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Serum Leucine-rich Alpha-2 Glycoprotein Is a Disease Activity Biomarker in Ulcerative Colitis

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Background: Reliable biomarkers for monitoring disease activity have not been clinically established in ulcerative colitis (UC). This study aimed to investigate whether levels of serum leucine-rich alpha-2 glycoprotein (LRG), identified recently as a potential disease activity marker in Crohn’s disease and rheumatoid arthritis, correlate with disease activity in UC. 

Methods: Serum LRG concentrations were determined by enzyme-linked immunosorbent assay (ELISA) in patients with UC and healthy controls (HC) and were evaluated for correlation with disease activity. Expression of LRG in inflamed colonic tissues from patients with UC was analyzed by western blotting and immunohistochemistry. Interleukin (IL)-6-independent induction of LRG was investigated using IL-6-deficient mice by lipopolysaccharide (LPS)-mediated acute inflammation and dextran sodium sulfate (DSS)-induced colitis. 

Results: Serum LRG concentrations were significantly elevated in active UC patients compared with patients in remission ($P < 0.0001$) and HC ($P < 0.0001$) and were correlated with disease activity in UC better than C-reactive protein (CRP). Expression of LRG was increased in inflamed colonic tissues in UC. Tumor necrosis factor alpha (TNF-α), IL-6, and IL-22, serum levels of which were elevated in patients with active UC, could induce LRG expression in COLO205 cells. Serum LRG levels were increased in IL-6-deficient mice with LPS-mediated acute inflammation and DSS-induced colitis. 

Conclusions: Serum LRG concentrations correlate well with disease activity in UC. LRG induction is robust in inflamed colons and is likely to involve an IL-6-independent pathway. Serum LRG is thus a novel serum biomarker for monitoring disease activity in UC and is a promising surrogate for CRP. 

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Key Words: IBD, ulcerative colitis, biomarker, leucine-rich alpha-2 glycoprotein, DSS
disease activity, only modest to absent CRP responses are observed in systemic lupus erythematosus (SLE), dermatomyositis, Sjögren’s syndrome, or UC, although active inflammation is present.9–11 In UC, endoscopic disease activity may predict future clinical symptoms,12 but direct endoscopic or radiologic visualization of the degree of inflammation is rarely performed in outpatients with inactive or mild disease. Therefore, alternative biomarkers, which can conveniently and precisely monitor disease activity during therapy in inflammatory diseases, are required for the determination of adequate treatment.

By using a quantitative proteomic approach, we have previously reported that serum levels of leucine-rich alpha-2 glycoprotein (LRG) were elevated in patients with active RA and serum LRG levels were correlated with disease activity of not only RA but also CD, suggesting that serum LRG is a serological biomarker for monitoring disease activity.13 LRG is a 50 kDa glycoprotein and contains repetitive sequences with a leucine-rich motif, first purified from human serum.14,15 LRG has been reported to be expressed by the liver cells and neutrophils;16,17 however, its function remains unclear. To date, the relationship between serum LRG levels and disease activity in UC has not been assessed. In this study we investigated serum LRG expression levels in UC patients and evaluated their correlation with clinical disease activity. Serum LRG levels were significantly increased in the active UC patients. LRG expression was upregulated in the inflamed colonic mucosa of UC possibly through stimulation by various cytokines including tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, and IL-22, the expression of which are increased in active UC. Moreover, we show that serum LRG correlates more strongly than CRP with disease activity in UC. Therefore, serum LRG may be a useful disease activity biomarker for UC.

**MATERIALS AND METHODS**

**Patients and Sera**

Sera were obtained from patients with UC (n = 82), appendicitis (n = 13), and diverticulitis (n = 4) and surgical or biopsy samples were obtained from patients with UC (n = 10) from Osaka University Hospital (Osaka, Japan) and the Department of Surgery, Osaka Rosai Hospital, respectively. Sera from healthy controls (HCs) (n = 50), age/sex-matched with UC patients, were used. Diagnosis of UC was based on conventional clinical, radiological, endoscopic, and histopathological criteria. Clinical activities were determined using the Clinical Activity Index (CAI) for UC.18 Clinical remission was defined as CAI < 6.19 In addition to CAI, the endoscopic findings were also graded according to Matts’ criteria.20 Endoscopic remission was defined as Matts’ score ≤ 2. Detailed patient characteristics are presented in Table 1. For Caucasian patients with UC, sera (n = 30) were obtained from the Department of Medicine, University of North Carolina Hospital (Chapel Hill, NC). Sera from HCs (n = 19), age/sex-matched with UC patients, were used. Detailed patient characteristics are presented in Table 2, while data of disease activity of UC is not available.

