Tissue Factor-Dependent Chemokine Production Aggravates Experimental Colitis

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Tissue Factor–Dependent Chemokine Production Aggravates Experimental Colitis

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Tissue factor (TF) is traditionally known as the initiator of blood coagulation, but TF also plays an important role in inflammatory processes. Considering the pivotal role of coagulation in inflammatory bowel disease, we assessed whether genetic ablation of TF limits experimental colitis. To this end, wild-type and TF-deficient (TFlow) mice were treated with 1.5% dextran sulfate sodium (DSS) for 7 d, and effects on disease severity, cytokine production and leukocyte recruitment were examined. Clinical and histological parameters showed that the severity of colitis was reduced in both heterozygous and homozygous TFlow mice compared with controls. Most notably, edema, granulocyte numbers at the site of inflammation and cytokine levels were reduced in TFlow mice. Although anticoagulant treatment with daiparin of wild-type mice reduced local fibrin production and cytokine levels to a similar extent as in TFlow mice, it did not affect clinical and histological parameters of experimental colitis. Mechanistic studies revealed that TF expression did not influence the intrinsic capacity of granulocytes to migrate. Instead, TF enhanced granulocyte migration into the colon by inducing high levels of the granulocyte chemoattractant keratinocyte-derived chemokine (KC). Taken together, our data indicate that TF plays a detrimental role in experimental colitis by signal transduction–dependent KC production in colon epithelial cells, thereby provoking granulocyte influx with subsequent inflammation and organ damage.

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INTRODUCTION

Inflammatory bowel diseases are chronic and recurrent disorders of the gastrointestinal tract, of which both etiology and pathogenesis remain only partly understood (1–3). Crohn’s disease and ulcerative colitis, the two major forms of inflammatory bowel disease, are characterized by intestinal inflammation as a result of transmural infiltration of granulocytes, lymphocytes, monocytes or macrophages and plasma cells, accompanied by the excessive production of free oxygen radicals, ultimately leading to mucosal disruption and ulceration (4).

Inflammatory bowel disease patients have an approximately three-fold increased risk of venous thrombosis compared with age, sex and geographically matched controls (5), and these thrombotic complications significantly contribute to morbidity and mortality (6,7). The frequency of venous thrombosis in patients suffering from inflammatory bowel disease lies between 1 and 8%, whereas postmortem studies show thrombotic events in up to 40% of patients (8). Thrombotic risk increases with active disease, although >30% of patients have inactive disease at the time thrombosis is diagnosed (6). Overall, it is thus apparent that a “hypercoagulable state” is frequently observed during inflammatory bowel disease and that coagulation abnormalities are an intimate part of the clinical picture of inflammatory bowel disease (9). However, whether exacerbated coagulation participates in the etiology of inflammatory bowel disease remains controversial. It is interesting to note that the risk of inflammatory bowel disease is lower than expected in patients with inherited bleeding disorders (10) and that several observational studies show a beneficial effect of anticoagulant treatment (11–14). However, a randomized clinical trial did not confirm this beneficial effect of heparin treatment (15). In line with a limited effect of blood coagulation in the etiology of inflammatory bowel disease, prothrombotic factor V Leiden mice were indistinguishable from wild-type animals in dextran sul-
fate sodium (DSS)-induced experimental colitis (16).

