44/06). For all patients, informed consent was obtained. If the patient was incapable of making decisions because of sedation or altered mental status, informed consent was obtained postrecovery. Blood was drawn within 1 h postinjury on admission to the emergency room at the University Hospital Ulm, and the thrombin–antithrombin complex (TAT) as well as the anaphylatoxin C5a concentrations were determined (see above). Plasma levels of TAT were measured using a sandwich ELISA. TAT was captured in wells coated with anti-human thrombin, and HRP-coupled anti-human antithrombin Ab was used for detection (both Abs from Enzyme Research Laboratories, South Bend, IN). A standard prepared by diluting pooled human serum in normal citrate-phosphate-dextrose plasma was used. The standard was calibrated using TAT complexes produced from purified thrombin and antithrombin. Values were expressed as milligrams per liter.

Statistical analysis

All values are expressed as mean ± SD. Data sets of ELISA and chemotaxis assays were analyzed by Kruskal-Wallis ANOVA on ranks;

differences in the mean values among the experimental groups were then compared using the multiple comparison procedure (Dunn's method). For correlation analysis, the Pearson coefficient was determined. Results were considered statistically significant when $p < 0.05$.

Results

C3 cleavage by human thrombin *in vitro*

As recently described, thrombin is capable of cleaving C5 and generating C5a in the absence of C3 (6). To evaluate whether thrombin also cleaves C3 into C3a, human C3 (5 μg ; concentration: 100 $\mu\text{g}/\text{ml}$) was incubated with increasing concentrations of thrombin (0–10 $\mu\text{g}/\text{ml}$) at 37°C for 90 min or indicated time periods. Whereas the complement concentration used did reflect physiological conditions, the concentrations of thrombin added appeared supraphysiological. However, in certain pathophysiological conditions, such as multiple tissue injury, levels of activated coagulation factors have been proposed to be much higher, especially during the propagation phase and when applied therapeutically (21). Furthermore, in the present mechanistic *in vitro* experiments as in similar studies (22), higher concentrations of the activated coagulation factors were used to maximize the signal. The amount of C3a generated was then analyzed by Western blot and ELISA techniques. As indicated in Fig. 1, the production of C3a was found to be time and concentration dependent on the amount of thrombin added. No C3a was detected when C3 was incubated in DPBS in the absence of thrombin. Using polyclonal Abs to human C3a, a single band at the predicted molecular mass of glycosylated C3a was found (Fig. 1A). Identical results were obtained when mAb to human C3a was employed (data not shown). The data were confirmed by parallel ELISA analysis of the samples (Fig. 1B). To determine the time dependency of thrombin-mediated cleavage, 5 $\mu\text{g}/\text{ml}$ thrombin was incubated with 100 $\mu\text{g}/\text{ml}$ C3 as a function of time (0–480 min). C3 cleavage appeared to reach a plateau after ~60 min of incubation (Fig. 1C). HMC-1 cells were used to assess biological (chemotactic) activity of the thrombin-induced cleavage product, which positively correlated with the amount of C3a generated (data not shown). To compare the thrombin-induced C3 cleavage with that of C5, cleavage of C5 was assessed using the same *in vitro* conditions, which in

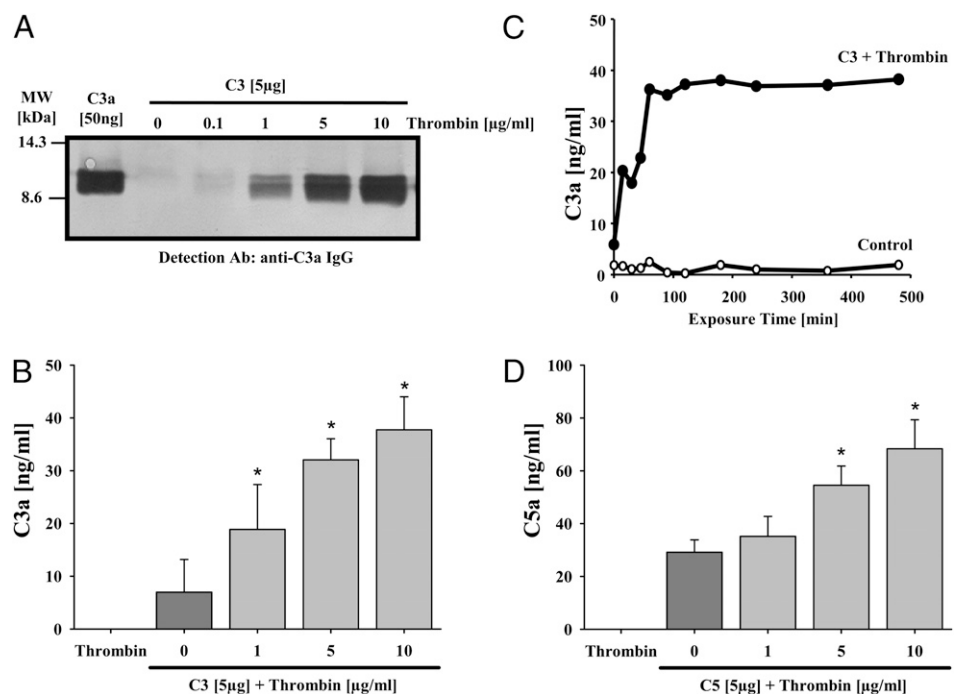
agreement with previous findings (6) resulted in significant generation of C5a as detected by ELISA (Fig. 1D).

