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Factor XII in Inflammation and Wound Healing

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Abstract

Purpose of review.—This review describes the contribution of coagulation factor XII (FXII) in sterile inflammation and wound healing with a focus on recently identified roles for zymogen FXII in neutrophil functions.

Recent findings.—Recent studies have identified an important role for FXII in neutrophil trafficking. In particular, following neutrophil activation, autocrine FXII signals through its receptor urokinase plasminogen activator receptor (uPAR) on the neutrophil surface to upregulate neutrophil functions. The sum of these activities leads to neutrophil adhesion, chemotaxis, and neutrophil extracellular (NET) formation. Downregulating FXII-mediated signaling in neutrophils is associated with improved wound healing.

Summary.—These recent findings add to our understanding of the sophisticated role of FXII *in vivo* and create new avenues of research for the treatment of chronic inflammatory diseases.

Keywords

factor XII; uPAR; neutrophils; inflammation; wound healing; neutrophil extracellular traps

INTRODUCTION

Factor XII (FXII) is the zymogen of serine protease, factor XIIa (FXIIa). FXII enzymatic activities are well-established and FXIIa inhibition has emerged as a potential target to prevent thrombosis without increasing bleeding risk [1–5]. In contrast, few zymogen FXII-initiated functions have been appreciated. Older reports indicate that FXII deficiency is associated with decreased migration of inflammatory cells into skin windows [6]. In human plasma, FXII and FXIIa assemble on the surface of neutrophils [7] and induce neutrophil aggregation [8]. In the central nervous system, FXII through urokinase plasminogen activator receptor (uPAR), induces a cytokine shift in dendritic cells contributing to neuroinflammation [9]. In acute respiratory distress syndrome, increased levels of FXII modulate the production and release of proinflammatory cytokines in the lung and correlate

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CONFLICTS OF INTEREST

None

with clinical outcome [10]. These FXII functions occur independently of plasma kallikrein and support that FXII directly engages in cellular proinflammatory responses [10]. In contrast to other components of the coagulation system, FXII has two epidermal growth factor domains and has mitogenic activity in endothelial and smooth muscle cells [11,12]. These combined data indicate that zymogen FXII influences cell biology independent of its protease function.

Here, we review the role of FXII in neutrophil trafficking at sites of sterile inflammation. We will focus on its contribution to wound healing and potential therapeutic applications in chronic inflammatory disease states.

FXII DEFICIENCY FACILITATES WOUND REPAIR

We recently reported our findings examining the role of FXII in neutrophil proinflammatory responses. We found that FXII deficient (*F12*$^{-/-}$) mice exhibit significantly faster wound closure rates compared to wild type (WT) mice [13]. Histologically, the re-epithelialization rate was significantly greater in *F12*$^{-/-}$ wounds compared to WT wounds. In regards to the composition of wounds, immunofluorescence studies indicated a substantial decrease in neutrophils with decreased neutrophil elastase and reduced citrullinated histone H3, a NET marker, compared to WT wounds.[14] Although bradykinin (BK) is considered the major inflammatory mediator of contact system activation and can directly influence leukocyte function[15,16], we did not observe decreased neutrophil recruitment in skin wounds when we examined bradykinin B2 receptor-deleted (*Bdkrb2*$^{-/-}$) mice and *Bdkrb2*$^{-/-}$ mice treated with a bradykinin B1 receptor antagonist, R715. These combined data indicated that reduced neutrophil recruitment in FXII deficient states had a beneficial effect on wound healing and this finding was independent of reduced bradykinin formation.