Tissue factor (TF), a 47-kDa transmembrane glycoprotein, initiates blood coagulation via formation of an enzymatic complex with factor (F)VIIa, eventually leading to the activation of thrombin and the formation of fibrin (17,18). Beyond its well-established role in coagulation, TF has been suggested to play a paramount role in inflammatory conditions. Indeed, TF may activate protease activated receptor (PAR) signaling leading to the production of (among others) inflammatory cytokines (19,20). The increase of both interleukin (IL)-6 and IL-8 levels in coagulating blood (21) is indicative of TF expression and cytokine production either through direct cellular effects or via downstream coagulation factors. Because lipopolysaccharide-induced IL-6 and keratinocyte-derived chemokine (KC) (a mouse analog of IL-8) production in cultured macrophages and in anticoagulated whole blood is lower in heterozygous TF-deficient cells than in wild-type cells, one might argue that TF influences cytokine production without intervention of other coagulation factors (22). The in vivo significance of TF for host defense is most evident because TF blocking agents reduce the risk of lipopolysaccharide-induced mortality. For instance, pretreatment with site-inactivated FVIIa delays death upon administration of lethal amounts of Escherichia coli sepsis (23). Moreover, immunization of mice with a polyclonal TF antibody attenuates coagulopathy and mortality in a lethal Escherichia coli sepsis model in baboons (23). Additionally, TF-dependent signal transduction induces KC production in colon epithelial cells, thereby provoking granulocyte influx with subsequent inflammation and organ damage.

**MATERIAL AND METHODS**

**Animals**

TFlow mice, previously generated (27) and provided by Dr. Mackman (University of North Carolina, Chapel Hill, NC, USA), were bred at the animal care facility of the Academic Medical Center. Homozygous TFlow mice are mice deficient for murine TF that are rescued from embryonic lethality by introducing a human TF minigene containing the human TF promoter and human TF cDNA leading to TF activity of around 1% (relative to mouse TF). Heterozygous TFlow mice express 50% murine TF and also contain the human TF minigene. C57BL/6J wild-type mice were purchased from Charles River (Someren, the Netherlands). All mice were bred and maintained at the animal care facility at the Academic Medical Center according to institutional guidelines, with free access to food and water. Animal procedures were carried out in compliance with the Institutional Standards for Humane Care and Use of Laboratory Animals. All mice were housed in the same temperature-controlled room with alternating 12-h light/dark cycles. Mice at an age of 8–10 wks were used in the colitis model as described below.

**Induction of Colitis and Assessment of Disease Progression**

Mice received 1.5% DSS (molecular weight of 40 kDa; TdB Consultancy, Uppsala, Sweden) in filter-purified drinking water for 7 d ad libitum as described before (16). Control mice received filtered water alone. For the anticoagulant experiments, mice received 80 IU/kg/d dalteparin (based on [28]). Body weight loss (calculated as the percentage difference between the original weight and the weight at the time of sacrifice) and stool consistency were used for clinical assessment of disease severity.

**Histological Analysis**

Histological analysis was performed essentially as described before (29,30). In detail, the longitudinally divided colons were rolled up, fixed in 4% formalin and embedded in paraffin for routine histology. The following parameters were scored in a blinded fashion by a pathologist: (i) percentage of area involved, (ii) number of follicle aggregates, (iii) edema, (iv) fibrosis, (v) erosion/ulceration and (vi) crypt loss and infiltration of (vii) mono- and (viii) polymorphonuclear cells. Percentage of area involved and crypt loss were scored on a scale ranging from 0 to 3 as follows: 0, normal; 1, <10%; 2, 10–50%; and 3, >50%. Erosions were defined as 0 if the epithelium was intact, as 1 for the involvement of the lamina propria, as 2 for ulcerations involving the submucosa and as 3 when ulcerations were transmural. The severity of the other parameters was scored on a scale of 0–3 as follows: 0, absent; 1, weak; 2, moderate; and 3, severe. The score ranges from 0 to a maximum of 24 points.

**Cytokines and Chemokines Measurement**

IL-6, monocyte chemoattractant protein (MCP)-1 and tumor necrosis factor
(TNF-α) were measured in colon homogenates using the BD Cytometric Bead Array Mouse Inflammation Kit (Becton Dickinson, Lincoln Park, NJ, USA) as described before (31). Detection limits were 10 pg/mL. KC was measured using an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions with a detection limit of 31.25 pg/mL.

