Factor Xa as an Interface Between Coagulation and Inflammation

Molecular Mimicry of Factor Xa Association with Effector Cell Protease Receptor-1 Induces Acute Inflammation In Vivo

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Abstract

Coagulation proteases were tested in a rat model of acute inflammation. Subplantar injection of Factor Xa (10–30 μg) produced a time- and dose-dependent edema in the rat paw, and potentiated carrageenin-induced edema. In contrast, the homologous protease Factor IXa was ineffective. This inflammatory response was recapitulated by the Factor Xa sequence L^{83}FTRKL^{88}(G), which mediates ligand binding to effector cell protease receptor-1 (EPR-1), while a control scrambled peptide did not induce edema in vivo. Conversely, injection of the EPR-1–derived peptide S^{123}PGKPGNQNSKNEPP^{137} (corresponding to the receptor binding site for Factor Xa) inhibited carrageenin-induced rat paw edema, while the adjacent EPR-1 sequence P^{136}PKKRERERSSHCYP^{150} was without effect. EPR-1-Factor Xa–induced inflammation was characterized by fast onset and prominent perivascular accumulation of activated and degranulated mast cells, was inhibited by the histamine/serotonin antagonists cyproheptadine and methysergide, but was unaffected by the thrombin-specific inhibitor, Hirulog. These findings suggest that through its interaction with EPR-1, Factor Xa may function as a mediator of acute inflammation in vivo. This pathway may amplify both coagulation and inflammatory cascades, thus contributing to the pathogenesis of tissue injury in vivo. (*J. Clin. Invest.* 1997. 99:2446–2451.) Key words: coagulation • leukocytes • inflammation • Factor Xa • tissue injury

Introduction

It has been appreciated for more than two decades that activation of coagulation is invariably linked to immune-inflammatory responses in vivo. From the earlier histopathological detection of fibrin in delayed-type hypersensitivity (1) and on tissue macrophages (2), recent experiments have demonstrated that systemic defibrinogenation drastically attenuates inflammatory responses to purulent bacterial infections (3), implanted biomaterials (4), and Ig-mediated acute nephrotoxic nephrites (5) in vivo.

While dysregulation of coagulation contributes to vascular injury (6) and atherosclerosis (7), the molecular basis of the interface between coagulation and inflammation is not completely understood. Recent experimental evidence has suggested that in addition to their role in hemostasis (8), proteases of the clotting and fibrinolytic cascades may also transduce intracellular signals and modulate immune-inflammatory responses (9). Proteolytic activation of the thrombin receptor on leukocytes increased cytosolic free Ca^{2+} (10), and stimulated MAP kinase activation, protein tyrosine phosphorylation (11), and the release of chemotactic (12) and inflammatory (13) cytokines. Occupancy of the urokinase receptor regulated macrophage adherence (14), gene expression (15), and chemotaxis (16). Finally, consistent with its mitogenic (17) and signaling (18) properties, binding of Factor Xa to effector cell protease receptor-1 (EPR-1)[1] participated in leukocyte activation (9), brain pericyte- (19), platelet- (20), and endothelial cell-thrombin formation (21), and aortic smooth muscle cell proliferation (22).

In an effort to reinvestigate the interface between coagulation and inflammation, we have found that Factor Xa, but not Factor IXa, induced an acute inflammatory response in vivo, and that this pathway was recapitulated by small synthetic peptides mimicking ligand binding to EPR-1.

Methods

Coagulation proteins and synthetic peptides. Coagulation Factors IXa and Xa were purchased from Calbiochem Corp. (La Jolla, CA) or Haematologic Technologies (Essex Junction, VT), and used in previous studies. Synthetic peptidyl mimicry of EPR-1 recognition of Factor Xa, as identification of the interacting sequences implicated in ligand binding, was reported previously (21). The partially overlapping peptides AG1 (S^{123}PGKPGNQNSKNEPP^{137}) and AG2 (P^{136}PKKRERERSSHCYP^{150}) were synthesized from the sequence of the A1 chimera in the extracellular domain of EPR-1, containing the receptor binding site for Factor Xa (21). Inhibition of Factor V/Va–independent endothelial cell prothrombin activation by the AG1 peptide, but not by AG2, was as described (21). The identification of the inter-EGF sequence L^{83}FTRKL^{88}(G) in Factor Xa (residue in parentheses added to the natural sequence) as a binding site for EPR-1 based on synthetic peptidyl mimicry, a sequence-specific antibody and gain-of-function of a recombinant Factor IX chimera containing the Factor X sequence 83–88, was reported previously (23). A scrambled peptide KFTGRLL for the Factor X 83-33 sequence was synthesized and used as a control in these experiments. All peptides were characterized by HPLC and mass spectrometry and dissolved in sterile saline.