**Quantification of Serum LRG and Cytokines**

Human serum LRG and mouse serum LRG were quantitated by human LRG assay kit (IBL, Fujioka, Japan) and mouse LRG assay kit (IBL, Fujioka, Japan). These enzyme-linked immunosorbent assay (ELISA) assays were performed

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**TABLE 1. Characteristics of Patients with Ulcerative Colitis (UC)**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients with UC</th>
<th>Patients with Appendicitis and Diverticulitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (male:female)</td>
<td>82 (41:41)</td>
<td>17 (8:9)</td>
</tr>
<tr>
<td>Age, yr, mean (SD)</td>
<td>40.1 (15.7)</td>
<td>33.1 (13.7)</td>
</tr>
<tr>
<td>Age at diagnosis, yr, mean (SD)</td>
<td>34.7 (15.6)</td>
<td>33.1 (13.7)</td>
</tr>
<tr>
<td>Bowel surgery (including appendectomy), N (%)</td>
<td>7 (8.54)</td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salazosulfapyridine or mesalazine, N (%)</td>
<td>66 (80.5)</td>
<td></td>
</tr>
<tr>
<td>Steroids, N (%)</td>
<td>16 (19.5)</td>
<td></td>
</tr>
<tr>
<td>Immunomodulators, N (%)</td>
<td>3 (3.7)</td>
<td></td>
</tr>
<tr>
<td>Disease location (N)</td>
<td>37/30/15</td>
<td></td>
</tr>
<tr>
<td>CRP, mg/dL, mean (SD)</td>
<td>0.884 (1.967)</td>
<td>8.47 (7.69)</td>
</tr>
<tr>
<td>WBC cells/μL, mean (SD)</td>
<td>6716 (2317)</td>
<td>12307 (3603)</td>
</tr>
<tr>
<td>CAI, mean (SD)</td>
<td>4.71 (4.89)</td>
<td></td>
</tr>
<tr>
<td>Matts’s score, mean (SD)</td>
<td>2.27 (0.89)</td>
<td></td>
</tr>
</tbody>
</table>
in duplicate. The intraassay coefficients of variations for human LRG and mouse LRG were ≤7.98% and ≤8.93%, respectively. For the quantification of IL-6, TNF-α, and IL-22 in human serum samples, the human IL-6 Ultra Sensitive ELISA kit (Biosource International, Camarillo, CA), human TNF-α Ultra Sensitive ELISA kit (Invitrogen, Carlsbad, CA), and human IL-22 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) were used following the manufacturer’s guidelines.

**Western Blot Analysis**

Frozen colon tissue samples were lysed in RIPA buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 1 x protease inhibitor cocktail; Nacalai Tesque, Kyoto, Japan) and 1 x phosphatase inhibitor cocktail (Nacalai Tesque) followed by centrifugation (13,200 rpm, 4°C, 15 minutes), after which the supernatants were stored at −80°C until use. Extracted proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously.21 Samples transferred onto PVDF membranes were treated with a rabbit antihuman LRG polyclonal antibody (Proteintech Group, Chicago, IL) or a rabbit anti-GAPDH polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used as described previously.22

**Immunohistochemistry**

Immunohistochemical analyses were performed according to a method described in our previous report.22 Briefly, rabbit antihuman LRG polyclonal antibodies were used as the primary antibody. After incubation with the primary antibodies, the sections were treated with biotin-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and avidin-biotin-peroxidase complexes (Vector Laboratories). Immunoreactive cells were visualized with a diaminobenzidine substrate (Merck, Darmstadt, Germany) and were counterstained with hematoxylin.

