Original Article

Fibrinogen-like protein 2 prothrombinase may contribute to the progression of inflammatory bowel disease by mediating immune coagulation

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Abstract: Inflammation and coagulation are interdependent processes that enable each process to activate and propagate the other in inflammatory bowel disease (IBD). Thus, we investigated the role of a novel immune coagulant, fibrinogen-like protein 2 prothrombinase (FGL2), in patients with IBD. 83 IBD patients and 40 normal controls were enrolled, and trinitro-benzene-sulfonic acid (TNBS)-induced colitis mice were used. Expression of FGL2 in the intestine was detected by immunohistochemistry. Using serial sections, staining was performed to detect tumor necrosis factor α (TNF-α) expression, and to demonstrate co-localization of FGL2 with macrophages and fibrin. Correlations between FGL2 expression with some common laboratory parameters were examined. FGL2 was seen primarily in inflammatory infiltrating cells, mainly macrophages, and microvascular vessels and had a strong co-localization with fibrin deposition. IBD patients and mice had increased expression of FGL2 compared with controls. Furthermore, FGL2 expression was correlated with intestinal and plasmatic TNF-α expression, mean platelet volume (MPV), platelet count (PLT), platelet-crit (PCT), and fibrinogen. Our data indicate that FGL2 may mediate immune coagulation in IBD patients. It may be considered as a novel molecule that contributes to the onset and development of IBD.

Keywords: Inflammatory bowel disease, fibrinogen-like protein 2 prothrombinase, coagulation, inflammation

Introduction

Inflammatory bowel disease (IBD) comprises a group of chronic and relapsing-remitting immune-mediated disorders of the gastrointestinal tract, which mainly includes Crohn’s disease (CD) and ulcerative colitis (UC). It has been shown that the coagulation cascade and fibrinolysis are activated in patients with IBD and increased thromboembolic events are extra-intestinal and potentially life-threatening complications [1-3]. The prothrombotic state in IBD may be promoted by the inflammatory response and cytokines [4]. As subsequent anticoagulant mechanisms fail to control the clotting process, the pro-inflammatory state is maintained or even increased [5-7]. There is growing evidence that inflammation and coagulation are two interdependent processes, which is crucial for the progression of IBD [8, 9].

Fibrinogen-like protein 2 prothrombinase (FGL2), also called fibroleukin, has been identified as a new member of fibrinogen-related protein superfamily. FGL2 has been shown previously to have the attributes to a serine protease capable of directly cleaving prothrombin into thrombin and then initiate a cascade coagulating reaction [10]. Several studies have indicated that FGL2 is involved in fulminant hepatitis, spontaneous abortion and xenograft rejection by mediating “immune coagulation”, such as fibrin deposition and microthrombus formation [11-15].

While the IBD-associated prothrombotic state has been linked to the inflammatory response,
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**Materials and methods**

**Patients and observation**

The study was performed at the Department of Gastroenterology and Hepatology of the First Affiliated Hospital of Wenzhou Medical University from December 2006 to December 2015. All the patients were followed up for 6-12 months. Informed consent was obtained for each patient included in the study. The research protocol was reviewed and approved by the clinical research Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University.

IBD patients were diagnosed according to the article written by Lennard-Jones JE [16]. 83 Patients with active disease enrolled in our study had not received any steroid medications for their disease. For patients with CD, severity of disease was classified in accordance with the CDAI (activity indices of CD) score [17]. Active disease was defined as a CDAI score ≥150, in inactive disease <150. Disease activity in UC patients (activity indices of UC, UCAI) was evaluated by using of the Truelove-Witts criteria [18]. For statistical purposes, the classification was quantitatively modified [19]. Active disease was defined as score >3 and inactive disease as a score ≤3. Disease activity was evaluated at the time of sample collection. 40 normal controls from the normal mucosal areas of healthy subjects or the patients with colonic polyp were included.

The characteristics of patients and controls are shown in Table 1.

**Table 1. Characteristics of the patients and control groups**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>NC</th>
<th>IBD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>40</td>
<td>83</td>
</tr>
<tr>
<td>Male</td>
<td>22</td>
<td>42</td>
</tr>
<tr>
<td>Female</td>
<td>18</td>
<td>41</td>
</tr>
<tr>
<td>Mean age, years</td>
<td>42 (20-79)</td>
<td>41.7 (15-79)</td>
</tr>
<tr>
<td>Smoking</td>
<td>10</td>
<td>29</td>
</tr>
<tr>
<td>Oral contraceptive use</td>
<td>4</td>
<td>13</td>
</tr>
</tbody>
</table>

NC: normal control; IBD: Inflammatory bowel disease.

