KININOGEN (HK) DEFICIENCY MODULATES CHRONIC INTESTINAL INFLAMMATION IN GENETICALLY SUSCEPTIBLE RATS

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ABSTRACT

Genetically susceptible Lewis rats injected in the intestinal wall with peptidoglycan-polysaccharide (PG-APS) polymers develop chronic granulomatous enterocolitis concomitant with activation of the kallikrein-kinin system. To elucidate the role of high molecular weight kininogen (HK) in chronic enterocolitis, we backcrossed Brown-Norway rats having a HK deficiency with Lewis rats for 5 generations. Two new strains were produced, wild type F5 (F5WT) and HK deficient (F5HKd), each with a ~97% Lewis genome. The HK values of F5WT rat plasma and F5HKd rat plasma were 0.62±0.20 units/ml and 0.08±0.03 unit/ml respectively. In PG-APS injected rats, chronic inflammation was measured using gross gut score, histologic inflammation, liver granuloma and white blood cell count. The mean gross gut scores were significantly lower in the F5HKd than the F5WT rats. Plasma T-kininogen was significantly less in F5HKd. These results indicate the importance of the kallikrein-kinin system in this model of chronic enterocolitis and systemic inflammation.
KEY WORDS: Inflammatory Bowel Disease, Experimental Colitis, Kallikrein-Kinin System, Contact System.
INTRODUCTION

Inflammatory bowel diseases (IBD), including Crohn’s disease and ulcerative colitis, are disorders characterized by local and systemic chronic inflammation with unpredictable relapses and remissions (1). These disorders are immunologically mediated and have a genetic component (1,2). In animal models, the aggressiveness and chronicity of the inflammatory process is dependent on the genetic background of the host (3).

Previous studies from our laboratory have used a rat model of acute and chronic granulomatous enterocolitis that permits detailed examination of the mechanisms of the genetically determined intestinal and systemic inflammation (4). Bacterial cell wall polymers, peptidoglycan – polysaccharide from group A streptococci (PG-APS), were injected intramurally to induce intestinal inflammation (5,6). Acute inflammation at the injected area was observed in all rat strains included, but the spontaneous reactivation of chronic granulomatous inflammation was restricted to genetically susceptible Lewis and Sprague-Dawley rats and did not occur in Buffalo and Fisher rats (4,6,7). Female Lewis rats, the highest responders, develop acute intestinal inflammation that peaks 1-2 days after PG-APS injection, and gradually decreases over the next 10 days. The enterocolitis spontaneously reactivates beginning on day 14, accompanied by peripheral erosive arthritis, granulomatous hepatitis, normochromic anemia, and leukocytosis (4). The histological findings of transmural inflammation, fibrosis and granulomas, the pattern of macrophage and TH1-derived cytokines resemble Crohn’s disease (8,9). The inflammatory response initiated by PG-APS is similar to that present in human IBD in that it is mediated by leukocyte activation with liberation of cytokines, eicosanoids, oxygen radicals, nitric oxide, and activation of complement, coagulation and fibrinolytic cascades, (5,10,11).

The plasma kallikrein-kinin system (KKS), or contact system is comprised of factor XII,
prekallikrein (PK), and factor XI (FXI), which are the zymogens of proteases, and high molecular weight kininogen (HK), which serves as the cofactor of contact activation and is the genesis of bradykinin release (12,13). Bradykinin is a potent inflammatory mediator which exerts its biological effects by activating constitutive B2 and inducible B1 receptors on endothelial cells, smooth muscle cells, epithelial cells, and fibroblasts (14). This potent inflammatory peptide enhances vasodilation, increases vascular permeability, and influences intestinal motility and electrolyte secretion (14,15), all of which are features of Crohn’s disease.

Negatively charged biological products such as endotoxin (16), or activated blood or endothelial cells are able to activate zymogen factor XII, the initial component of the contact system to factor XIIa. Factor XIIa converts PK to kallikrein and coagulation Factor XI to Factor XIa. In addition, Factor XIIa is an agonist for neutrophils (17), and monocytes (18), and is capable of initiating the classical complement cascade (19). Kallikrein in the presence of HK produces neutrophil chemotaxis (16), aggregation (20), and oxygen consumption (21), and induces elastase release (22). Kallikrein stimulates the fibrinolytic system by converting prourokinase to urokinase (23) and activates the alternative complement pathway (24).