**Mice**

C57BL/6 mice were purchased from Clea Japan (Tokyo, Japan). C57BL/6-background IL-6-deficient mice were kindly provided by Professor Yoichiro Iwakura (Laboratory of Molecular Pathogenesis, Center for Experimental Medicine, Institute of Medical Science, University of Tokyo, Tokyo, Japan). Mice were maintained under specific pathogen-free conditions. C57BL/6 and IL-6-deficient mice were used at 7–9 weeks of age. All experiments were conducted according to the institutional ethical guidelines for animal experimentation.

**LPS-mediated Acute Inflammation**

To induce acute inflammation, wildtype (WT) mice and IL-6-deficient mice were injected intraperitoneally with 0 or 10 mg/kg LPS (Escherichia coli LPS, Sigma, St. Louis, MO) dissolved in 500 μL phosphate-buffered saline (PBS). Blood was collected at before and 24 hours after LPS injection and the serum was separated by centrifugation and stored at −30°C until used for ELISA analysis.

**Induction of Colitis**

For induction of colitis, WT mice and IL-6-deficient mice were given 3% dextran sodium sulfate (DSS) (m/w 36,000–50,000; MP Biomedicals, Solon, OH) dissolved in drinking water provided ad libitum for 5 days, followed by provision of ordinary water for 20 days.

**Assessment of Severity of DSS-induced Colitis**

WT mice were weighed daily from day 0 to day 25. Changes in body weight were calculated as follows: body weight change (%) = [(weight on a given day (days 0–13) − weight on day 0)/weight on day 0] * 100. Blood was collected from WT mice on days 5, 7, 10, 15, and 25 after DSS administration or day 0 by cardiac puncture under anesthesia and on days 0 and 10 from IL-6-deficient mice. The serum was separated by centrifugation and stored at −30°C until used for ELISA analysis.

**Cell Culture**

The human colonic adenocarcinoma COLO205 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT) and 1% penicillin–streptomycin (Nacalai Tesque) at 37°C under a humidified atmosphere of 5% CO2.

For the analysis of LRG protein induction, COLO205 cells were stimulated with various concentrations of cytokines for 24 hours and culture supernatant were concentrated using Amicon Ultra-4 10K MWC (Millipore, Bedford, MA). Concentrated supernatants were used for western blot analysis. Full-length human LRG cDNA was inserted into pcDNA3.1/V5-His-TOPO vector (Invitrogen) and designated pcDNA3.1-LRG-V5-His. pcDNA3.1-LRG-V5-His vector was transfected
Quantitative Real-time Reverse-transcription Polymerase Chain Reaction (RT-PCR) Analysis

For the quantification of mRNA levels of LRG, various mouse organs were analyzed by real-time RT-PCR as described previously.23 Levels of mouse LRG and mouse hypoxanthine phosphoribosyltransferase (HPRT) levels were determined by the 7900HT Real-time PCR system (Applied Biosystems, Foster City, CA) using specific primers: murine LRG forward 5'-ATCAAGGAAAGCCTCCAGGAT-3'; reverse 5'-CAGCTGCGTCAGGTTGG-3' and murine hypoxanithine phosphoribosyltransferase (HPRT) forward 5'-TCAGTCAACGGGGACATAAA-3'; reverse 5'-GGGGCTGTACTGCTTAACCAG-3'.

Statistics

The Mann–Whitney U-test or one-way analysis of variance (ANOVA) followed by a Scheffe’s test were used for statistical analyses. Two-tailed Student’s t-test was used for significant differences in LRG expression between identical patients with UC in active and remission disease stage. One-way ANOVA followed by a Dunnett’s test was used for multiple comparison of the difference of serum LRG levels at various timepoints after DSS treatment in mice. Pearson’s test was used to analyze the relationship between LRG and CRP, IL-6, or CAI. For drawing of receiver operating characteristic (ROC) curves and estimation of the area under the ROC curve (AUC) statistics, the software Excel Statistics 2010 (Social Survey Research Information, Tokyo, Japan) was used to quantify the ability to differentiate between remission and active by CAI. \( P < 0.05 \) was considered significant.

Ethical Considerations

Informed consent was obtained from all donors and all studies involving human subjects were approved by the Institutional Review Boards of the National Institute of Biomedical Innovation, Osaka University Hospital, the Department of Surgery, Osaka Rosai Hospital, and the University of North Carolina.