Laboratory parameters of patients and controls

Venous blood samples were collected from all the participants with tubes containing 3.8% trisodium, then centrifuged (2000 rpm) for 10 min at 4°C, and finally plasma was separated. Plasma levels of ESR and C-reaction protein (CRP), PLT, MPV, PCT, prothrombin time (PT), activated partial thromboplastin time (APTT), TNF-α, and fibrinogen were routinely measured.

**TNBS-induced colitis in mice**

6- to 8-week-old BALB/c male mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd (License Number: SCXK (Shanghai) 2012-0002). Mice were kept under specific pathogen-free conditions (Laboratory Animal Research Center of Wenzhou Medical University), and all procedures were conducted ethically according to the Guide for the Care and Use of the Administration Committee of Experimental Animals of Wenzhou Medical University. After a week of adaptive feeding, mice were weighed and anesthetized by intraperitoneal injection with 80 ml of ketamine/xylazine solution per 10 g body weight. TNBS was dissolved in alcohol (50:50 vol/vol), 100 μl of TNBS solution (vol (TNBS)/vol (alcohol) was 50:50) was administered intra-rectally (via 3.5 F-catheter) to mice maintained for 60 s in a vertical position. The dose was 100 mg/kg body weight. The insert depth was 4 cm proximal to the anus. Control mice received 100 μl of 0.9% saline intra-rectally [20]. Sample size in each group was 6.

Clinical and macroscopic analysis of colitis in mice

The clinical analysis of colitis in mice was monitored daily for body weight, diarrhea, and hemafecia [25]. Loss of body weight was calculated as percent difference relative to initial body weight. Diarrhea was scored as follows: 0, normal; 2, loose stools; and 4, diarrhea that remained adhesive to the anus. Bleeding scores were assessed as follows: 0, negative hemoccult; 2, positive hemoccult; and 4, obvious bleeding [23]. Mice were euthanized 3 days after TNBS administration. The colon was removed and opened longitudinally. The macroscopic damage was measured by a blinded observer with the following score system [5,
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Histology in patients and mice

For histological analysis, mucosal biopsy specimens, from macroscopically inflamed areas of patients, and normal controls from the normal areas of healthy subjects were obtained. Patients with a colonic polyp were seen at the Department of Gastroenterology and Hepatology of First Affiliated Hospital of Wenzhou Medical University, and samples were collected in this study from December 2006 to December 2015. Furthermore, the colons of mice were removed and divided into fragments after sacrifice. The colonic fragments (0.5 cm) of patients, controls and mice were fixed in 4% paraform, dehydrated, embedded in paraffin, sectioned (4 μm thickness) and stained with hematoxylin and eosin. The pathological slices were observed under a light microscope by two blinded pathologists.

Immunohistochemistry staining FGL2 expression in local intestine

Mucosal specimens of patients and controls and the colonic fragments of mice were fixed with 4% paraformaldehyde, processed into paraffin, sectioned, and deparaffinated in xylene and rehydrated through ethanol to water. Nonspecific binding was blocked by sequential incubation of the sections in citrate buffer for 4 min at 100°C and 16 min at 20°C, and then 3% hydrogen peroxide solution for 15 min followed by 5% normal goat serum in PBS at 37°C for 60 min. Thereafter incubation in a monoclonal antibody from mouse to human FGL2 (Abnova, Taiwan) was done at a dilution of 1:500 in PBS at 4°C for 16 h. After washing with PBS, sections were incubated with immunoperoxidase-conjugated rabbit IgG fraction to mouse IgG Fc (Zhongshan Company, Beijing, China) at 37°C for 30 min, followed by three washes in PBS. Finally, the sections were incubated with 3,3′-diaminobenzidine chromagen and counterstained with hematoxylin. Negative control was set in the experiment. For the evaluation of FGL2 expression, a total of 10 random fields across each section were selected to conduct the semi-quantitative analysis of the mean absorbance at a magnification of ×200.