To evaluate the role of HK in intestinal and systemic inflammation, we created a kininogen deficient Lewis rat by back crossing the brown Norway (Katholiek strain) HK deficient rat (25) to normal Lewis rats (Table I). Preliminary studies indicated that brown Norway rat did not develop chronic granulomatous inflammation after PG-APS injection. After five generations we produced two new rat strains, one with <10% kininogen and a 97% Lewis genetic background and a wild type rat with normal levels of HK and 97% Lewis background. We compared the ability of intramural PG-APS to induce chronic gastrointestinal, joint and systemic inflammation in rats with normal vs. deficient plasma levels of kininogen.
METHODS

Production of high molecular weight kininogen deficient Lewis rat. The Brown-Norway Katholiekg rat strain has an Ala to Ser (aa 145, sequence without signal peptide) substitution in rat HK, which leads to a secretion defect of the molecule in hepatocytes resulting in plasma levels of HK ranging from 5-10% of normal (25). Since the Brown Norway Katholiekg strain was resistant to PG-APS induced enterocolitis (data not shown), we produced an HK- deficient rat strain susceptible to chronic inflammation by backcrossing Lewis rats for 5 generations with a offspring of a Brown-Norway Katholiekg x Lewis cross (Table 1). At the end of 5 backcrosses to Lewis rats, the Brown Norway gene pool has been diluted 1/2^5 or = 3%. Therefore the Lewis gene pool now represents 97% of the total gene pool. Two new strains were produced: F5 HKd (deficient in HK) and F5WT (not-deficient in HK), each with 97% Lewis genetic material.

PG-APS Polymers. Purified, sterile PG-PS fragments from the cell walls of group A, type 3, strain D58 streptococci (Streptococcus pyogenes) were prepared as described previously (4).

Experimental protocol and treatment. Two groups of female specific-pathogen crossed rats of approximately 155g were used, HK deficient (F5HKd), n=9 and; wild type (F5WT) rats, n = 5. The intestines of each animal were exposed by laparotomy with sterile technique, and PG-APS were injected (15mg/gbw) as previously described (4), intramurally into 5 sites of the terminal ileum and cecum. Animals were euthanized 21 days after surgery.
Quantification of intestinal inflammation. Intestinal inflammation was quantified by gross, biochemical and immunological and histological methods validated for this model (4,6). At necropsy, performed by a blinded observer, the gross gut score was calculated using the sum of 0-4 scores, of four independent parameters, including: intestinal wall thickening, adhesion, mesenteric contraction and serosal nodules (granulomas). The maximum score was 16. Components of a blinded histologic inflammatory score, was based on the number of infiltration of mononuclear cells (macrophages and lymphocytes) (chronic inflammation), neutrophils and edema (acute inflammation). The presence of crypt abscesses and granulomas were noted. The maximum score was 24 (acute 0-4 and chronic 0-4 in 2 sections of the cecum).

Systemic inflammation. Rats were weighed prior to surgery and at necropsy. At necropsy, liver and spleen weights were recorded. Hepatic granulomas were quantified with a score of 0-4. Joint diameters were measured with calipers (4). Cardiac blood was collected for hematological measurements (WBC, hematocrit and hemoglobin) and assays of inflammatory proteins at euthanasia.

Assays of contact activation in vitro. Prekallikrein function levels were performed by a microtiter, amidolytic assay using a chromogenic substrate, S-2302 (Pro-Phe-Arg-p-nitroanilide, Chromogenix, Moindal, Sweden) as described in our laboratory (26). HK coagulant activity was evaluated by our modification of an APTT test assay (27,28), using total kininogen deficient plasma purchased from George King (Overland Park, KS) (29). In addition, FXI and FXII coagulant activity was performed by a similar method using deficient plasma obtained from George King.

T-kininogen determination. Plasma T-Kininogen was measured by sandwich enzyme-linked immunoabsorbent assay, as described previously (30).
**Statistical analysis** was performed using the unpaired Student’s test. P values <0.05 were considered significant. *p<0.05, **p<0.02, ***p<0.01  Values represent the means ± SD or SEM if noted in the figure legends.