RESULTS

Serum LRG Levels Are Increased in Active UC Patients

We quantified serum LRG concentrations by ELISA using sera from patients with UC. Serum LRG concentrations were significantly elevated in the active UC patients (CAI \( \geq 6 \)) (14.24 ± 8.08 \( \mu \)g/mL) compared with HC (3.07 ± 1.42 \( \mu \)g/mL; \( P < 0.0001 \)) (Fig. 1A). There was also a significant difference between LRG serum levels in patients with active UC (CAI \( \geq 6 \)) (14.24 ± 8.08 \( \mu \)g/mL) compared with UC in remission (CAI <6) (5.34 ± 2.60 \( \mu \)g/mL; \( P < 0.0001 \)) (Fig. 1A). To determine whether serum LRG levels are increased in non-IBD disease controls, we quantified serum LRG levels in patients with appendicitis and diverticulitis.
diverticulitis. Elevated serum LRG levels were also observed in appendicitis and diverticulitis (16.83 ± 6.50 µg/mL) compared with HC (3.07 ± 1.42 µg/mL; \( P < 0.0001 \)) (Fig. 1A), suggesting that serum LRG levels are also increased in acute intestinal inflammation.

When UC were classified into three categories based on disease extent, significantly higher serum LRG concentrations were observed in active patients with extensive colitis (14.34 ± 7.89 µg/mL) compared with remission (4.96 ± 2.68 µg/mL; \( P < 0.0001 \)) and HC (3.07 ± 1.42 µg/mL; \( P < 0.0001 \)) and active patients with left-sided colitis (15.41 ± 9.16 µg/mL) compared with remission (5.91 ± 2.41 µg/mL; \( P = 0.0003 \)) and HC (3.07 ± 1.42 µg/mL; \( P = 0.001 \)) (Fig. 1B). Nonetheless, there was no clear difference between active patients with proctitis and HC, possibly due to the low number of patients in this group. In patients with UC in remission, serum LRG levels in all of three disease extent categories were comparable with HC (Fig. 1B). Significantly elevated serum LRG levels were also detected in a Caucasian UC cohort (9.46 ± 8.44 µg/mL) compared with HC (4.42 ± 1.91 µg/mL; \( P < 0.0005 \)) (Fig. 1C). In this Caucasian UC cohort, serum LRG levels were also significantly elevated in patients with extensive colitis (9.54 ± 8.05 µg/mL) compared with HC (4.42 ± 1.91 µg/mL; \( P < 0.05 \)) and left-sided colitis (10.90 ± 9.16 µg/mL) compared with HC (4.42 ± 1.91 µg/mL; \( P < 0.02 \)) (Fig. 1D). However, a clear difference was not observed between patients with proctitis and HC (Fig. 1D). These results suggest that serum LRG levels were elevated in active UC.

**Serum LRG Levels Are Correlated with Disease Activity in UC Patients**

We investigated the correlation between serum LRG levels and disease activity (CAI) in UC patients. A positive correlation was observed between LRG and CAI (\( r = 0.731, P < 0.00001 \)) (Fig. 2A). This correlation was stronger than that observed between CRP and CAI (\( r = 0.654, P < 0.00001 \)) (Fig. 2A). When patients with UC were classified into active and remission according to the endoscopic findings, significantly elevated serum LRG levels and CRP levels were observed in patients with active UC compared with patients in remission (\( P < 0.005 \), respectively) (Supporting Fig. 1A). While serum LRG levels were significantly correlated with CRP levels in patients with UC (\( r = 0.850, P < 0.00001, n = 82 \)) (Supporting Fig. 2A), such a correlation was not found when a CRP-negative subgroup (CRP <0.2, \( n = 51 \)) was analyzed (\( r = 0.101, P = 0.481 \)) (Supporting Fig. 2B). In this CRP-negative group, serum LRG levels were significantly correlated with CAI (\( r = 0.416, P = 0.00241 \)) (Supporting Fig. 2C); however, significant correlation was not found between CRP and CAI (\( r = -0.0896, P = 0.532 \)) (Supporting Fig. 2D). Additionally, in the CRP-negative group elevated serum LRG levels were detected in patients with endoscopically active UC compared with patients with UC in remission (\( P = 0.0442 \)) (Supporting Fig. 1B). These findings in patients with low CRP may explain a better correlation of CAI with LRG than that with CRP.