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1. *Abbreviations used in this paper:* EPR-1, effector cell protease receptor-1; PAS, periodic acid Schiff.

Rat paw edema formation. The experimental procedures for agonist-induced edema in vivo have been described previously (24). Briefly, groups of five to six male Wistar rats (Charles River Laboratories, Milano, Italy) weighing 120–150 g each were lightly anesthetized with enflurane and treated with subplantar injections of sterile saline or various concentrations (1–30 μg) of Factors IXa, Xa, or the EPR-1– or Factor Xa–derived peptides (10–300 μg), in a total volume of 100 μl. In potentiation experiments, animals were injected with a suboptimal concentration of carrageenin (0.1%, 100 μl/paw) simultaneously with concentrations of Factor Xa (1–3 μg) which do not cause edema, before evaluation of rat paw edema. To assess the potential contribution of EPR-1 in carrageenin-induced edema, animals were injected with a maximal concentration of carrageenin of 1% (100 μl/paw) simultaneously with 10–100 μg of the EPR-1–derived peptide AG1 or AG2 (21). Paw volume was determined using a hydroplethismometer (Ugo Basile, Milano, Italy) immediately before the subplantar injection of the various agonists, and sequentially at 15-min time intervals for the first hour of the experiment, and hourly thereafter for up to 5 h (carrageenin-induced edema only). The increase in paw volume under the various experimental conditions tested was evaluated as difference between the paw volume at each interval and the basal paw volume (24). In another series of experiments, animals were separately injected intraperitoneally with the histamine/serotonin antagonist cyproheptadine (5 mg/kg), the serotonin-specific antagonist methysergide (2 mg/kg), the mast cell amine-stabilizing agent cromolyn (10 mg/kg), or the thrombin-specific inhibitor Hirulog (5 mg/kg) (24) 30 min before administration of the Factor Xa peptide 83-88 (100 μg) or Factor Xa (10 μg) and determination of changes in rat paw volume. All edema experiments were repeated at least three times with similar results.

Histochemistry. Untreated animals, or rats injected with Factor Xa (10 μg) or the Factor Xa peptide 83-88, were killed 15 min after treatment. The paws were removed, fixed in 10% buffered formalin, demineralized, and tissue samples were embedded in paraffin blocks. 4-μm sections were cut, put on high adhesive slides, and stained with hematoxylin/eosin, periodic acid Schiff (PAS), or Giemsa before counterstaining and examination under light microscopy.

Statistical analysis. All results are reported as mean±SEM. The unpaired two-tailed Student's *t* test was used for statistical evaluation when two groups were compared. When control values were compared to more than one group, ANOVA followed by Dunnett's fort test was used. A value of $P < 0.05$ was taken as significant.

Results

Factor Xa induces acute inflammation in vivo. Subplantar administration of Factor Xa in rats was associated with a rapid increase in the paw volume, which peaked after 15 min from the injection, and progressively decreased to background levels of control animals injected with saline after a 45-min interval (Fig. 1 *A*). Edema formation under these experimental conditions was dose-dependent and maximal at 10 μg of added Factor Xa, with no additional increase in rat paw volume for Factor Xa concentrations up to 30 μg (Fig. 1 *A*). In contrast, comparable concentrations of the Factor Xa–homologous coagulation protease, Factor IXa, did not significantly increase the rat paw volume under the same experimental conditions (Fig. 1 *A*). The combination of a nonstimulating concentration of Factor Xa (3 μg) plus a suboptimal dose (0.1%) of carrageenin (25) resulted in a significantly increased rat paw edema when compared with that of control animals treated with carrageenin alone, with a peak response observed 2 h after injection (Fig. 1 *B*).