In vitro cleavage of C3 and C5 by human FXa

FXa, the junction of the extrinsic and intrinsic paths of the coagulation system, was evaluated for its capability to cleave C3. When human C3 and human FXa were incubated at 37°C for 90 min and subjected to Western blot analysis for C3a, a single band at 9 kDa was found. The intensity of the band initially correlated with the amount of FXa added, peaking at 2 $\mu\text{g}/\text{ml}$ FXa (Fig. 2A). When supraphysiological concentrations of FXa were used (20–100 $\mu\text{g}/\text{ml}$), the bands appeared at slightly lower molecular levels, suggesting further cleavage most likely at the C-terminal site of C3a. When the polyclonal Ab used for detection of C3a was preblocked with recombinant human C3a, the C3a bands were significantly fainter, confirming the specificity of C3a detection by the Western technique (data not shown). A similar *in vitro* cleavage pattern was also indicated by the ELISA data, in which the Abs employed might not detect further C3 cleavage products at lower molecular weights than the native C3a (Fig. 2B). Reflecting these results, FXa-generated C3a induced chemotaxis of HMC-1 cells in a concentration-dependent fashion, peaking at 2 $\mu\text{g}/\text{ml}$ FXa and declining at higher concentrations (Fig. 2C).

Subsequently, the capability of human FXa to cleave human C5 (5 μg ; concentration: 100 $\mu\text{g}/\text{ml}$) *in vitro* was evaluated. Samples were incubated at 37°C for 90 min and then subjected to Western blot and ELISA analysis for C5a. A C5 cleavage product was present at ~14 kDa that was reactive with anti-human C5a IgG (Fig. 2D). The band intensity was dependent on the concentration of FXa added. Confirmation of these results was obtained using a monoclonal anti-C5a IgG (data not shown). No evidence of autocleavage existed in the absence of human FXa. To further specify the cleavage product obtained, the anti-C5a detection Ab was preblocked with recombinant human C5a, which resulted in faint bands for the expected cleavage product (data not shown). The amount of C5a generated in the presence of increasing concentrations of FXa was confirmed using C5a ELISA analysis (Fig. 2E). Finally, functional analysis of the C5 cleavage product following FXa exposure was performed. The cleavage product

FIGURE 1. Thrombin-induced cleavage of C3 and C5. Human C3 (5 μg ; concentration 100 $\mu\text{g}/\text{ml}$) was incubated in DPBS in the absence or presence of increasing concentrations of human thrombin for 90 min. *A*, Western blot analysis of the C3 cleavage product in the presence of thrombin using polyclonal anti-C3a IgG as detection Ab. Equal protein loading was ensured for all samples. Depicted blot is representative of $n = 3$ experiments. *B*, ELISA assessment for C3a from samples coincubated with thrombin and C3 in DPBS ($n = 3$). *C*, C3 (100 $\mu\text{g}/\text{ml}$) and thrombin (5 $\mu\text{g}/\text{ml}$) were incubated as a function of time with subsequent evaluation for C3a by ELISA. *D*, Western blot analysis of C5 cleavage in the presence of thrombin. * $p < 0.05$ versus control.