When UC was classified by disease extent, a significantly higher positive correlation was detected between LRG and CAI than CRP and CAI both in extensive colitis (\( r = 0.690, P < 0.000001 \) and \( r = 0.580, P = 0.000168 \)) and left-sided colitis (\( r = 0.840, P < 0.000001 \) and \( r = 0.759, P < 0.000001 \)), but not in proctitis (Fig. 2B). Importantly, by analyzing sera obtained at active (CAI >6) and remission (CAI <6) disease stages from 10 identical UC patients, a significant decrease in serum LRG levels in remission was detected (Fig. 2C).

By generating an ROC curve, the sensitivity and specificity of serum LRG for remission and active by CAI were determined (Fig. 2D). The AUC for serum LRG levels was 0.901, whereas the AUC for CRP levels was 0.845. The cutoff value of serum LRG levels was 7.21 µg/mL (sensitivity = 84.0%, specificity = 82.5%). In contrast, when the cutoff value of CRP levels was set to 0.20, a maximum CRP value of normal range, the sensitivity was 80.0% and the specificity was 80.7%. These results emphasize the usefulness of monitoring serum LRG levels for the evaluation of the disease activity of UC.

**Expression of LRG Was Increased in Inflamed UC Colons**

Next, to investigate whether local inflammatory sites in patients with UC are a potential source of increased serum LRG we first looked at the expression of LRG in the colon by western blot analysis on inflamed and non-inflamed sites of surgically resected full-thickness colon specimens from patients with UC. Western blot analysis showed that LRG expression in colon tissues was increased in inflamed sites of active UC patients compared with non-inflamed colon tissues (Fig. 3A). Next, we tried to examine the localization of LRG. By immunohistochemistry, increased expression of LRG was detected in the cytoplasm of intestinal epithelial cells (IECs) in inflamed tissues (Fig. 3B–E). In contrast, expression of LRG was lower in non-inflamed tissues (Fig. 3B–E). These data suggest that inflamed colon tissue is a potential source of increased serum LRG in patients with UC.

**LRG Is Induced by Stimulation with TNF-α, IL-6, or IL-22**

It has been reported that IL-6 is an inducer of LRG expression.\(^{16}\) However, it is not clear whether LRG is induced by cytokines other than IL-6. At first we investigated the serum levels of IL-6, IL-22, and TNF-α, known
Figure 2. Serum LRG levels are correlated with disease activity better than CRP in patients with UC. (A) Serum levels of LRG correlated with CAI \( (n = 82; \ P < 0.000001; r = 0.731) \) better than CRP \( (n = 82; \ P < 0.000001; r = 0.654) \) in patients with UC. (B) Serum levels of LRG correlated with disease activity in extensive colitis \( (n = 37; \ P < 0.000001; r = 0.690) \) and left-sided colitis \( (n = 30; \ P < 0.000001; r = 0.840) \) better than CRP in extensive colitis \( (n = 37; \ P = 0.000168; r = 0.580) \) and left-sided colitis \( (n = 30; \ P < 0.000001; r = 0.759) \), while neither LRG \( (n = 15; \ P = 0.649; r = -0.128) \) nor CRP levels \( (n = 15; \ P = 0.360; r = -0.255) \) were correlated with disease activity in proctitis. (C) Compared with 10 identical active patients with UC, serum levels of LRG were decreased in remission. * \( P < 0.002 \) by Student’s t-test. (D) ROC curves for LRG and CRP for differentiation between UC patients with remission \( (n = 57) \) and active \( (n = 25) \) by CAI.
Indeed, ELISA analysis using sera from 82 UC patients revealed that serum TNF-α, IL-6, and IL-22 levels were significantly elevated in active UC patients compared with those patients in remission ($P = 0.0178$, $P = 0.00690$, and $P < 0.0001$, respectively) (Fig. 4A). Next, to investigate...
which proinflammatory cytokines induce expression of LRG we stimulated human colonic adenocarcinoma COLO205 cells with TNF-\(\alpha\), IL-6, or IL-22 for 24 hours. After cytokine stimulation, secretion of LRG protein into the culture media was analyzed by western blotting. Interestingly, LRG was induced not only by stimulation with IL-6, but also by TNF-\(\alpha\) and IL-22 in a dose-dependent manner (Fig. 4B). These results indicate that expression of LRG is induced by various proinflammatory cytokines including IL-6.