Fibrin deposition in local intestine

For fibrin detection, serial sections were stained with a rabbit-anti-fibrinogen Ab (Abcam, Cambridge, USA, at a dilution of 1:100). This reagent is known to react with fibrinogen and...
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Fibrin in mouse and human tissues. The technique used for detection of fibrin was the method as previously described.

TNF-α expression in local intestine

Serial sections were stained with a rabbit-anti-TNF-α Ab (Abcam, Cambridge, USA, at a dilution of 1:150). The technique used for detection of TNF-α was the method as previously described.

Immunoperoxidase staining of macrophages

A CD68 Ab (ProteinTech, at a dilution of 1:100) was used to detect macrophages using the similar methodology described above.

Statistical analysis

Statistical Analysis was carried out by SPSS16.0 software. All results were presented as mean ± SEM and P<0.05 were considered significant. The associations between FGL2 expression and disease activity indices or other Laboratory parameters, including CRP, ESR, PLT, MPV, PCT, fibrinogen, D-dimer, PT, APTT, TNF-α were examined by non-parametric correlation (Spearman r).

Results

TNBS-induced mice develop serious clinical manifestation with general and histological changes similar to IBD patients

The TNBS-induced colitis mice acquired a severe sickness characterized by body weight loss, diarrhea, defecate occult blood (OB) or hemafecia after TNBS administration (Figure 1A-C). Subsequently, the colonic samples were generally observed and scored by a blinded...
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observer (Figure 1D, 1I, 1J). We observed that the colon of IBD model was accompanied by hyperemia, edema, ulcers, necrosis, and adhesion. The clinical manifestation and general changes were similar to patients with IBD. Simultaneously, histological examination showed that the changes of TNBS-induced mice covered decrease of goblet cells, loss of crypts, damage of crypts, infiltration by inflammatory cells, and extensive destruction of the mucosal layer (Figure 1H) compared with normal controls (Figure 1G). Similarly, IBD patients had the same histopathology (Figure 1E, 1F). Fibrin deposition was detected in the matrix and around the FGL2-positive infiltrating cells and microvascular vessels (Figure 2G, 2H) and was strongly co-localized with FGL2 expression.

**Correlations between FGL2 expression and inflammation**

There was association between FGL2 expression and inflammatory parameters. There was a strong correlation between FGL2 expression and TNF-α expression in the intestine and plasma of IBD patients \( (r=0.628, P<0.001; r=0.425, P<0.001) \). But the enhanced FGL2 expression was not correlated with ESR levels or CRP levels. Moreover, an amount of TNF-α expression was observed in areas of inflammatory infiltration and in the vessels region of IBD patients (Figure 2E, 2F) and colitis mice (Figure 2A, 2B). Spearman-rank correlation coefficient values and the two-tailed significance of the correlation between FGL2 expression and inflammation parameters in IBD patients are shown in Table 2.

**Correlations between FGL2 expression and coagulation parameters**

Intestinal FGL2 expression in patients with IBD negatively correlated with platelet count \( (r=0.330, P<0.01) \), MPV \( (r=-0.304, P<0.01) \) and PCT \( (r=-0.404, P<0.001) \), but was not correlated with the levels of PT or aPTT. There was cor-

<p>| Table 2. Correlation of FGL2 expression with inflammation parameters |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Pearson Correlation</th>
<th>TNF-α (intestine)</th>
<th>TNF-α (plasma)</th>
<th>ESR (mm/h)</th>
<th>CRP (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGL2</td>
<td>0.00146 ± 0.00115</td>
<td>0.628***</td>
<td>0.425***</td>
<td>0.161 n.s.</td>
</tr>
<tr>
<td></td>
<td>-0.175 n.s.</td>
<td>48.4 ± 92.2 pg/ml</td>
<td>46.4 ± 34.3</td>
<td>23.8 ± 32.0</td>
</tr>
</tbody>
</table>

IBD: Inflammatory bowel disease; ESR: sedimentation rate; CRP: C-reaction protein; TNF-α: tumor necrosis factor α. All results were expressed as mean ± SD. A level of \( P<0.050 \) was considered to be statistically significant. ***\( P<0.001 \); n.s. = not significant.