**Colon Tissue Culture**

Colon sections, collected from wild-type and TFlow mice, were washed in Hanks’ balanced salt solution (HBSS) medium containing penicillin and streptomycin, after which colon sections of approximately 2 mm were placed in RPMI 1640 medium containing 1% fetal calf serum, penicillin and streptomycin. Colon sections were stimulated with FVIIa (10 and 100 nmol/L) for 24 h, after which the supernatant was collected for analysis of KC levels.

**Patients**

Rest material of ulcerative colitis patients was used for immunohistochemistry according to the guidelines of the Medical Ethical Committee of the Academic Medical Center (AMC), University of Amsterdam, Amsterdam, the Netherlands.

**(Immuno)histochemistry**

Colon sections were harvested, fixed, paraffin embedded and stained with hematoxylin and eosin according to routine procedures. For immunohistochemistry, 4-μm sections were deparaffinized, endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide for 15 min at room temperature and antigen retrieval was performed for 10 min at 100°C in 10 mmol/L sodium citrate buffer, pH 7.4 (PAR-2 and TF stainings), or for 15 min at 37°C with 0.025% pepsin in 0.1 mol/L HCl (granulocyte staining) or for 30 min at 37°C with 0.1% trypsin. After subsequent washing at room temperature and rehydration in phosphate-buffered saline, sections were blocked with TENG-T (100 nmol/L Tris-HCl, pH 8.0, 50 mmol/L EDTA, 1.5 mmol/L NaCl, 2.5% gelatin, 0.5% Tween) for 10 min (lymphocyte antigen 6 complex, locus G [LY-6G]) or with 10% normal goat serum in phosphate-buffered saline for 30 min (PAR-2) or with normal rabbit serum for 10 min (fibrin) and subsequently incubated with antibodies against LY-6G (fluorescein isothiocyanate labeled) (1:1,000; Pharmingen, San Diego, CA, USA), TF (1:50; American Diagnostica product number 4509; Stamford, CT, USA), PAR-2 (1:80; SAM11; Santa Cruz, CA, USA) or fibrinogen (1:1,000; biotinylated goat anti-mouse fibrinogen antibody; Accurate Chemical and Scientific, Westbury, NY, USA [32]) overnight at 4°C. For LY-6G staining, the slides were first incubated for 15 min with a rabbit–anti-FITC antibody (1:1,000), after which the slides were incubated with the appropriate horseradish peroxidase–conjugated secondary antibodies. For fibrin stainings, slides were incubated for 30 min with avidin-biotin-peroxidase complex (DAKO K0355). For all stainings, 3,3′-diaminobenzidine (DAB) staining was used to visualize peroxidase activity. Slides were photographed with a microscope equipped with a digital camera (Leica CTR500; Leica Microsystems, Wetzlar, Germany). For granulocyte stainings, we scored the number of positive cells in 10 different fields (200x magnification).

**Granulocyte Migration**

Granulocytes were isolated from EDTA-anticoagulated blood by polymorph-prep density gradient centrifugation (Axis-Shield, Oslo, Norway). Granulocytes were collected, diluted and labeled with CellTracker Green as described before (33,34). Briefly, the fluorescent dye was incubated with the cells for 30 min at 37°C. Cells were washed once in medium to remove excessive soluble dye and then incubated for 1 h at 37°C in medium without dye. Subsequently, cells (1 x 10^7) were transferred to 3 mmol/L pore size HTS FluoroBlok Cell Culture inserts, which were inserted in fitting 24-well plates containing IL-8 as the chemoattractant in the presence or absence of FVIIa. Promptly, fluorescence values representing the number of cells on the bottom side of the insert were read at 37°C on a Series 4000 CytoFluor Multi-Well Plate Reader (PerSeptive Biosystems, Framingham, MA, USA). The raw fluorescence data were corrected for background fluorescence and fading of the fluorophore; the data were then plotted with GraphPad Prism 4.

**Data Analysis**

Statistical analyses were performed using one-way analysis of variance, followed by the Mann-Whitney U test. All values are reported as means ± SE. Statistical significance was set at P < 0.05.