Synthetic peptidyl mimicry of Factor Xa–induced inflammation. Previous experiments demonstrated that the inter-EGF sequence Leu^{83}-Leu^{88} in Factor Xa mediates ligand binding to

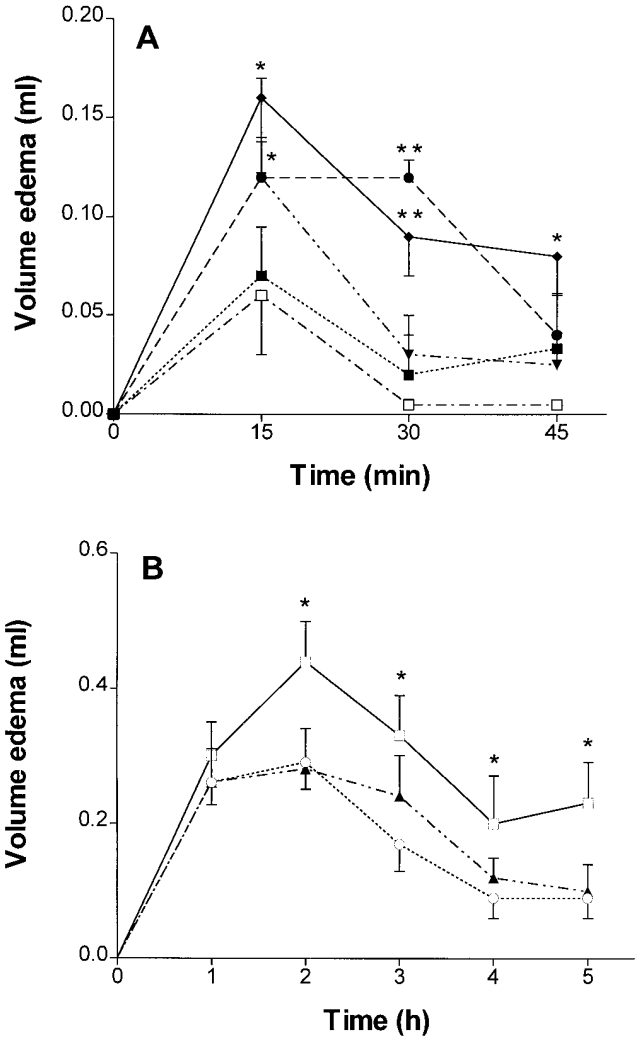

Figure 1. Factor Xa–induced edema in vivo. (*A*) Groups of five to six animals were injected in the paw with 1 μg (▼), 10 μg (◆), or 30 μg (●) Factor Xa, 10 μg Factor IXa (■), or control saline (□), in a total volume of 100 μl. Edema formation under the various conditions tested was determined using a hydroplethismometer at 15-min intervals from the injection. (*B*) Animals were injected with a suboptimal concentration of carrageenin (0.1%) in the absence (○) or in the presence of 1 μg (▲) or 3 μg (□) Factor Xa, before determination of edema at the indicated time intervals. For both panels, data are the mean±SEM of at least three independent experiments.

EPR-1 (23). The possibility that this interacting motif may mimic the inflammatory effect of Factor Xa in vivo was investigated. Administration of the Factor Xa peptide 83-88 produced a dose-dependent edema of the rat paw, which was maximal at 300 μg of peptide and peaked 15 min after injection, remaining sustained for 45–60 min (Fig. 2 *A*). In contrast, no statistically significant changes in rat paw volume were observed after injection of 100-μg concentrations of a control scrambled peptide of the 83-88 sequence (Fig. 2 *A*). Similar to the potentiating effect of Factor Xa on carrageenin-induced edema, intravenous administration of the Factor Xa peptide 83-88 (3 mg/kg) significantly increased (32–39%) the magnitude of rat paw edema induced by suboptimal concentrations of carra-