**RESULTS**

**Comparisons of contact system plasma proteins in rat strains.** One ml of Lewis rat plasma is defined as containing 1.00 Units/ml (U/ml) of rat HK. The parent Lewis strain had a mean plasma HK concentration of 1.00±0.14 U/ml, while the Brown-Norway strain had a HK concentration of 0.09± 0.01 U/ml. The new strains produced (F5WT) had HK = 0.63± 0.20 U/ml and (F5HKd) had HK = 0.08±0.03 U/ml (Figure 1). Thus, we produced two new strains of rats composed of 97% Lewis genetic background with and without a severe deficiency of HK.

Other plasma contact factors were examined in these strains (Figure 2). The factor XI levels in the noninflamed parental Lewis strain and Brown-Norway strains showed no significant differences (1.00±0.14 U/ml, and 1.18±0.01 U/ml respectively). The factor XI level was decreased in F5WT (0.70± 0.05 U/ml) but did not differ significantly from the Lewis parental strain. The F5HKd contained a factor XI value significantly lower than observed in the F5WT. Prekallikrein levels in the parent Lewis and Norway rats also were not significantly different (1.00±0.05 and 0.73±0.05 U/ml respectively). F5WT prekallikrein levels (0.98±0.04 U/ml) were similar to the Lewis rat. The F5HKd (0.52±0.04 U/ml) had a significantly lower prekallikrein concentration than the F5WT strain. The F5WT and F5HKd strains contained the same 97% genetic background, therefore lower factor XI and prekallikrein levels in the F5HKd rats are presumably a consequence of the lower functional HK levels in the plasma with which factor XI and prekallikrein associates. This decreased level of prekallikrein is also observed in
HK deficient human plasma (32). Factor XII was not significantly different in all four strains. The similarity of the measured factor XII levels in all the groups indicates that the sample preparation methods did not affect the other protein values measured in our samples.

**Effect of HK deficiency on experimental inflammation.** In previous studies from our laboratory (4) we used a Lewis rat model of chronic granulomatous enterocolitis induced by intramural PG-APS injection into the cecum and ileum. Intramural injection of PG-APS in the two new rat strains, F5WT and F5HKd, induced chronic inflammation in both strains compared with the HSA-injected controls (Figure 3). However, there were significant differences in the values of many of the measured pathological parameters between the two new rat strains with normal vs. deficient plasma kininogen levels. These differences reflect decreased inflammation in the intestine and liver after PG-APS injection in the rats with low baseline kininogen levels. The gross gut score was significantly decreased (p=0.024) in the F5HKd strain (7.8±1.5) compared to the F5WT strain (13.0±0.1). The total intestinal histological score compared to the F5WT rats (19.2±1.5) was significantly decreased in the F5HKd rats (13.5±1.8). Likewise, the liver granuloma score was significantly decreased in F5HKd rats (1.7±0.3) compared to the F5WT (3.6±0.3). The WBC was significantly elevated (p=0.028) in the F5WT strain (37.1±5.3 x10³ cell/µl) compared to the F5HKd (24.1±3.0 x10³ cell/µl).

No significant differences were found between F5WT and F5HKd rats injected with PG-APS in liver weight, spleen weight, hematocrit, hemoglobin, or joint diameter (not shown).

Plasma T-kininogen, an acute phase protein unique to rats, was measured
antigenically (30) as another indicator of the inflammatory response in these rats (Figure 4). The levels were significantly increased in both PG-APS treated F5 groups, compared to their untreated values (p<0.001). When PG-APS injected rats were compared, the F5HKd group had a significantly lower T-kininogen level relative compared to the F5WT rats (p=0.023).

Examination of microscopic sections of the cecum showed that the degree of cellular infiltration and inflammatory changes were increased in F5WT rats injected with PG-APS (Figure 5 B). The kininogen deficient group (F5HKd) group showed a minimal to moderate thickening of the mucosa with moderate mononuclear cell infiltration in the lamina propria and submucosa and fibrosis (Figure 5 A). There was a relative absence of neutrophils, crypt abscesses and necrosis. In contrast, the F5WT group showed marked thickening of the submucosa with crypt abscesses in the mucosa, necrotic granulomas in the submucosa and severe chronic inflammation and fibrosis (Figure 5B).