**RESULTS**

**TFlow Mice Are Protected against DSS-Induced Colitis**

The development of DSS-induced colitis was determined in wild-type, heterozygous and homozygous TFlow mice. Importantly, wild-type mice developed colitis, as evident from weight loss, reduced length of the colon and increased weight of the colon (Figure 1). Although the TFlow mice did develop colitis as well, it was clearly not as severe as in the wild-type mice. In detail, TFlow mice showed a clear reduction in weight loss compared with wild-type mice. Moreover, the colon length of wild-type mice that received DSS decreased by 23%, whereas the colon length decreased by about 10% for both homozygous and heterozygous TFlow mice. Finally, the colon weight increased by around 57% in wild-type animals, while in the TFlow animals, the colon weight increased by approximately 18% in homozygous TFlow animals and 27% in heterozygotes.

**Reduced Inflammation and Coagulation in TFlow Mice**

To confirm the macroscopic data showing that TFlow mice are protected against DSS-induced colitis, we next scored edema, crypt loss, granulocytes, mononuclear cells, fibrosis and ulceration on HE-
shown in Figure 3A, dalteparin treatment in DSS-induced colitis. As we evaluated the effect of anticoagulant mechanisms by which TF is detrimental, discriminate between these two potential or by its cellular signaling properties. To colon. This is particularly interesting because TF is known to influence cell migration (mainly in the setting of tumor cells) (35,36). Consequently, we determined whether TFlow granulocytes (obtained from TFlow mice) are affected in their migratory response compared with wild-type granulocytes. However, FVIIa-stimulated wild-type and TF granulocytes did migrate to IL-8 to a similar extent (data not shown). Thus, TF seems not to alter the intrinsic capacity of granulocytes to migrate into inflamed tissues. An alternative explanation for the increased influx of granulocytes in wild-type versus TFlow colon might be the local expression of granulocyte chemoattractants (IL-8, or its murine homolog KC). Indeed, KC levels are high in colon homogenates of wild-type mice treated with DSS but not in both heterozygous and homozygous TFlow animals (Figure 4A). To support the hypothesis that low numbers of granulocytes in the colon of TFlow mice are due to impaired TF-dependent KC production, we first analyzed expression levels of TF and its cognate receptor PAR-2 in溃疡性结肠炎 (Figure 4B) and Crohn’s disease patients (data not shown). As shown in Figure 4B, TF and PAR-2 expression is most prominent on colonic epithelial and goblet cells, although TF is also expressed on the inflammatory infiltrate. Next, we determined whether the colon would produce KC in response to FVIIa. Indeed, ex vivo stimulation of small colon sections obtained from wild-type animals with FVIIa significantly induced KC production in a dose-dependent manner (Figure 4C). Similar experiments using TFlow colon sections did not show increased KC production after FVIIa stimulation (Figure 4C). Finally, we stimulated colon sections with a PAR-2 agonist peptide and observed that PAR-2 activation also led to KC production, thereby suggesting an important role for TF/FVIIa/PAR-2 signaling in local KC production (data not shown).

**DISCUSSION**

We studied the role of TF in DSS-induced colitis, and we have shown that TF deficiency protects mice from DSS-

**Anticoagulant Treatment Does Not Limit DSS-Induced Colitis**

TF may exert its aggravating effect in colitis by either its procoagulant activity or by its cellular signaling properties. To discriminate between these two potential mechanisms by which TF is detrimental, we evaluated the effect of anticoagulant treatment in DSS-induced colitis. As shown in Figure 3A, dalteparin treatment did significantly reduce DSS-induced colonic levels of fibrinogen to similar levels as observed in DSS-treated heterozygous TFlow mice. Interestingly, however, dalteparin treatment did not protect mice against DSS-induced colitis as opposed to TFlow mice. As shown in Figure 3B, clinical parameters of experimental colitis tested (body weight loss, colon length and colon weight) were not significantly different in dalteparin-treated versus -untreated control mice. However, the reduced cytokine levels do not diminish tissue damage, as evident from similar scores for edema, crypt loss, granulocytes, mononuclear cells, fibrosis and ulceration (Figure 3D). Ly-6G stainings do not show any difference in the number of granulocytes in dalteparin-versus control-treated mice (Figure 3E), although KC levels were reduced in the dalteparin-treated mice.