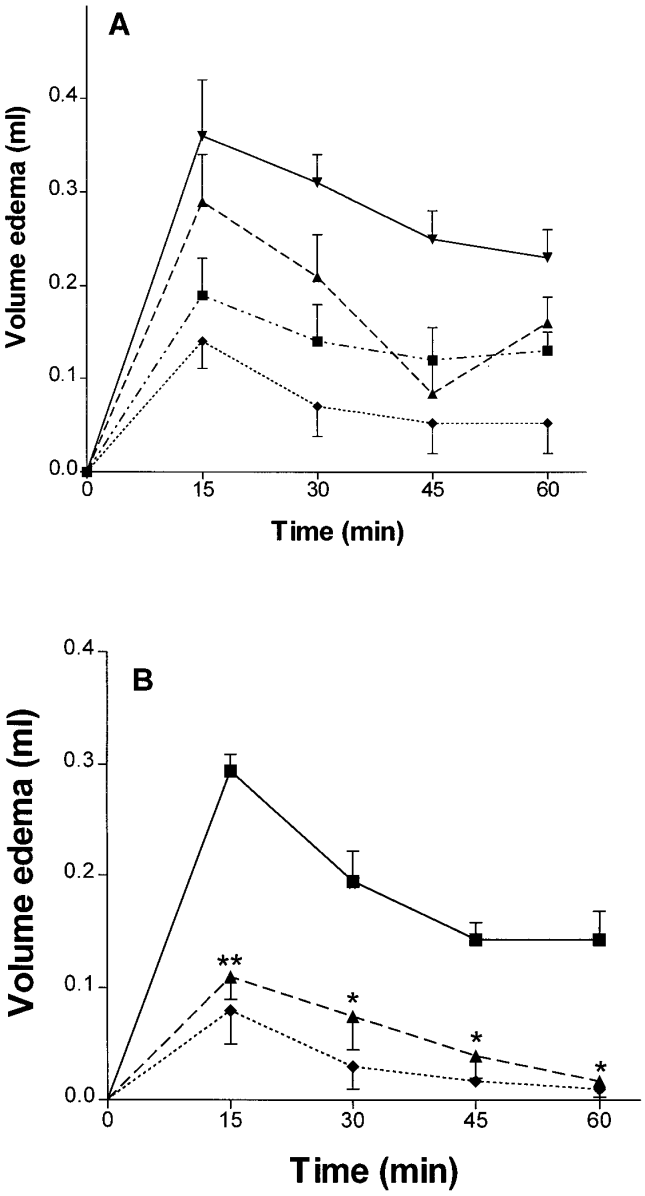

Figure 2. Effect of Factor Xa peptide 83-88 on rat paw edema. (*A*) Animals were injected with 10 μg (■), 100 μg (▲), or 300 μg (▼) of the Factor Xa peptide $L^{83}FTRKL^{88}$(G), or with 100 μg of control scrambled peptide KFTGRLL (◆), and paw edema was determined at the indicated time intervals. (*B*) Animals were injected with 100 μg of the Factor Xa peptide 83-88 in the absence (■) or in the presence of the histamine/serotonin antagonist cyproheptadine (◆), or the serotonin-specific antagonist methysergide (▲), before determination of paw edema at the indicated time intervals.

geenin at 3 and 4 h after injection, respectively (not shown). The mechanism(s) of Factor Xa–induced inflammation was investigated. First, in vivo administration of the serotonin/histamine antagonist cyproheptadine or the serotonin antagonist methysergide inhibited rat paw edema induced by the Factor Xa peptide 83-88 at all time points examined (Fig. 2 *B*). A partial reduction in edema formation was also observed after administration of the mast cell amine stabilizer cromolyn (not shown). In contrast, injection of the thrombin-specific inhibitor Hirulog (5 mg/kg) did not reduce the magnitude or the duration of rat paw edema induced by 10 μg Factor Xa (not shown), while it inhibited thrombin-dependent edema in vivo (24).

Morphologic analysis of Factor Xa–induced inflammation in vivo. Histochemical analysis of hematoxylin/eosin staining of the rat paw injected with Factor Xa or the Factor Xa peptide 83-88 revealed a complete disruption of tissue architecture and loss of organization characteristic of interstitial edema (24), as compared with control tissue sections of untreated animals (Fig. 3, *A–C*). Relevant histological features of the inflammatory response induced by Factor Xa or the Factor Xa peptide 83-88 included prominent perivascular accumulation of activated and partially degranulated mast cells and fibroblasts identified by PAS and Giemsa staining (Fig. 3, *D* and *E*, *arrows*, *insets*).