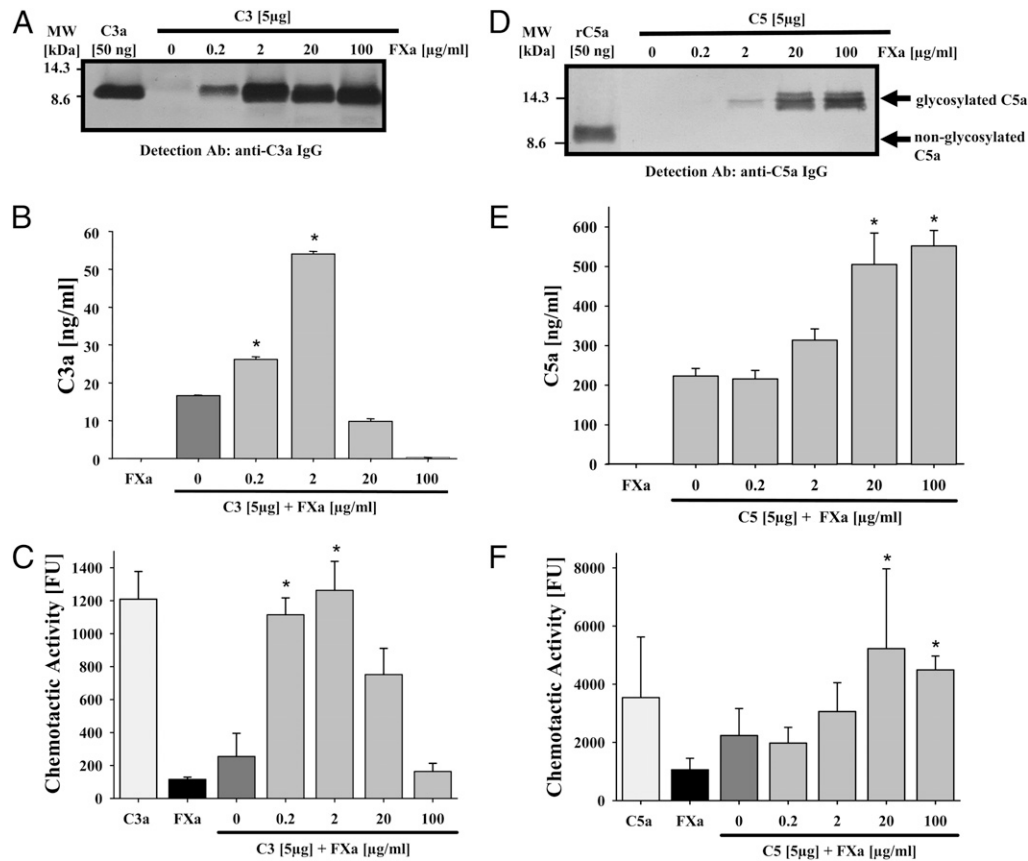


FIGURE 2. FXa-mediated C3 and C5 cleavage with subsequent generation of C3a and C5a. Human C3 or C5 (5 µg; concentration 100 µg/ml) were incubated in DPBS in the absence or presence of increasing concentrations of FXa for 90 min at 37°C. *A*, Western blot detection of C3a following exposure of C3 to FXa. Equal protein loading was ensured for all samples. Blots are representative of at least three independent experiments. *B*, ELISA evaluation for C3a following coincubation of FXa and native C3. * $p < 0.05$ versus control ($n = 3$). *C*, Chemotaxis assay using HMC-1 cells to determine biological activity of C3-cleavage product postexposure to FXa ($n = 6$). Recombinant human C3a (100 ng/ml) served as a positive control. *D*, Western blot analysis of samples following exposure of C5 to FXa using anti-C5a IgG. Nonglycosylated, rC5a served as a positive control. Blot is representative of three independent experiments. *E*, ELISA assessment of the preparations described in *D*. * $p < 0.05$ versus control. *F*, Evaluation of C5 + FXa preparations for chemotactic activity using human neutrophils. Recombinant human C5a (100 ng/ml) served as a positive control. For each experimental condition, $n = 6$.

showed clear evidence of chemotactic attraction for human blood neutrophils (Fig. 2*F*).

Identification of FXa-induced cleavage products as C3a and C5a

To identify the FXa-induced cleavage products on a molecular level, MALDI-TOF MS analyses were performed. C3 (100 µg/ml) or C5 (100 µg/ml) were incubated with FXa (10 µg/ml). The molecular mass of the C3-cleavage product generated by FXa exactly matched the predicted molecular mass for native C3a of 9090 Da (Fig. 3*A*). As an internal control, C3a expressed with a histidine tag (MRGSHHHHHHGS) from *Escherichia coli* revealed an identical molecular mass of 9090 Da after subtracting the mass of the histidine tag (data not shown).

Due to the N-linked glycosylation of C5a, deglycosylation of C5a was necessary prior to MS analysis. Purified C5a from human plasma (kindly provided by Complement Tech, Tyler, TX) was used as a standard. The molecular mass of the deglycosylated C5a standard (Fig. 3*B*, upper panel) was identical to the FXa-induced cleavage product C5a (Fig. 3*B*, bottom panel).

C3 and C5 cleavage by human plasmin

As the central factor of the fibrinolysis cascade, plasmin not only cleaves fibrin, but also fibronectin, thrombospondin, laminin, and von Willebrand factor (23). To determine whether the fibrinolysis system also interacts with the complement cascade, human plas-

min was incubated with human C3 or C5 as a function of concentration. Plasmin was found to cleave C3 into C3a fragments, as detected by Western blot analysis using anti-C3a-IgG (Fig. 4*A*) and quantitative ELISA measurements (Fig. 4*B*). Plasmin-induced

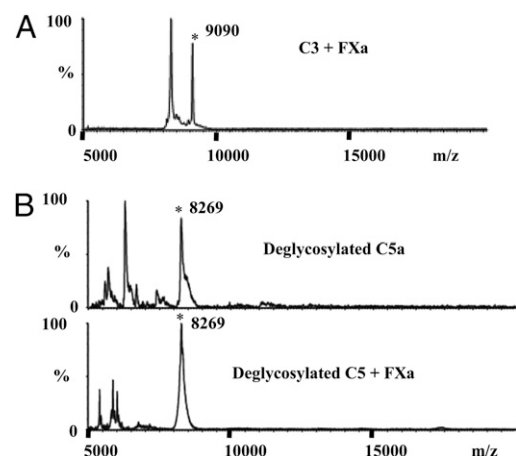


FIGURE 3. MS analysis of FXa-induced cleavage products of C3 and C5. MALDI-TOF MS analysis of the C3 and C5 cleavage products following FXa incubation. *A*, Mass sizes of C3a produced by FXa-induced C3 cleavage. *B*, Mass sizes of deglycosylated, recombinant human C5a (upper panel) and C5a generated by incubation of C5 with FXa (lower panel).

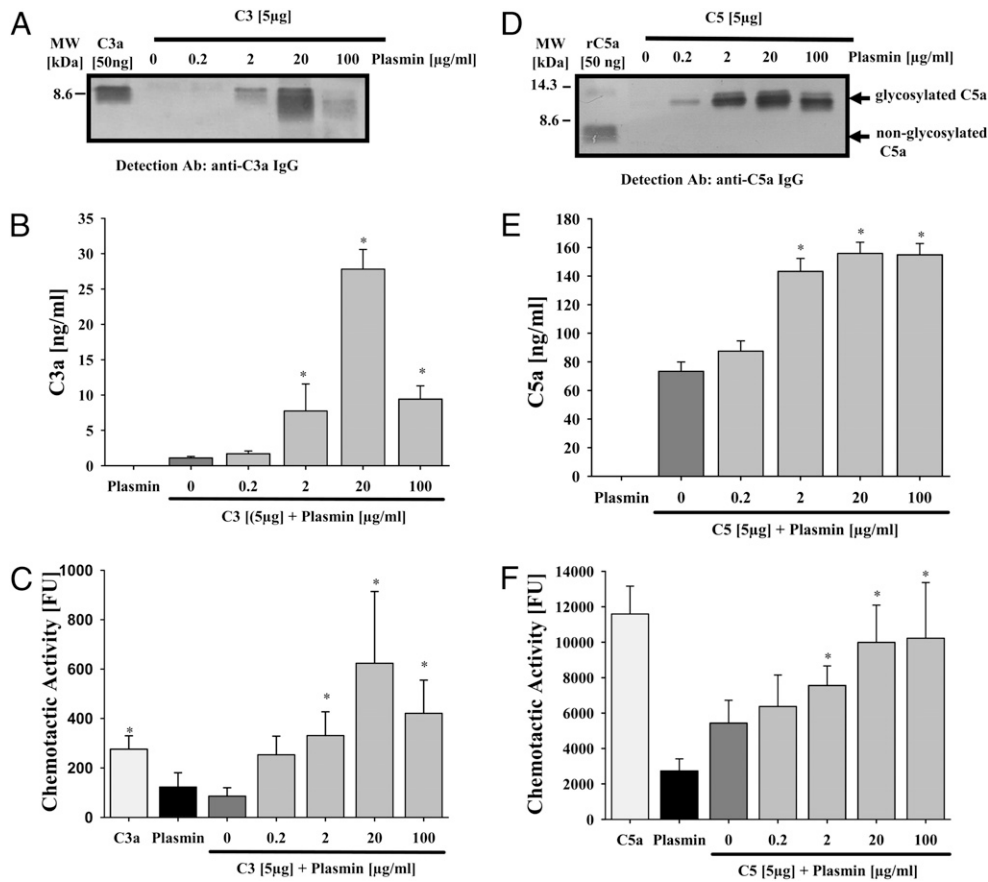


FIGURE 4. Plasmin-induced C3 and C5 cleavage. Human C3 or C5 (each at 5 μg; concentration 100 μg/ml) were incubated in DPBS in the absence or presence of increasing concentrations of plasmin for 90 min (37°C). *A* and *D*, Western blot analysis of C3 or C5 samples following exposure to plasmin. As a positive control, 50 ng of rC3a or rC5a was used. Depicted blots are representative of $n = 3$ independent experiments. *B* and *E*, C3a- or C5a-ELISA measurements of C3 or C5 samples after plasmin incubation ($n = 3$). *C*, Analysis of chemotactic activity of C3 preparation following incubation with increasing amounts of plasmin using HMC-1 cells. Recombinant human C3a (100 ng/ml) served as positive control ($n = 6$). *F*, Following incubation of C5 with increasing amounts of plasmin, samples served as chemotactic stimulus for isolated human neutrophils. C5a (100 ng/ml) was used as a positive control. $n = 6$ per experimental condition. * $p < 0.05$ versus control.

generation of C3a peaked at 20 μg/ml plasmin and decreased at higher concentrations (Fig. 4A, 4B). In addition, plasmin-derived C3a dose-dependently induced chemotactic activity of HMC-1 cells (Fig. 4C), suggesting biological activity for the C3 cleavage product obtained.

When human plasmin was incubated with human C5, a concentration-dependent cleavage of C5 was found, as assessed by Western blotting and ELISA (Fig. 4D, 4E). Maximal plasmin-induced C5a-generation was found at plasmin concentrations above 2 μg/ml. Again, generated C5a was biologically active, as determined by neutrophil chemotaxis assays (Fig. 4F).

Characterization of the C3- and C5-proteolytic activity of coagulation factors

To determine whether the above-described clotting and fibrinolysis factors exhibit different degrees of catalytic activities, analysis of enzymatic activity was performed. Accordingly, increasing concentrations of C3 or C5 were used as a substrate, whereas a constant concentration of 80 nM each factor was added as the enzyme. Various coagulation factors (TF, FVIIa, FVII, plasminogen, and activated protein C) failed to generate C3 or C5 cleavage activity (data not shown). In contrast, FXa and plasmin most effectively cleaved both C3 and C5 (Fig. 5A, 5B). The enzymatic activity analyses ($n = 5$ for each) revealed the following order: FXa > plasmin > thrombin > FIXa > FXIa >

control for C3 and plasmin > FXa > FIXa = FXIa > thrombin > control for C5.