LOCAL FXII PRODUCTION IN NEUTROPHILS

An increased presence of contact system components including FXII, was recently demonstrated in bronchoalveolar lavage fluid from ARDS patients. Although loss of vascular integrity and dissemination of plasma proteins can in part explain these findings, the possibility of local intrapulmonary FXII production was raised [10]. The authors also refer to unpublished findings of intrapulmonary FXII production in patients with chronic lung pathologies such as idiopathic pulmonary fibrosis [10]. Similarly, in order to determine whether plasma-derived FXII directly contributes to leukocyte function, we targeted FXII production in the liver by *F12* siRNA [17]. We found that *F12* siRNA significantly reduced FXII coagulant activity to < 5% within 24 h. However, unlike *F12*$^{-/-}$ mice, *F12* siRNA-treated mice did not exhibit significant reduction in neutrophil recruitment into skin wounds [14]. Infusion of FXII into *F12*$^{-/-}$ mice such that the plasma FXII concentration was made physiologic, did not correct cell migration into the peritoneum after thioglycolate-induced peritonitis [14]. These studies indicated that decreased neutrophil migration in *F12*$^{-/-}$ mice is not dependent on plasma FXII. Gene sequencing confirmed the presence of FXII cDNA in murine and human neutrophils while confocal immunofluorescence studies provided evidence that following neutrophil activation, FXII translocates from the intracellular compartment to the neutrophil surface [13].

THE FXII-uPAR INTERACTION IN NEUTROPHILS

The presence of FXII in neutrophils prompted investigations to determine its role in neutrophil functions. One would expect that FXII is secreted from these cells. Indeed, we confirmed that following neutrophil activation by fMLP, FXII content decreased in the cell fraction and increased FXII was detected in the supernatant over time [13]. It was previously shown that neutrophils provide a circulating platform for components of the contact-phase system [7]. Therefore, we examined if secreted FXII remained bound on the neutrophil surface. In search of putative FXII receptors, we focused on uPAR given prior studies that uPAR is functionally important for leukocyte activities [18–22] and also serves as a receptor for FXII on endothelial cells [12]. Co-immunoprecipitation studies showed that FXII and uPAR interact on the neutrophil surface (unpublished data). Surface plasmon resonance studies showed that FXII bound to uPAR in a concentration-dependent manner. Binding kinetics showed a high-affinity interaction with k_{on} (association rate constant) of 2.03 ± 0.85 10^5 M^{-1} s^{-1}, k_{off} (dissociation rate constant) of 5.02 ± 2.82 10^{-3} s^{-1}, and K_D (equilibrium dissociation constant) of 37.1 ± 29.4 nM [14].

THE FXII-uPAR-pAkt2 SIGNALING AXIS

We sought to determine if the physiologic interaction between FXII and uPAR results in cell signaling. In neutrophils, the phosphoinositide 3-kinase (PI3K)/Akt signaling cascade is involved in adhesion, migration, degranulation and superoxide production [23]. In human and mouse neutrophils, only Akt1 and Akt2 are expressed but Akt1 has no defined role [23,24]. In contrast, Akt2 is the only isoform that translocates to the leading edge of neutrophils where it regulates key functions [23,24]. Akt2 KO mice have reduced neutrophil chemotaxis and impaired superoxide production in response to fMLP, C5a, and phorbol-12-myristate-13-acetate (PMA) [24]. Therefore, we examined if FXII and uPAR preferentially activate Akt2 by promoting Akt2 phosphorylation at Ser^{474}. In WT cells, $pAkt2S^{474}$ was substantially increased in response to fMLP and FXII/Zn^{2+} [14]. In *F12*$^{-/-}$ neutrophils, both fMLP and FXII/Zn^{2+} promoted $pAkt2S^{474}$. FXII-induced $pAkt2S^{474}$ proceeded through uPAR since *Plaur*$^{-/-}$ neutrophils did not exhibit $Akt2S^{474}$ phosphorylation in response to FXII/Zn^{2+} while this was partly preserved with fMLP [14]. These data showed that in neutrophils, the FXII-uPAR interaction induced Akt2 phosphorylation.

During these signaling studies, FXII remained a single chain (~ 78kDa) on reduced SDS-PAGE, suggesting that FXII did not auto-activate to FXIIa (two-chained) during its incubation with neutrophils. However, it was recently shown that single chain FXII exhibits weak proteolytic activity [25]. In order to conclusively determine if the effect of FXII on neutrophils is a zymogen property or an enzymatic activity we generated a double FXII mutant, termed FXII-D, that consists of two mutations: FXII Locarno (FXII-R353P mutation) [26] and FXII-S544A (an alanine substituting the active site serine) [25]. FXII-D lacks all enzymatic activity and is incapable of contact-activation [14]. Independent of its inability to generate enzymatic activity, FXII-D was able to promote pAkt2. These studies, along with kinetic enzymatic assays, showed that FXII-mediated signaling in neutrophils is a zymogen FXII function.

Given that the plasma concentration of FXII is ~ 450 nM and since FXII action on neutrophils relates to its zymogen form, the question arises as to why FXII-uPAR do not interact *constitutively*. Indeed, there are rate-limiting steps that regulate FXII-uPAR binding. It is ingrained in our understanding that uPAR is membrane-anchored on the surface of neutrophils; one can assume that this association occurs through the entire life cycle of neutrophils. However, it was previously shown that in resting neutrophils, uPAR molecules are stored in two distinct intracellular compartments: easily mobilizable secretory vesicles and specific granules [27]. Stimulation of neutrophils with various agonists results in a rapid increase in the expression of uPAR and is accompanied by the translocation of increasing amounts of uPAR to the neutrophil plasma membrane [27]. This step-wise appearance of FXII receptor binding sites on activated neutrophils can explain in part, why the FXII-uPAR interaction is not operating continuously. Another key aspect to consider is FXII's cell-binding ability. *In vivo*, circulating FXII is not constitutively bound to cells in the intravascular compartment; it does so, only when the local *free* zinc ion concentration rises significantly from physiologic plasma levels of ~ 20 nM [28,29] to a micromolar (μM) range. The heavy chain of FXII contains four zinc binding sites which are in close proximity to its artificial surface- and cell-binding regions [30–37]. The source of extracellular zinc was previously shown to derive from activated cells such as platelets and endothelial cells [37,38]. Neutrophils themselves contain zinc-sensing receptors and may potentiate the mobilization of zinc towards the extracellular compartment during inflammation. To this end, surface plasmon resonance studies confirmed that FXII did not interact with uPAR in the absence of zinc. In sum, FXII-uPAR complex formation is a tightly regulated process *in vivo* [14].

Since FXII signals in neutrophils, we examined if it regulates cell activities. FXII-mediated signaling had not been previously studied in neutrophils. When cells were stimulated with fMLP, we found that *F12*$^{-/-}$ neutrophils had significantly reduced adherence to integrin binding glycoproteins fibronectin and fibrinogen. Using a novel microfluidic chamber and time lapse microscopy, we determined that FXII is a very potent chemotaxin [14]. Dissecting the mechanisms involved downstream of pAkt2, we found that FXII-uPAR upregulate the surface expression of αMβ2 integrin, increase intracellular Ca^{2+}mobilization, and promote histone citrullination and NET formation [14]. When neutrophils were pretreated with LRG20, a uPAR Domain II inhibitory peptide that blocks the FXII-uPAR interaction, pAkt2 formation and extracellular DNA release were significantly reduced in FXII-stimulated WT neutrophils [14].

In sum, data show that the FXII-uPAR interaction contributes to cell adhesion, chemotaxis, and NETosis (Figure 1). uPAR itself is a regulator of integrin activity modulating their affinity and avidity [18,19]. In endothelial cells, occupancy of uPAR by FXII promotes the formation of uPAR-integrin complexes that subsequently activate intracellular signaling pathways (outside-in signaling) [12]. Whether this lateral association between FXII-uPAR and αMβ2 integrin directly contributes to neutrophil functions is the focus of on-going investigations in our lab. Importantly, what the aforementioned studies support is that the FXII-uPAR interaction in neutrophils *precedes* NET formation. Finding a pathway for NET formation through FXII is novel and therapeutically applicable given the current paradigm

that their relationship lies solely on contact activation of circulating FXII on the surface of preformed NETs [39].

HARNESSING THE FXII-uPAR AXIS IN NEUTROPHILS - THERAPEUTIC POTENTIAL

Efficient wound repair requires the coordinated effort of many different cell types. Following hemostasis, a healing wound typically goes through phases of inflammation, proliferation and remodelling/scar formation [40]. The inflammatory phase, is an important part of the wound healing response. Neutrophils are the first inflammatory cells to be recruited to the wound site. Their primary function is to prevent infection but their ability to generate cytokines and growth factors allows them to do more than simply sterilize wound sites [40]. In fact, studies demonstrated that a variety of genes encoding proteins that recruit more neutrophils, promote angiogenesis, and stimulate keratinocyte and fibroblast proliferation is upregulated in wound-resident neutrophils compared to circulating neutrophils [41].

While neutrophils are an integral part of the inflammatory response, recent evidence suggests that timely resolution of inflammation is equally important for wounds to progress through subsequent phases of healing [42]. Therefore, continued recruitment, or buildup of active neutrophils, can prolong inflammation and contribute to the development of chronic wounds. Animal models show that excess neutrophil influx into wound sites impairs keratinocyte migration and proliferation [43]. In addition, the persistence of neutrophils in wounds leads to unrestricted proteolytic activity mediated by neutrophil granular enzymes leading to matrix disruption and proteolysis of growth factors and their receptors [44]. Indeed, neutrophil elastase was previously shown to be markedly increased in the exudate of non-healing human wounds [45]. Circulating neutrophils from diabetic humans are primed to produce NETs [46] and NETosis delayed diabetic wound healing in mice and humans [47]. In contrast, high levels of alpha1-antitrypsin, an *in vivo* neutrophil elastase inhibitor, are a biomarker of successful wound healing [44]. Altogether, these data support that limiting the activity of neutrophils may be beneficial for the treatment of chronic, non-healing wounds.

Our prior studies showed that a) the effect of FXII on neutrophils is a bone marrow-endowed function and b) the degree of inflammatory component in wounds is dependent upon neutrophil FXII [14]. More recently, we examined the crosstalk between neutrophils and keratinocytes in a purified system *in vitro*. We determined that addition of stimulated WT neutrophils to skin epithelial cells (SECs) resulted in significant inhibition of wound closure (unpublished data). Addition of stimulated $F12^{-/-}$ neutrophils to adherent SECs, resulted in considerably accelerated wound closure compared to WT neutrophils. To confirm that loss of FXII-uPAR activity in neutrophils alone accelerates SEC migration, WT neutrophils were pre-treated with LRG20, a uPAR Domain II peptide, before they were stimulated with fMLP or PMA and added to SECs. We found that SEC migration rate was significantly increased in the presence of LRG20-treated neutrophils compared to activated WT neutrophils. These studies confirm that a) wound closure is dependent on the activation state of neutrophils; b)

downregulation of FXII-mediated signaling in neutrophils, restricts neutrophil activation and improves epithelial cell migration.

How can these findings be translated therapeutically? Systemic inhibition of FXII could adversely affect endothelial cell proliferation [12], prekallikrein activation [25], and the structure of fibrin [48]. Similarly, ubiquitous uPAR inhibition can impair access of other ligands and affect fibrinolysis. To circumvent these issues, a potential approach is to selectively abrogate the FXII-uPAR interaction specifically on the surface of neutrophils. Our on-going studies show that site-specific inhibition of FXII-uPAR binding, can effectively curb neutrophil responses while preserving systemic FXII functions and uPAR availability to other ligands (unpublished data).

We found that FXII signaling in neutrophils upregulates the surface expression of αMβ2 integrin, previously shown to be the main receptor for neutrophil adhesion on fibrinogen and also responsible for stable heterotypic cell-cell interactions with platelets [49–51]. It would be interesting to determine if FXII's action on neutrophils, and downstream integrin surface expression, creates additional binding sites between neutrophils, platelets, and fibrin to facilitate thrombus propagation. Recognition of such a function for FXII would be novel and complementary to its enzymatic activity.

Overall, targeted abrogation of the FXII-uPAR interaction can be therapeutically relevant in disease states where neutrophils contribute to disease. However, one should consider the elucidation of tissue-specific neutrophil recruitment patterns. In this context, a recent study identified that the neutralization of cathepsin G in mouse models did not affect neutrophil extravasation during acute lung inflammation but instead, specifically limited atherogenic leukocyte recruitment [52]. Tissue-specific neutrophil recruitment patterns have been identified for the kidney, lung and liver [53], and should be taken into consideration when designing anti-inflammatory strategies. Similarly, future studies should examine if simultaneous silencing of FXII-uPAR and possible lateral partners of their complex, are more efficient at overriding redundant neutrophil trafficking cues.

Finally, one cannot avoid addressing the long-awaited question as to physiologic role of FXII. Why does it exist and why has it persisted *in vivo*. Animal studies appear to support the notion that FXII deficiency comes with experimentally demonstrated "benefits" only. To the consternation of investigators in the field, FXII deficiency is not associated with obvious disease that makes it easy to show why investigation on it is important and relevant. However, this very fact may make FXII a better target for disease modification. It is also worth noting a key point. A systematic assessment of FXII deficient individuals is presently lacking. This is primarily due to the fact that these individuals do not constitute a "patient" population per se as they do not suffer from an obvious disorder e.g. bleeding tendency. In addition, it is inherently more difficult to capture positive phenotypic changes (such as faster wound healing) among individuals, than negative traits. A detailed epidemiologic study is lacking to conclusively determine beneficial (or not) characteristics in these individuals. Unless such study is completed, the role of FXII in physiology cannot be presumed to be non-existent. Indeed, FXII is highly conserved among mammals. One would assume this is due to its participation in important functions. Prior studies from our group and others

show that FXII is a growth factor promoting endothelial cell proliferation and *in vitro* and *in vivo* angiogenesis [12]. This FXII function is independent of its catalytic and contact activation properties. Being part of the kallikrein kinin system (KKS), FXII contributes to liberation of bradykinin, a biologic peptide that regulates vessel tone, intravascular nitric oxide (NO), prostacyclin (PGI_2) and tissue plasminogen activator liberation [54–56]. We estimate that FXIIa accounts for ~50% of constitutive plasma bradykinin levels. In fact, a gain-in-function mutation in FXII is mechanistically associated with Type III angioedema. Given that single-chain FXII has been proposed as an activator of prekallikrein [25], it may be an initiator of activation of the KKS independent of contact activation. All these studies support that FXII and its various forms contribute to both physiologic and pathologic events.

We believe that our studies on FXII functions in neutrophils, as neutrophils themselves, ought to be considered separately in non-sterile vs. sterile inflammatory states. During infection(s), neutrophil recruitment constitutes the first line of defense against pathogens. As inflammation progresses, DNA associated with NETs functions as a “contact” substrate, promptly activating FXII leading to fibrin formation. The fibrin scaffold generated by this synergism between the coagulation pathway (FXII and the extrinsic pathway) and neutrophils, entraps microbes within microvessels limiting the systemic spread of infection while enhancing the clearance of pathogens by activated leukocytes [57,58]. The process appears to be more complex *in vivo* and pathogen-specific. FXII deficiency confers protection from *Klebsiella pneumoniae* sepsis but not from *Streptococcus pneumoniae* sepsis [59]. Therefore, FXII in specific infectious settings, can be viewed as an important contributor to innate immune functions.

From a teleological viewpoint, these functions appear to have emerged as part of the host immune response to invading pathogens. However, there has been a recent epidemiological transition associated with decreased incidence of infections and the development of chronic inflammatory diseases. In these scenarios of sterile inflammation, neutrophils have proven to damage host tissues, contribute to the development of autoimmunity and lead to a multitude of adverse outcomes [47,60–62]. In these settings, the contribution of FXII to neutrophil activation may be maladaptive and targeting its activity is therapeutically relevant.

CONCLUSION

Recent studies have identified a novel signaling pathway in neutrophils that involves autocrine FXII and its receptor uPAR, that upregulate neutrophil functions. The FXII-uPAR axis becomes operative after initial cell activation and appears to sustain neutrophil primeness and propagates neutrophil pro-inflammatory responses. Targeted abrogation of the FXII-uPAR interaction downregulates neutrophil activation, resolves perpetuating inflammation and promotes wound healing. Future studies are required to establish its therapeutic potential in other disease states driven by excess sterile inflammation.