Participation of EPR-1 in acute inflammatory responses in vivo. A potential accessory role of EPR-1 in carrageenin-induced edema in vivo was investigated. Administration of increasing concentrations of the functionally blocking EPR-1–derived peptide AG1 S^{123}PGKPGNQNSKNEPP^{137} (21) inhibited in a dose-dependent manner carrageenin-induced rat paw edema at 2- and 3-h intervals (Fig. 4). In contrast, comparable concentrations of the adjacent EPR-1 sequence AG2 P^{136}PKKRERERSSHCYP^{150} did not reduce carrageenin-induced edema under the same experimental conditions (Fig. 4).

Discussion

In this study, we have shown that Factor Xa, but not the homologous protease Factor IXa, induced an acute inflammatory response in the rat paw, and potentiated carrageenin-induced edema in vivo. This pathway was mediated by binding of Factor Xa to its cellular receptor EPR-1 (26), as judged by the ability of small synthetic peptides mimicking this interaction to induce inflammation and modulate carrageenin-induced edema, in vivo.

Compelling experimental and clinical evidence suggests that coagulation and inflammation are intimately connected processes (6, 27). While the participation of leukocytes in coagulation has been extensively demonstrated and recognized as a major pathogenetic risk factor in vascular diseases (7), recent experimental findings have also identified a direct role of clotting and fibrinolytic proteases in intracellular signal transduction and modulation of inflammatory cell responses. This concept was further reinforced by the recent identification of various cellular protease receptors as modulators of second messengers (11, 18), cytokine gene expression (12, 15), and white blood cell motility (16). In this context, the vascular Factor Xa receptor EPR-1 (26) has been characterized for its role in platelet- (20), brain pericyte- (19), and endothelial cell-thrombin formation (21), leukocyte activation (9), and smooth muscle cell proliferation (22). Here, binding of Factor Xa to EPR-1 resulted in a novel nonhemostatic function of this protease, i.e., the induction of an acute inflammatory response in vivo. The molecular basis of this pathway appears to reside in the ability of Factor Xa to promote recruitment of mast cells and rapid discharge of vasoactive mediators, as judged by the prominent perivascular accumulation of activated and partially degranulated mast cells in situ, and by the inhibition of edema formation by histamine/serotonin antagonists. Consistent with this model, anti–EPR-1 mAbs B6 and 12H1 prominently re-