**Kallikrein kinin system.** As observed previously in the Lewis rat (4), injection of PG-APS caused a marked decrease in the F5WT rat plasma HK concentrations from initial normalized levels (1.0±0.2U/ml) to (0.2±0.1 U/ml). This observation indicates an extreme in vivo activation of the contact system as a consequence of intestinal PG-APS injection (p=0.04)(Figure 6). No change occurred from the low HK values in F5HKd rats with PG-APS injection. Significant differences (p<0.05) occurred in both plasma Factor XI and prekallikrein between the treated F5WT and F5HKd rats but when corrected by normalization for the pretreatment values there were no difference in the percentage change. No significant differences were found in the factor XII levels in any of these
groups. We have determined that a 5% presence of HK in vitro is able to support sustained prekallikrein activation compared to normal HK values (33). This observation may explain the lack of significant differences in the values of factor XI and prekallikrein between these two rat strains upon PG-APS treatment.

**DISCUSSION**

Inflammation is accompanied by activation of the KKS and resulting alterations of its component plasma proteins. The KKS is one of the participants in the pathogenesis of inflammatory reactions involved in cellular injury, including coagulation, fibrinolysis, kinin formation, complement activation, cytokine secretion and release of proteases. Protein components of the kallikrein-kinin system bind to and interact with leukocytes, platelets and endothelial cells. The formation of plasma kallikrein has at least two effects: neutrophil stimulation and bradykinin release. KKS activation has been demonstrated in various experimental and human disease states caused by bacterial infection and/or immunological injury (34), including ulcerative colitis and Crohn’s disease. Direct involvement of the KKS in the pathogenesis of the rat acute and chronic enterocolitis (27,35) acute arthritis (36) and experimental human sepsis (37) has been documented by previous studies from our laboratory.

We have described kallikrein-kallikrein activation (4) in a well described, Lewis rat inflammation model using a bacterial cell wall polymer (PG-APS) injected intramurally to induce intestinal inflammation. We correlated changes in the KKS system with pathophysiological measurements of the inflammation (4). Both the acute and chronic changes in genetically susceptible Lewis rat are accompanied by evidence of activation of the KKS, yet this system is not activated during acute inflammation in
Buffalo rats, which is equal in intensity to that of Lewis rats (4). Of considerable interest, Buffalo rats fail to develop the chronic, T cell-mediated phase of enterocolitis nor systemic inflammation after PG-APS injection (4,6). In vitro studies show more rapid degradation of kininogen and liberation of bradykinin in Lewis rat plasma vs. Buffalo plasma (4). These results agree with our present observations that our F5HKd rats deficient in kininogen have less active chronic inflammation than our F5WT rats with normal kininogen levels.

A specific boronic acid inhibitor of plasma kallikrein (P 8720) modulates the chronic local and systemic inflammation in Lewis rats injected with PG-APS (35). The P8720 treated group showed a highly significant decrease in all components of the gross, and histologic inflammatory score and liver and spleen enlargement, leukocytosis and arthritis associated with chronic intestinal inflammation. There was a significant decrease in plasma FXI, and HK in the untreated group as compared with the P8720 treated group. This study strongly implicated kallikrein activation in the pathogenesis of chronic PG-APS-induced inflammation. Results using this pharmacological inhibitor are not definitive due the possible confounding influence of hepatic toxicity and because inhibition of kallikrein activity will also prevent HK activation, since plasma kallikrein cleaves HK to release the biologically active peptide bradykinin. Therefore it is impossible to determine whether the kallikrein inhibitor modulates inflammation by acting directly on kallikrein to prevent bradykinin formation.

Parental Lewis rat Factor XI was decreased in the F5HKd strain prior to injection of PG-APS. Prekallikrein levels (an important participant in the KKS system) appeared normal in the F5WT strain and reduced in the F5HKd strain. The reduced level of
prekallikrein in this deficient strain is similar to what is found for prekallikrein levels in human patients with HK deficiency (32). We have shown in these human individuals this difference can be overcome by adding HK in vitro indicating that this result is due to the participation of HK in the assay to detect PK activity (33).