Serum complement activation by Fxa: inhibition by anticoagulants

To determine whether the results obtained in vitro can be transferred into a more complex biological system, the ability of FXa to activate the complement system in serum was investigated ex vivo. We also sought to determine whether the in vitro cleavage process is capable of assembling the TCC ex vivo, which was assessed by the CH50. Human serum from healthy volunteers (50 μl) was incubated in the absence or presence of increasing concentrations of FXa (0–100 μg/ml) and analyzed by ELISA for generated C3a and C5a. Serum levels of C3a and C5a were both found to be increased in a concentration-dependent manner on incubation with FXa (Fig. 6A, 6B). Similarly, serum incubation with FXa (100 μg/ml) resulted in significant generation of TCC (384 ± 24 ng/ml TCC), reflected functionally by a small but significant decrease in serum hemolytic activity (Fig. 6C).

The specificity of FXa-dependent anaphylatoxin generation in serum was further evaluated using fondaparinux and enoxaparin, both selective inhibitors of FXa. Serum incubation with FXa in the copresence of increasing concentrations of fondaparinux led to a significantly suppressed production of C5a (Fig. 6D). Similar results were found when a different inhibitor of FXa protease

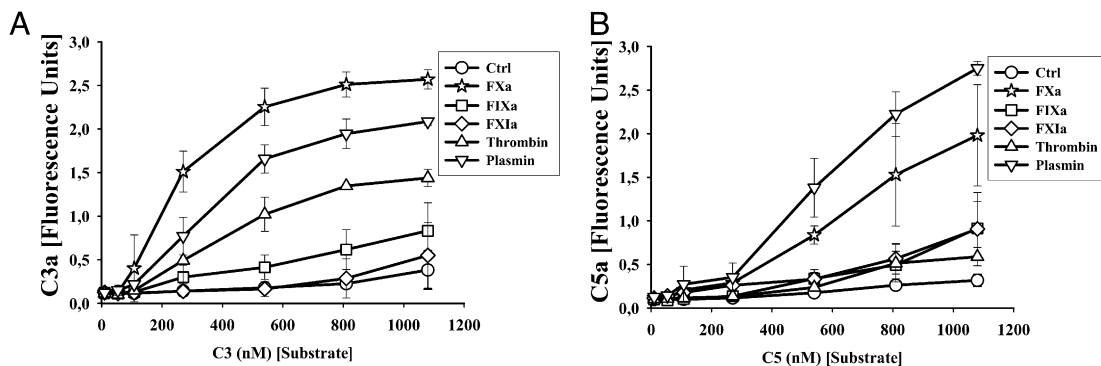


FIGURE 5. C3- and C5- proteolytic activity of various coagulation factors (FXa, FIXa, FXIa, plasmin, thrombin). *A* and *B*, C3- and C5- proteolytic activity of various coagulation/fibrinolysis factors (each at 80 nM) were assessed by ELISA measurements of C3a and C5a generated within 90 min in the presence of increasing concentrations of the substrate (10, 54, 108, 270, 540, 810, and 1080 nM C3 or C5). Every proteolytic activity value represents the average of duplicate measurements based on five independent experiments.

activity, enoxaparin, was used (Fig. 6E). Additional experiments substituting human plasma for human serum demonstrated similar patterns of C3a and C5a generation in the presence of FXa (data not shown). Collectively, these data suggest functional *ex vivo* cleavage of C3 and C5 by FXa.

For a first transfer of the reported *ex vivo* findings to a relevant clinical setting, plasma from 12 polytrauma patients within the first hour after trauma was analyzed for activation of the coagulation and complement systems (for detailed patients' demography, see Ref. 24). In accordance with a previous study in multiple injured patients (25), very early activation (and dysfunction) of the coagulation system was found with significant formation of TAT complexes. A subsequent analysis determined whether these changes in TAT complexes were associated with complement activation. Early post-trauma (within 1 h postinjury), there was a positive correlation between the generation of the TAT complexes and the anaphylatoxin C5a concentrations in plasma (correlation coefficient $r = 0.697$; $p = 0.012$; $n = 12$; Fig. 6F). The present *in vitro* and *in vivo* results are summarized in a simplified scheme (Fig. 7).

Discussion

In the current study, we investigated the interaction between the coagulation/fibrinolysis cascades and the complement system *in vitro* and *ex vivo*. Exposure of C3 to thrombin resulted in time- and concentration-dependent generation of C3a *in vitro*. C5 was also cleaved by thrombin to produce C5a. In parallel experiments, incubation of C3 and C5 with either FXa or plasmin resulted in generation of C3a and C5a. The resulting cleavage products exhibited intact chemotactic activity and were indistinguishable from native C3a or C5a when assessed by MS. Incubation of serum or plasma with FXa resulted in a concentration-dependent generation of C3a and C5a as well as decreased hemolytic serum activity.