Expression of LRG Through an IL-6-independent Pathway Is Demonstrated in LPS-mediated Acute Inflammation and DSS-induced Colitis

CRP is one of the representative acute phase proteins in humans and CRP production is primarily dependent on liver by circulating IL-6. To examine the possible differences in induction mechanisms between LRG and CRP, particularly with regard to the involvement of IL-6, we took advantage of murine models. We first assessed whether LRG is induced in WT mice by injecting LPS, an inducer of proinflammatory cytokines from macrophages, because CRP is poorly induced in mice during acute inflammation. At 24 hours after intraperitoneal injection of LPS, serum samples were prepared and serum LRG levels were determined by ELISA. Compared with WT mice, significant elevation of serum LRG levels were detected in LPS-administered WT mice (Fig. 5A), suggesting that LRG is induced during acute inflammation in mice as in humans.

We next used a murine IBD model to investigate induction mechanisms of LRG during colonic inflammation. DSS-induced colitis is often used as a murine model of UC.\(^{27}\) We induced colitis in WT mice by treating them with 3% DSS for 5 days and measured changes in relative body weight. Body weight began to decrease at day 5, showed greatest reduction at day 9, and recovered at 18 days after DSS treatment (Fig. 5B). We analyzed changes in serum LRG levels by ELISA before and 5, 7, 10, 15, and 25 days after DSS treatment. Consistent with body weight loss, serum LRG levels were significantly elevated at 5 days after DSS treatment (Fig. 5B). Serum LRG levels remained high until day 15, but decreased at day 25. Delayed normalization of serum LRG levels is likely due to the prolonged inflammation at inflamed tissue sites. Additionally, a long half-life of serum LRG might also be involved in this, since our preliminary data suggest that the half-life of serum human LRG levels are about two times longer than that of CRP (data not shown). To investigate which organs produce LRG in DSS-induced colitis, RNA was extracted from colon, liver, and spleen before and 9 days after DSS treatment. By quantitative PCR analysis (Fig. 5D), expression of LRG was significantly increased in liver (\(P = 0.00106\)) and spleen (\(P = 0.0376\));
However, the strongest induction was observed in colon ($P = 0.000126$).

To investigate whether LRG induction is dependent on IL-6 or not, we analyzed serum LRG levels in IL-6-deficient mice. Interestingly, basal LRG levels in IL-6-deficient mice were similar to those in WT mice and LRG was robustly induced by LPS administration in IL-6-deficient mice (Fig. 5A). Moreover, increased serum LRG levels were also detected in the active stage (day 9) of DSS-induced colitis in IL-6-deficient mice (Fig. 5E). Importantly, the increase of serum LRG in IL-6-deficient mice was similar to that in WT mice (Fig. 5A, E). These findings indicate that LRG expression can be induced in the absence of IL-6.
In this study we first demonstrated that serum LRG levels were significantly increased in sera of active UC patients compared with patients in remission and HC. Serum LRG is likely elevated in diverse racial groups, because we detected increased serum LRG levels not only in Japanese patients (Fig. 1A) but also in Caucasian patients with UC (Fig. 1C,D) and CD (data not shown). In addition, levels of serum LRG were significantly correlated with disease activity in UC and the correlation was stronger than CRP. Moreover, by analyzing ROC curve and AUC, serum LRG levels showed higher AUC than CRP and serum LRG levels represented superior sensitivity and specificity to CRP for remission and active of UC by CAI (Fig. 2D), indicating that LRG is a useful marker to evaluate disease activity in UC.

In the normal state, serum LRG is thought to be produced from liver and LRG is abundantly found in the sera of HC. In colonic inflammation, we found that the expression of LRG is increased in the inflamed mucosa of UC patients and mice with DSS colitis, suggesting that inflamed tissue can be a source for production of LRG (Fig. 3). The increased expression of LRG in inflamed tissue has previously been observed in appendix during acute appendicitis. Increased expression of LRG in inflamed tissue has previously been observed in appendix during acute appendicitis. Moreover, in acute inflammatory disorders, including appendicitis and diverticulitis, increased expression of serum LRG was observed (Fig. 1A). These results indicate that the elevated expression of LRG at inflamed sites and in sera occurs in various acute and chronic inflammatory disorders. Therefore, increased serum LRG levels are not suitable for use as a specific diagnostic marker of IBD.

CRP is the most common serum marker used to evaluate disease activity in inflammatory diseases. However, serum CRP is primarily dependent on liver production induced by circulating IL-6. Compared with CD and RA, only modest to absent CRP responses are observed in UC, despite active inflammation in colon. Indeed, our cohort of 82 UC patients, analyzed in this study, included five patients with normal value of CRP while having active disease (Fig. 2A). However, our study demonstrated that serum LRG levels were significantly increased in active UC patients' sera and correlated better with disease activity of UC than CRP levels (Figs. 1A, 2A). Particularly, in the group of patients with negative CRP (CRP <0.2), significant correlation was observed between serum LRG levels and CAI (Supporting Fig. 2C). Similarly, among CRP-negative patients serum LRG levels were significantly elevated in those with endoscopically active UC, compared with UC in remission (Supporting Fig. 1B). In addition, serum LRG levels were decreased after therapy (Fig. 2C), suggesting that LRG is a useful serological biomarker for evaluating disease activity and therapeutic effect in UC.

Better correlation of serum LRG levels with disease activity of UC than CRP might be explained in part by the differences in induction mechanisms between LRG and CRP. While the expression of CRP is essentially dependent on IL-6, several cytokines may compensate for the absence of elevated IL-6 in induction of LRG expression. Accordingly, expression of LRG in COLO205 cells was induced not only by IL-6 but also by TNF-α and IL-22 (Fig. 4B), all of which were increased in sera of UC patients (Fig. 4A). Expression of LRG was strongly induced by IL-22 in COLO205 cells, correlating with enhanced STAT3 (Tyr705) phosphorylation by IL-22 compared with IL-6 (data not shown). Thus, inflammatory cytokines such as TNF-α and IL-22 may mediate LRG expression in the absence of IL-6. Moreover, using DSS-induced colitis in IL-6-deficient mice, we could demonstrate an IL-6-independent pathway for LRG induction (Fig. 5E). Because promoter regions of human and mouse LRG share high sequence homology and contain putative binding sites for transcription factors such as C/EBP, MZF1, and STAT, it is conceivable that the similar IL-6-independent mechanisms of LRG induction are also involved in humans. Future studies are required to fully elucidate the induction mechanisms of LRG in both humans and mice.

In the three disease categories of UC based on extent of disease, serum LRG levels tended to be low in proctitis compared with extensive colitis and left-sided colitis (Fig. 1B). In addition, correlation between serum LRG levels and disease activity did not reach significance in proctitis (Fig. 2B). Although the low number of patients with active proctitis may preclude the proper evaluation of LRG levels, limited inflamed area of proctitis may also be a reason for slight increases of serum LRG levels in these patients. Given the increased production of LRG in inflamed colonic mucosa, fecal LRG might be a more sensitive disease biomarker for UC including proctitis. Optimization for the measurement of fecal LRG is currently under way in our laboratory.

This study also highlights the potential usefulness of LRG in evaluating murine colitis. Our results indicate that serum LRG levels increase as the disease progresses in a DSS-induced colitis model (Fig. 5B,C). In addition, the LRG expression is significantly upregulated in the colon with DSS-induced colitis (Fig. 5D). Thus, LRG in mice can be an objective disease activity marker for colitis models and may be useful for preclinical studies of IBD.

In conclusion, serum LRG levels reflect disease activity of UC better than CRP, especially in patients with low CRP. In the inflammatory condition, LRG is expressed in the inflamed tissue and expression of LRG is regulated by mechanisms different from that of CRP. These findings suggest that serum LRG is a novel and potential serologic biomarker for evaluating disease activity of UC.

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