Unfortunately functional plasma prekallikrein activity is also low in the presence of HK deficiency, therefore limiting assessment of the comparative roles of HK or plasma kallikrein in intestinal and systemic inflammation. However, the consistent ability of a selective kallikrein inhibitor and the endogenous HK in the present deficiency study to modulate intestinal and systemic inflammation firmly implicates the KKS in general and the participation of prekallikrein and HK in the pathogenesis of chronic immune-mediated enterocolitis. F5HKd rats developed significantly less chronic intestinal inflammation, hepatic granulomas and leukocytosis than F5WT rats following intestinal PG-APS injection consistent with our previous observations with pharmacological blockade of kallikrein (38). However, HK deficiency did not protect against PG-APS-induced arthritis in Lewis rats in contrast to prevention of acute and chronic phases of arthritis with P8720 (38). The reasons for this discrepancy are unclear. One possibility is the effect of kallikrein to stimulate neutrophils distinct from HK cleavage and bradykinin liberation. The difference may reflect a nonspecific effect of the pharmacologic inhibition or a requirement for more aggressive blockade of the KKS in arthritis than enterocolitis. However, a specific bradykinin receptor 2 inhibitor attenuated PG-APS-induced arthritis but not enterocolitis (39) and treatment with recombinant IL-1 receptor antagonist and IL-10 more easily suppressed arthritis than intestinal inflammation in this model (6,8).
In summary, our results indicate that relative plasma HK deficiency in genetically susceptible Lewis rat results in decreased chronic enterocolitis, hepatic granulomas and leukocytosis, but no change in arthritis. Together with our previously reported inhibition of intestinal and systemic inflammation following selective pharmacologic blockade of plasma kallikrein, we demonstrate an important role for the KKS in the pathogenesis of chronic granulomatous inflammation. These observations lay the foundation for selective inhibition of kallikrein and bradykinin in human IBD, particularly Crohn’s disease, which shares a number of pathologic and immunologic features with experimental PG-APS-induced granulomatous inflammation.
AKNOWLEDGMENTS

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Figure Legends

**Figure 1** Plasma HK concentration in the parent rat strains (Lewis and Brown Norway – Katholiek) and the two created F5 rat strains (F5WT and F5HKd). ***p<0.001 vs. F5WT values. Mean ± SD.

**Figure 2** Examination of selected plasma contact factors in the parental rat strains (Lewis and brown Norway-Katholiek) and derived F5 rat strains. *** p<0.001 vs. F5WT values. Mean ± SD.

**Figure 3** Comparison of the gross gut score, total histological score, liver granuloma score and white blood cell count were blindly determined in the F5 rats 21 days after PG-APS injection. The short bar in the first column represents mean normal value of non-PG-APS treated parental Lewis rats which were albumin-injected, as reported in a previous study (35). The p values are comparison to F5WT. Gross Gut Score p = 0.024. Liver Granuloma Score p = 0.002. Mean ± SEM.

**Figure 4** Plasma T-kininogen antigenic levels of the F5 strains. Pretreatment and PG-APS injected comparisons. A significant difference (p=0.023) was observed between levels in the F5WT (solid bars) and F5HKd (open bars) PG-APS injected rats. Mean ± SD.

**Figure 5** (A) Chronic colitis in the kininogen deficient (F5HKd) rat 21 days after PG-APS. Note the moderate degree of mononuclear cell infiltration in the lamina propria and submucosa (SM), with fibrosis, but relative absence of neutrophils, crypt abscesses (CA)
and necrosis.

( B ) Chronic colitis in the F5WT rat 21 days after treatment. Note crypt abscess marked thickening of the submucosa and necrotic granulomas (NG).

**Figure 6.** Kallikrein-kinin system values. Contact factor levels in the two F5 strains were compared for significant difference observed between the pretreatment animal values or post-treatment animal values (21 days). Significant differences were observed in the factor XI and prekallikrein between the post treatment group. However the differences may be related to the significant differences in the pretreatment comparisons. Comparisons between the pre and post treatment in each group are as follows: F5WT: HK and PK, p < 0.001, factor XI, p = 0.305; F5HKd, HK and PK, p < 0.001, XI, p = 0.003. Mean ± SD.
Table 1

Animal Backcrossing

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HH = HK dominant = Lewis Rat
hh = HK recessive = Brown-Norway Katholiek Rat
Figure 1

HK (U/ml)

Lewis (HH)  Norway Katholiek (hh)  F5WT (HH)  F5HKd (hh)

***

Lewis (HH) has a significantly higher HK level compared to Norway Katholiek (hh) and F5WT (HH). F5HKd (hh) has a lower HK level than Lewis (HH) but higher than Norway Katholiek (hh).