<p>| Table 3. Correlation of FGL2 expression with coagulation parameters |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Pearson Correlation</th>
<th>Plt ( (10^9/l) )</th>
<th>MPV (fl)</th>
<th>PCT (µg/l)</th>
<th>PT (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGL2</td>
<td>0.00146 ± 0.00115</td>
<td>-0.330**</td>
<td>-0.304**</td>
<td>-0.404***</td>
</tr>
<tr>
<td></td>
<td>-0.005 n.s</td>
<td>7.77 ± 1.25</td>
<td>14.2 ± 1.93</td>
<td>39.2 ± 4.69</td>
</tr>
<tr>
<td></td>
<td>0.002 n.s</td>
<td>4.23 ± 1.22</td>
<td>301.8 ± 119.6</td>
<td></td>
</tr>
</tbody>
</table>

Plt: platelet count; MPV: mean platelet volume; PCT: platelet-crit; PT: prothrombin time; APTT: activated partial thromboplastin time. All results were expressed as mean ± SD. A level of \( P<0.050 \) was considered to be statistically significant. *\( P<0.05; ** P<0.01; *** P<0.001; n.s. = no significance.

FGL2 expression was increased in patients and mice with IBD

Compared with normal controls, there was significantly up-regulation of FGL2 expression in inflamed mucosal specimens from patients and mice with IBD (Figure 2C, 2D, 2J, 2K). The expression of FGL2 was not only in the areas of inflammatory infiltration and the vessels, but also in the mucosal lamina propria. There was no significant difference between the mean ages of patients and controls. Gender distribution was similar in patients and controls.

Cellular source of FGL2 and fibrin deposition in IBD patients

By serial section staining, the majority of CD68+ cells showed high expression of FGL2 protein in patients with IBD (Figure 2K, 2L).
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relation between FGL2 expression and fibrinogen level (r=0.224, P<0.05). Spearman-rank correlation coefficient values and the two-tailed significance of the correlation between FGL2 expression and coagulation parameters in IBD are shown in Table 3.

Discussion

Despite numerous studies, to date, the exact pathogenesis of IBD has not been clearly elucidated. The most prevailing factors include host genetic susceptibility and immune abnormalities [21-23]. However, recently, a large number of studies that focus on the role of endothelial damage and coagulation disturbances as IBD-triggering factors [24-27]. Inflammation and coagulation are interdependent processes that enable each process to activate and propagate the other [6-8]. The persistent pro-coagulant state likely involves endothelial cells, leukocytes, and platelets, which are activated in response to the inflammatory stimulus. The activated endothelial cells, leukocytes, and platelets during inflammation phase exhibit an increased tissue factor expression, downregulation of the anticoagulant protein C pathway, and generation/activation of coagulation factors (e.g., factor Xa), as well as enhanced thrombin production [28-30]. In addition, several findings have demonstrated a role for cytokines in mediating inflammation-induced coagulation or thrombosis, such as interleukin (IL)-1β, TNF-α and IL-6. Blocking antibodies of IL-1β or TNF-α, or lacking the gene for the TNF receptor (TNF r/-) largely abolish the extra-intestinal thrombosis associated with experimental IBD [31, 32].

Our study first demonstrates the role of FGL2 in IBD patients. We found that there was significantly higher FGL2 expression in patients and mice with IBD than normal controls. The up-regulated expression of FGL2 in IBD patients may be promoted by inflammatory response or pro-inflammatory cytokines like TNF-α [32]. This is consistent with our result that there was parallel high expression of intestinal TNF-α. FGL2 was primarily seen in macrophages and endothelial cells in IBD patients and was colocalization with fibrin deposition. This conforms to the cellular source and coagulable characteristic of FGL2 as previous studies [11-15]. Disease activity indices, ESR, and CRP are important for disease assessment in IBD. Platelet count, MPV, PCT, PT, and APTT are also biomarkers of coagulation and fibrinolysis as measures of disease activity in active IBD [9]. In our results, correlation of FGL2 expression with CDAI or UCAI, ESR, and MPV, PCT, as well as fibrinogen was found. All these findings support our assumption that FGL2 may participate in inflammation-induced coagulation and thrombosis in IBD through a clotting-dependent pathway.

In conclusion, our study of FGL2 expression in IBD has important theoretical implications for the understanding of the “inflammation-coagulation” crosstalk that may result in novel diagnostic and therapeutic strategies that target the inflammation-induced hypercoagulable state. Measurement of FGL2 may be used as a
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helpful biomarker in the pathogenesis and disease assessment of IBD. To confirm this, we have now initiated further investigation of the role of FGL2 in patients with IBD and in experimental IBD models.

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Disclosure of conflict of interest

None.

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