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Key points

- Recent studies have identified distinct FXII functions in sterile inflammation that occur independently on its protease activity.
- Autocrine FXII-uPAR-pAkt2 upregulate neutrophil functions and promote neutrophil trafficking at sites of sterile inflammation.
- Continued recruitment, or buildup of active neutrophils, can prolong inflammation and contribute to the development of chronic wounds. Targeting FXII-mediated signaling in neutrophils improves wound healing potential.
- Selective abrogation of the FXII-uPAR interaction in neutrophils can be therapeutically effective in chronic wounds and potentially, in other chronic inflammatory disease states.

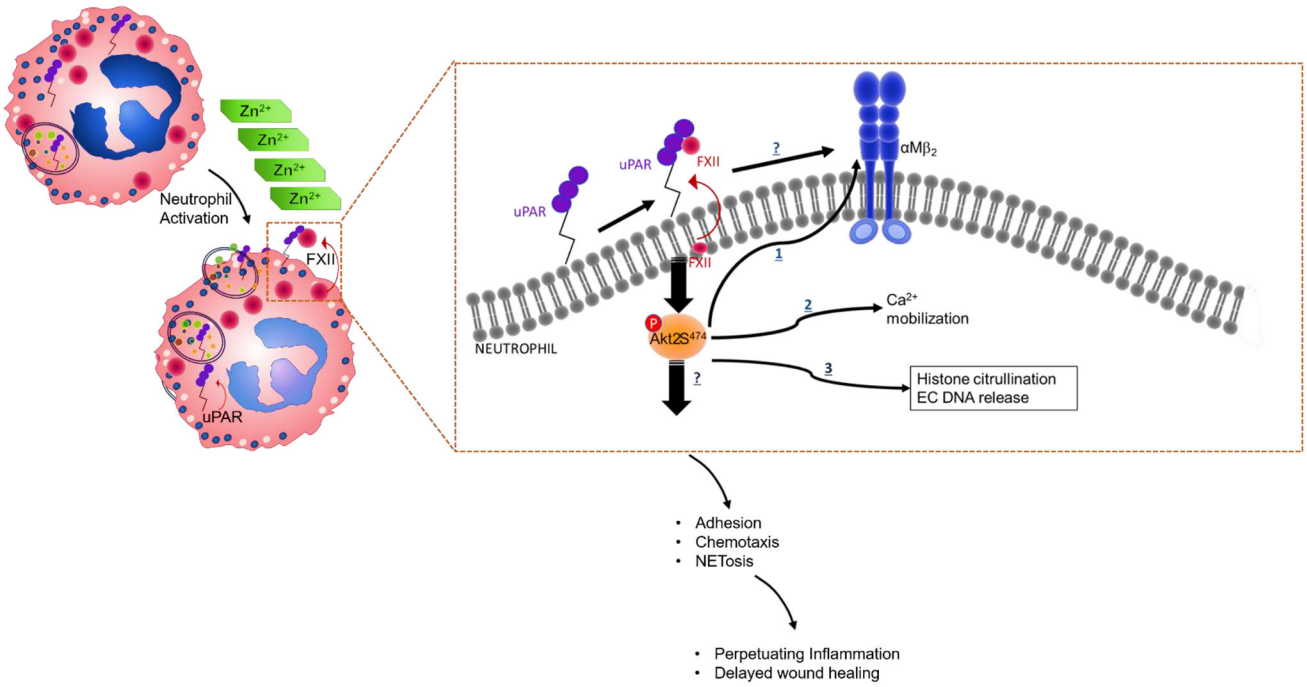

Figure 1: Role of FXII in inflammation and wound healing.

Following initial neutrophil activation, autocrine FXII and uPAR interact on the neutrophil surface to promote Akt2S^{474} phosphorylation. Propagation of FXII-mediated neutrophil activities leads to 1) surface expression of αMβ2 integrin, 2) increase in intracellular calcium (Ca^{2+}), and 3) histone citrullination and extracellular (EC) DNA release. The sum of these activities sustains neutrophil proinflammatory responses and contributes to delayed wound healing. Neutrophil-derived FXII activities are distinct from plasma FXII functions of contact activation on the surface of preformed NETs. Areas of active investigation to further delineate the FXII-uPAR interactome are marked as “?”.