**TF-Dependent Signaling in Colon Epithelial Cells Induces KC Levels**

It is well accepted that granulocytes play a detrimental role during the development of inflammatory bowel disease. Interestingly, a major difference between wild-type animals and TFlow mice in response to DSS-induced colitis is the influx of granulocytes into the inflamed colon. This is particularly interesting because TF deficiency reduces the severity of clinical indices of DSS colitis in mice. (A) TFlow mice fed 1.5% (w/v) DSS in their drinking water for 7 d were protected against body weight loss compared with wild-type mice. The TFlow mice presented smaller changes in colon length (B) and colon weight (C) (shown in mg/cm) than wild-type mice. Data are means ± SEM (n = 7). *P < 0.05, **P < 0.01, ***P < 0.001. **Figure 1. TF deficiency reduces the severity of clinical indices of DSS colitis in mice. (A) TFlow mice fed 1.5% (w/v) DSS in their drinking water for 7 d were protected against body weight loss compared with wild-type mice. The TFlow mice presented smaller changes in colon length (B) and colon weight (C) (shown in mg/cm) than wild-type mice. Data are means ± SEM (n = 7). *P < 0.05, **P < 0.01, ***P < 0.001. **Figure 2E, DSS induces the level of fibrinogen in both wild-type and TFlow animals. Interestingly, however, fibrinogen levels were significantly reduced in TFlow compared with wild-type mice. As shown in Figure 2C, Ly-6G stainings confirm the reduced number of granulocytes in the TFlow mice. In line with these reduced granulocyte numbers, DSS-induced inflammatory cytokine production was dramatically diminished in wild-type mice compared with both heterozygous and homozygous TFlow mice (Figure 2D). To assess local coagulant activity, we scored the levels of fibrinogen in the colon of DSS-treated or untreated control animals. As shown in Figure 2E, DSS induces the level of fibrinogen in both wild-type and TFlow animals. Interestingly, however, fibrinogen levels were significantly reduced in a dose-dependent manner in the TFlow mice.
induced experimental colitis. Both homozygous as well as heterozygous, TF$^{low}$ mice have significantly reduced scores for both clinical and histological parameters compared with wild-type mice. The reduced severity of experimental colitis correlated with reduced levels of proinflammatory cytokines in the TF$^{low}$ mice, whereas the number of granulocytes at the site of inflammation was also significantly reduced in TF$^{low}$ mice. These data are in perfect agreement with previous studies showing that a monoclonal TF-blocking antibody limits experimental colitis in wild-type animals (26), and our data thus underscore the importance of TF in inflammatory bowel disease.

To determine whether TF would aggravate experimental colitis by activating blood coagulation, wild-type mice were treated with the low–molecular weight heparin dalteparin. The selected dose of dalteparin is within the dose range used in patients and has previously been shown to effectively block coagulant activity and to limit murine diabetic nephropathy (28). However, dalteparin treatment did not ameliorate DSS-induced colitis, as evident from similar increases in colon weight, decreases in colon length and DSS-induced disease activity scores between treated and untreated animals. Although dalteparin treatment reduced fibrin levels and limited inflammatory cytokine production as efficient as heterozygous TF deficiency, anticoagulant treatment also did not limit granulocyte influx into the inflamed area. Thus, it seems that the procoagulant activity of TF does not significantly contribute to the development of experimental colitis. This result is particularly interesting considering the ongoing controversy on the importance of activated blood coagulation in inflammatory bowel disease. Indeed, a vascular component to the pathogenesis of inflammatory bowel disease was already proposed in 1934 (37), and small observational studies did show dramatic responses in patients with ulcerative colitis (11–14). Nine out of ten patients on combined heparin and sulphasalazine therapy became asymptomatic,
whereas patient number 10 had a partial response (12). Nadroparine treatment alone significantly improved endoscopic and histological signs of inflammation in 20 out of 25 patients (11), whereas dalteparin treatment resulted in complete remission in 6 out of 12 patients (13). Although a small clinical randomized trial could not confirm these beneficial effects (15), the inclusion criteria of mainly mildly affected patients and the short duration of LMWH treatment did not allow confirmative conclusions.