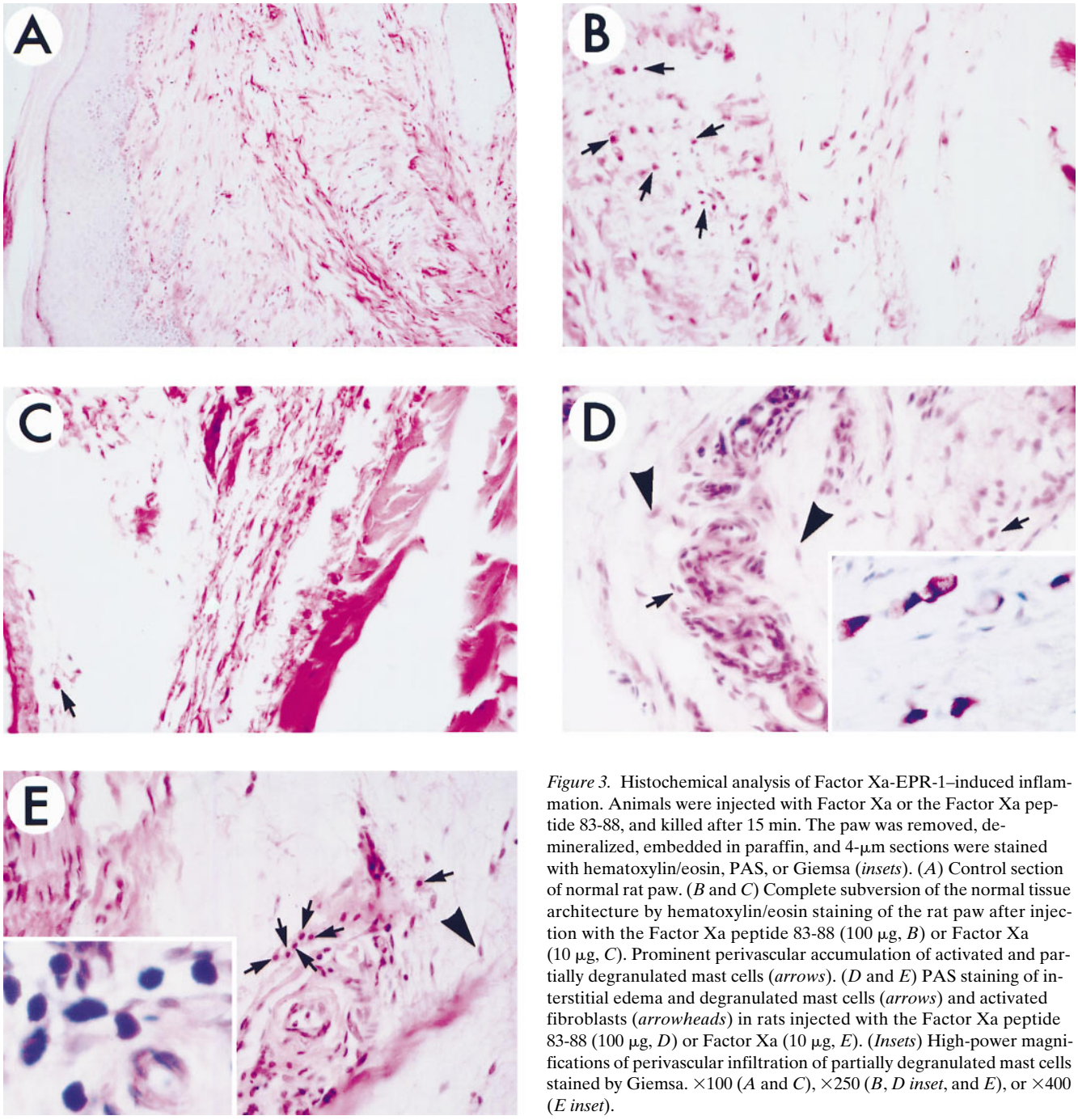

Figure 3. Histochemical analysis of Factor Xa-EPR-1–induced inflammation. Animals were injected with Factor Xa or the Factor Xa peptide 83-88, and killed after 15 min. The paw was removed, demineralized, embedded in paraffin, and 4-μm sections were stained with hematoxylin/eosin, PAS, or Giemsa (*insets*). (*A*) Control section of normal rat paw. (*B* and *C*) Complete subversion of the normal tissue architecture by hematoxylin/eosin staining of the rat paw after injection with the Factor Xa peptide 83-88 (100 μg, *B*) or Factor Xa (10 μg, *C*). Prominent perivascular accumulation of activated and partially degranulated mast cells (*arrows*). (*D* and *E*) PAS staining of interstitial edema and degranulated mast cells (*arrows*) and activated fibroblasts (*arrowheads*) in rats injected with the Factor Xa peptide 83-88 (100 μg, *D*) or Factor Xa (10 μg, *E*). (*Insets*) High-power magnifications of perivascular infiltration of partially degranulated mast cells stained by Giemsa. ×100 (*A* and *C*), ×250 (*B*, *D inset*, and *E*), or ×400 (*E inset*).

acted with murine MC/9 mast cells by flow cytometry (our unpublished observations). At variance with the paradigm of protease-activated receptors (5, 28), but analogous to the recognition of the urokinase receptor (29), the interaction of Factor Xa with the A1 extracellular region of EPR-1 (21) did not require an intact catalytic active site in the ligand. Rather, EPR-1 recognition of Factor Xa was mediated by the inter-EGF sequence Leu^{83}-Leu^{88}, which acquired receptor binding specificity in the active protease, but not in the zymogen Factor X (23). Accordingly, this peptide sequence recapitulated the pathway of Factor Xa–mediated inflammation, inducing rat paw edema and accumulation of activated and degranulated mast cells in situ. While Factor Xa modulation of vascular cell gene expression and aortic smooth muscle cell proliferation required binding to EPR-1 followed by a secondary step of ligand-dependent proteolysis (22), other signaling events can be directly triggered by physical receptor occupancy, as shown by the ability of anti–EPR-1 mAbs to increase cytosolic Ca^{2+} in isolated T cells and to potentiate mononuclear cell proliferation (9).

The inability of the thrombin-specific antagonist Hirulog (24) to affect Factor Xa–induced inflammation in vivo confirmed the specificity of this pathway, and ruled out a potential participation of downstream activation of coagulation. On the

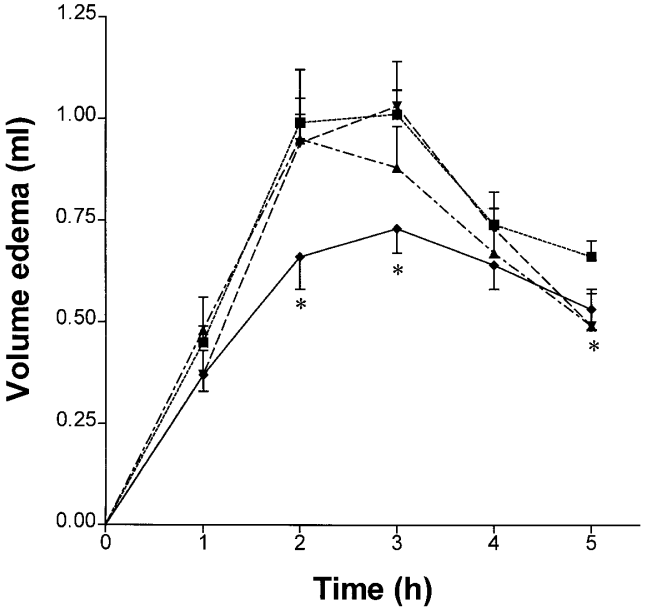

Figure 4. Participation of EPR-1 in carrageenin-induced edema in vivo. Animals were injected with 1% carrageenin simultaneously with 10 μg (▼), 30 μg (▲), or 100 μg (◆) of the EPR-1–derived AG1 peptide S^{123}PGKPGNQNSKNEPP^{137}, or 100 μg (■) of EPR-1–derived AG2 peptide P^{136}PKKRERERSSHCYP^{150}, before determination of paw edema at the indicated time intervals. Data are the mean±SEM of three independent experiments.

other hand, the ability of the EPR-1–Factor Xa interaction to enhance prothrombin activation on several vascular and perivascular cell types (19, 20, 23, 26) may provide an additional mechanism to potentiate this inflammatory response in vivo. Increase in vascular permeability (25) with extravasation of coagulation zymogens may favor monocyte/macrophage activation of Factor X to Xa (30) in a localized membrane microenvironment, with enhanced inflammation by Factor Xa (this study) or thrombin (24), generated through the EPR-1 pathway. In this context, the EPR-1–derived AG1 peptide, which comprised the receptor binding sequence for Factor Xa and inhibited EPR-1–dependent prothrombin activation (21), significantly reduced carrageenin-induced edema in vivo.

The potential pathophysiological relevance of these observations is reflected in the pivotal role of leukocytes in vascular injury and atherosclerosis (7). As a target of the Factor Xa–EPR-1 pathway described here, mast cells (31, 32) are ideally positioned to initiate and amplify a broad range of vascular and inflammatory cell responses, including acute angioedema or urticaria. Mast cell release of inflammatory mediators like histamine/serotonin, leukotrienes, PAF, and cytokines, triggers increased vascular permeability, enhanced leukocyte activation (33), upregulation of β_2 integrin function (34), and increased leukocyte rolling and adherence to endothelium (35, 36), thus further exacerbating tissue injury and disruption of the endothelial cell monolayer (7).

In summary, by mimicking the EPR-1 recognition of Factor Xa (26), we have identified a novel molecular interface between inflammation and coagulation that may contribute to vascular and tissue injury in vivo. Antagonists of this pathway, like the EPR-1–derived AG1 peptide (21), may prove beneficial to selectively target the inflammatory functions of blood proteases in vivo without affecting their normal hemostatic properties.

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