The coagulation/fibrinolysis cascades and the complement system appear to be triggered simultaneously by severe tissue injury (26, 27), acute trauma (28), or during systemic inflammation (9). Pathophysiologically, the formation of thrombin at the site of injury following the activation of the coagulation cascade may not only provide a physical barrier against invading micro-organisms (29), but also trigger the complement system. Locally produced anaphylatoxins help in activating cellular immune responses (30). Thus, the innate serine protease system, with its three major columns, coagulation, fibrinolysis, and complement, may be essential for both an effective protection against bleeding and invading pathogens. However, when the injurious load is excessive, uncontrolled and simultaneous activation of the complement and coagulation/fibrinolysis systems can occur. In particular, excessive generation

of C5a is known to have adverse effects during systemic infection (31, 32). Therefore, it is tempting to speculate whether various clotting factors might contribute locally and systemically to generate C3a and C5a, which, in turn act as chemoattractants for phagocytic cells to the site of inflammation, where these cells release their major arsenals of tissue-damaging proteases, reactive oxygen species, and cytokines/chemokines (15, 18, 33, 34). An important role for C5a/C5aR signaling has also been postulated in the fibrinolysis system (35). Finally, many proinflammatory cytokines can cause decreased levels of several anticoagulant proteins including thrombomodulin, the endothelial cell protein C receptor, and protein S (36), resulting in an inflammatory, procoagulant state. Mannan-binding lectin-associated serine protease 2, a protease that is characteristic of the lectin pathway of complement activation, can trigger coagulation by cleaving prothrombin into active thrombin (13). The procoagulant activities of complement are increased when anticoagulant mechanisms are inhibited; for example, the formation of a complex between C4b-binding protein and protein S results in a decrease in the availability of protein S to act as a cofactor for the anticoagulant protein C pathway (37). In addition, the thrombin-prothrombin complex activates carboxypeptidase B, which, in turn, blocks C5a to counteract the inflammatory mediators generated at the site of vascular injury (38). Profound effects of the complement system on the coagulation system and vice versa have also been found for some innate regulators, such as the C1-inhibitor or the thrombin activatable fibrinolysis inhibitor. The latter is a potent and broadly reactive carboxypeptidase (39), which is generated by the thrombomodulin–thrombin complex and reacts in an anticoagulant manner (40). It not only moderates fibrinolysis but also has some anti-inflammatory effects due to its ability to inactivate C3a and C5a by removing carboxy-terminal arginine residues from these components (41). Thus, we are now beginning to understand that, rather than acting as separate, independent cascades, the coagulation/fibrinolysis system and the complement cascade cross talk extensively with each other and mutually fine-tune their activation status (16, 17).

Previously, the presence of C3 was thought to be indispensable for the assembly of the C5-convertase to generate C5a. Surprisingly, the presence of C5a was recently discovered in activated C3-deficient serum, where enhanced levels of thrombin, a primary component of the activated coagulation system, induced C5 conversion to C5a (6). In the current study, we found evidence of thrombin-mediated cleavage of C3 into C3a in a dose- and time-dependent manner in complement-sufficient human serum. In line with these findings, Kalowski et al. (42) reported in 1975 that thrombin and thromboplastin injected into rabbits somehow led to

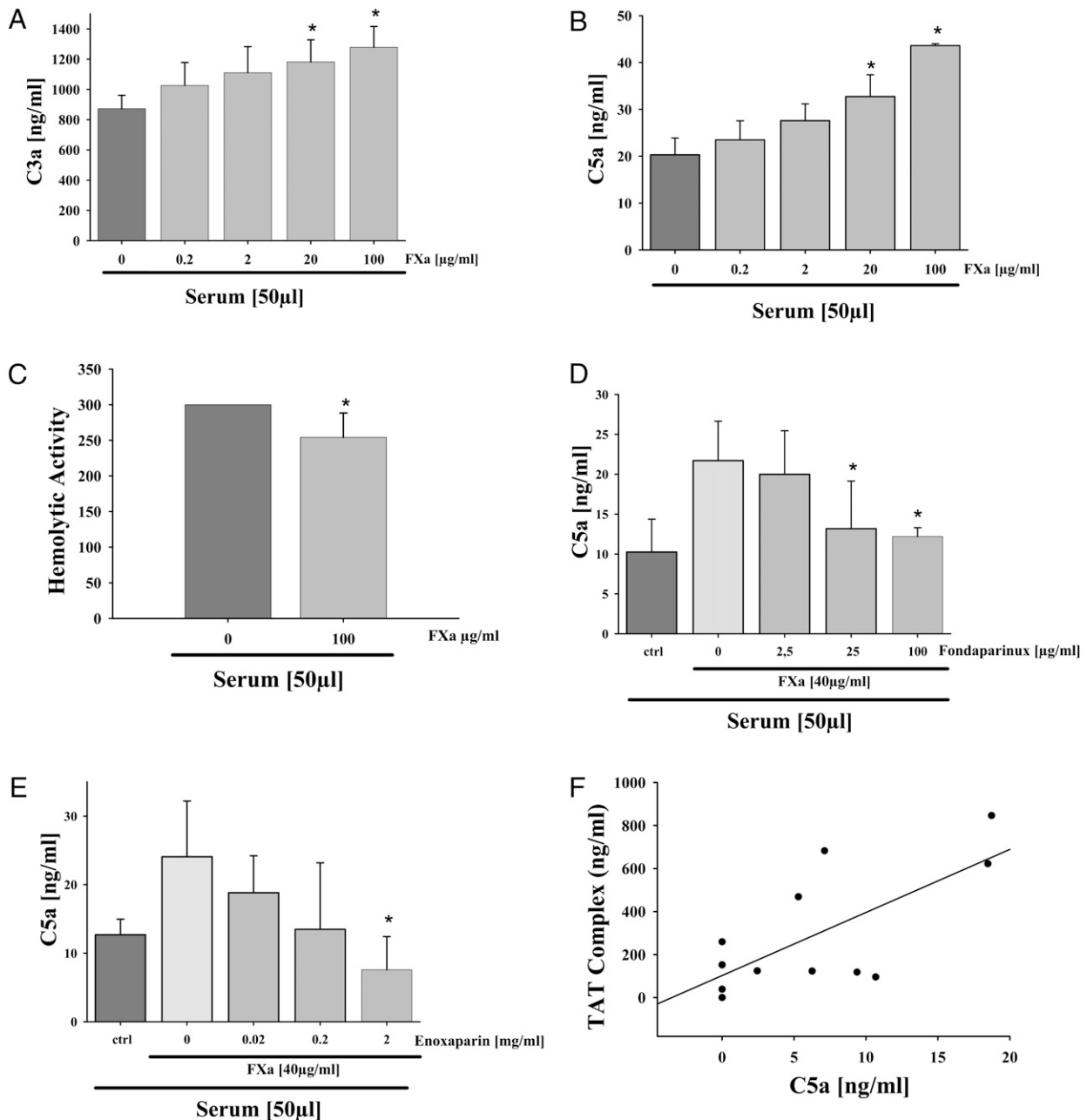


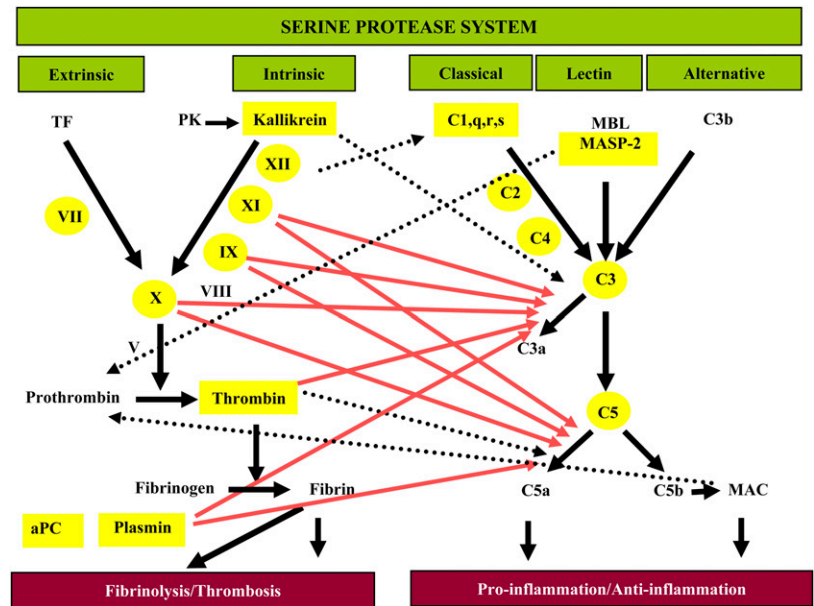
FIGURE 6. FXa-induced serum complement activation. Human serum was incubated in the presence or absence of increasing concentrations of FXa for 90 min at 37°C. Evaluation of serum samples for C3a (A) and C5a (B) concentration following FXa-incubation using ELISA analysis. * $p < 0.05$ versus control ($n = 3$). C, CH50 was assessed in human serum in the presence or absence of 100 $\mu\text{g/ml}$ FXa. Data are representative of 12 separate and independent experiments. * $p < 0.05$ versus control. ELISA analysis of serum samples for C5a postincubation with FXa (40 $\mu\text{g/ml}$) in the copresence of increasing amounts of the FXa-selective inhibitors sodium fondaparinux (D) or sodium enoxaparin (E). * $p < 0.05$ versus control; $n = 6$. F, Correlation analysis between generated TAT complexes and complement activation product C5a in the plasma of patients early (<1 h) after multiple injury.

activation of complement, which appeared to be partially dependent on platelets. Similar to thrombin, kallikrein and plasmin have been described to directly cleave C3 (43, 44).

Serine proteases are characterized by a serine residue at the active center of these proteases (45), which participates in the catalytic mechanism during peptide bond hydrolysis (46). Under physiological conditions, complement C3 and C5 are cleaved by the C3-convertase and C5-convertase at a single arginine-serine peptide bond at position 77 and 75 in the α -chain of C3 and C5 to release C3a and C5a, respectively (47, 48). Similarly, the coagulation factors FXa, FXIa, FIXa, plasmin, and thrombin are known to be potent serine proteases (49) with specific affinity for

the arginyl-X peptide bonds of their natural substrates (50), which led us to the above-proposed hypothesis. In the current study, our hypothesis was supported by the identification of thrombin-, FXa-FXIa-, FIXa-, and plasmin-mediated cleavage products of C3 and C5. The proteolytic effectiveness of these serine proteases to cleave C3 and C5 was further compared using a proteolytic activity analysis, identifying coagulation factor FXa as the most potent one. Interestingly, maximal C3 cleavage via FXa or plasmin was achieved at lower concentrations (2 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$, respectively) than the peak of C5 cleavage (20–100 $\mu\text{g/ml}$). In fact, FXa- and plasmin-mediated cleavage of C3 drastically declined at higher concentrations of these serine proteases. In this

FIGURE 7. Simplified model of the serine protease system. Depiction of the complex interplay between the coagulation/fibrinolysis cascades and the complement system. The serine proteases of the complement, coagulation, and fibrinolysis systems are all highlighted in yellow. The black dotted arrow bars show previously known interactions of these systems. The red arrows identify the new paths of complement activation by the coagulation/fibrinolysis factors resulting in the generation of C3a and C5a. aPC, activated protein C; MAC, membrane attack complex; MBL, mannan-binding lectin; PK, prekallikrein.



case, it is tempting to speculate that further cleavage on the C terminus of C3a occurred because the C3a/C3a-des-arg neopeptide Ab of the ELISA system failed to detect the cleavage product. This is supported by the significant loss of the chemotactic activity known to be specifically dependent on the C-terminal part of C3a. Thus, when components of the coagulation/fibrinolysis system are only moderately triggered, activation of the complement cascade may be mainly achieved through the cleavage of C3, whereas, when coagulation/fibrinolysis is massively activated, C5 may represent the primary target for cleavage.

The concentrations of coagulation factors required to cleave purified complement proteins appear to be supraphysiological, which might suggest that such interactions between complement and coagulation are less relevant to the *in vivo* situation. However, the data presented in this study do not support this conclusion. It has been clear from a number of studies that cofactors and biological surfaces exert vital effects on the activity of plasma enzymes (51, 52). The prothrombinase complex (consisting of FXa, FVa, and negatively charged surface) increases FXa activity to cleave prothrombin into thrombin 300,000-fold as compared with FXa and prothrombin alone. Furthermore, little is known about the pathophysiological concentrations of activated clotting/fibrinolysis factors produced locally and systemically upon excessive activation of the coagulation system (e.g., during hemorrhagic shock and disseminated coagulopathy after severe tissue trauma). In this regard, a C3a-like fragment has been found in blood clots, indicating local complement activation and deposition (53). It is striking that the clinically established anticoagulants enoxaparin and fondaparinux, targeting FXa, are highly capable of inhibiting complement activation. Thus, selective FXa inhibitors, and theoretically other anticoagulants, might also act as immune modulators, a barely understood new function.

To our knowledge, the current study represents the first systematic assessment of the communication between the complement system and the coagulation/fibrinolysis cascades, providing novel insights into their complex interplay. Limitations of our study include the fact that it remains to be determined if our findings can be extrapolated into a much more complex *in vivo* setting in humans. As an initial evaluation of the potential transferability of our *in vitro* findings, the present findings were assessed by a more elaborate *ex vivo* system using serum and plasma samples. Based

on significant changes to the CH50 and generation of TCC in the presence of FXa, it is likely that the activated coagulation system closely interacts with the complement cascade on various molecular levels *ex vivo*, generating not only anaphylatoxins but also the opsonin C3b and C5b-9. This could be mirrored by the present results in multiple injured patients of a very early synchronized appearance of coagulation (TAT complexes) and complement activation (C5a) products. However, because correlation is not necessary causative, these findings will have to be confirmed in a standardized experimental *in vivo* setting. In the current study, factors of the tissue factor pathway failed to affect the complement system, whereas serine proteases of the contact activation pathway were able to significantly activate the complement system. It is now becoming clear that a distinction between the intrinsic and extrinsic coagulation pathways in the coagulation cascade represents an antiquated description of test tube coagulation and does not reflect physiological *in vivo* coagulation (54). As a result, a cell-based model of coagulation has been developed (55), which appears to much better mimic the complex interactions of cellular and plasma components *in vivo*. The intricate interplay of the coagulation and fibrinolysis cascades with various cells further complicates their interactions with the complement system. Thus, unraveling the precise communications between coagulation/fibrinolysis, various cells, and the complement system will represent a major future scientific challenge. In addition, therapeutic interventions targeting one cascade might result in unfavorable, so far unanticipated adverse effects on the other cascade and the cells involved.

In conclusion, we provide the first evidence, to our knowledge, for paths that activate the complement cascade, intimately linking the coagulation/fibrinolysis cascades to the complement system (Fig. 7). Thus, rather than considering them as separate cascades, this study proposes a concept of a common serine protease network, stressing the extensive cross talk between both cascades.

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Disclosures

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