Probably the most important finding of our study is the large difference in influx of granulocytes into the inflamed colon between wild-type and TFlow mice in response to DSS treatment. This is particularly interesting, since granulocytes are of pivotal relevance in the clinical setting of inflammatory bowel disease, as evident from, for instance, the promising effect of Natalizumab and MLN02 for the treatment of Crohn’s disease and ulcerative colitis (38,39). Moreover, it is well known that TF induces migration of a large variety of cells (35,36). Although the presence of TF on granulocytes is under debate (40), we determined whether granulocytes derived from TFlow mice did have a hampered migratory capacity compared with granulocytes from wild-type mice. However, FVIIa-stimulated wild-type granulocytes migrated toward IL-8 as efficient as wild-type granulocytes, suggesting the observed differences in granulocyte influx are not due to the intrinsic migratory capacity of the granulocytes. Instead, we show that the low numbers of granulocytes in the colon of TFlow mice are most likely due to impaired TF-dependent KC production. Indeed, KC levels are significantly induced in wild-type mice treated with DSS but not in both homo- and heterozygous TFlow mice.
erozygous TFlow mice. In an apparent discrepancy, the absence of KC was previously shown to increase the susceptibility to DSS-induced colitis, mainly by preventing granulocyte infiltration into the inflamed colon (41). However, granulocyte numbers are tightly regulated during inflammatory conditions and both low as well as high numbers may be detrimental. Dalteparin treatment reduces KC levels in wild-type mice, but these levels are still significantly increased compared with TFlow animals. The decreased KC levels after anticoagulant treatment are most likely due to reduced thrombin levels. Indeed, it is well known that thrombin induces IL-8/KC levels in different cell types (42,43).

Interestingly, IL-8 (the human counterpart of KC) expression is significantly induced in the colonic mucosa of inflammatory bowel disease patients, and its expression is in direct proportion with the degree of inflammation (44,45), suggesting an essential role of IL-8 in the pathogenesis of inflammatory bowel disease. Moreover, colon epithelial cells are a known source of IL-8 in inflammatory conditions (46), and the FVIIa/TF complex was previously shown to induce IL-8 levels in several different cell lines (47–49). In agreement, we show that ex vivo FVIIa-dependent KC production is dramatically reduced in TFlow colon sections compared with colon sections of wild-type mice. These data suggest an important role for the TF/FVIIa complex in KC production and subsequent granulocyte influx during colitis.

Our data suggest that the TF/FVIIa complex aggravates experimental colitis depending on the signaling properties of TF. In line with this notion, activation of PAR-2 by either the TF/FVIIa complex or agonist peptides leads to IL-8 production in several cell lines, including epithelial cells of the gastrointestinal tract (47,50,51), but also in the whole colon section (this report). Moreover, promoter methylation of PAR-2 seems to correlate with the severity of ulcerative colitis (52), and PAR-2 deficiency was protective in DSS-, 2,4,6-trinitrobenzenesulfonic acid (TNBS)- and oxazolone-induced colitis (53). Most importantly, endogenous PAR-2 seemed to aggravate experimental colitis by inducing leukocyte recruitment toward the colon. In DSS-induced colitis, the lack of PAR-2 in hematopoietic cells did not protect the animals, suggesting an important role for the epithelial cells (54).

In conclusion, our data show that TF plays a detrimental role in experimental colitis. We suggest that TF-dependent PAR-2 activation in colon epithelial cells leads to local KC production, thereby provoking granulocyte influx with subsequent inflammation and organ damage.

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DISCLOSURE

The authors declare that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES