Role of Csk in intestinal epithelial barrier function and protection against colitis

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学 位 論 文 の 内 容 要 旨

Role of Csk in intestinal epithelial barrier function and protection against colitis

腸上皮バリア機能と大腸炎発症抑制における Csk の役割

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INTRODUCTION

The intestinal epithelium consists of a variety of epithelial cells, such as enterocytes, goblet cells, Paneth cells, and enteroendocrine cells, which arise from intestinal stem cells (ISCs) located at the base of crypts in the intestine and undergo continuous and rapid renewal. These intestinal epithelial cells (IECs) play a pivotal role in the maintenance of the integrity and barrier function of the intestinal epithelium. Dysfunctions of IECs are thought to participate in the disruption of the intestinal epithelial barrier, resulting in gastrointestinal diseases, such as colitis and colorectal cancer. The molecular mechanism underlying the maintenance of the intestinal epithelial barrier by IECs remains unclear, however.

The COOH-terminal Src kinase (Csk) is a tyrosine kinase and negatively regulates Src family kinases (SFKs)—including c-Src, Fyn, and c-Yes—are nonreceptor protein tyrosine kinases that play essential roles in the regulation of cell proliferation, survival, and cell-cell adhesion. We previously demonstrated that IEC-specific Csk-deficient mice (Csk CKO mice) manifested the increased proliferative activity and turnover of IECs, with the activity of Src, Fyn, and c-Yes being elevated in epithelial cells of the small intestine. By contrast, the significant reduction of ISCs was observed at the base of crypts in the small intestine of the mutant mice. The number of Paneth cells was decreased, whereas that of goblet cells was increased, in the small intestine of the mutant mice. These results thus suggested that Csk in IECs is important for homeostasis of the intestinal epithelium in the steady-state condition. The role of intestinal epithelial Csk in the barrier function of, or inflammatory condition of, the intestinal epithelium remains unclear, however.

To address this issue, we have here examined the effect of dextran sodium sulfate (DSS) on Csk CKO mice. DSS acts as a toxic agent for the colonic epithelium and causes epithelial cell injury, leading to the disturbance of intestinal epithelial barrier function and then the development of colonic inflammation (DSS-induced colitis) as well as regeneration.

MATERIALS AND METHODS

Mice – Villin-*cre* mice were crossed with $Csk^{fl/fl}$ mice to generate $Csk^{fl/+}$;villin-*cre* offspring, which were then crossed with $Csk^{fl/fl}$ mice to obtain $Csk^{fl/fl}$;villin-*cre* (Csk CKO) and $Csk^{fl/fl}$ (control) animals.

DSS-induced colitis – Male mice at 8-10 weeks of age were treated with 2% (wt/vol) DSS in drinking water for 7 days, followed by 2 days of regular drinking water.

Clinical and histological assessment of colitis – Mice were monitored daily for weight loss, stool consistency, and stool blood. The total score for these three parameters, ranging from 0 (normal) to 12 (severe), was determined.

For scoring of colonic inflammation by histological examination, the fresh colon was immediately fixed with 4% (wt/vol) paraformaldehyde in phosphate buffered saline (PBS). Paraffin-embedded sections (5 μm) were stained with Mayer's hematoxylin and eosin (H&E), and a combined score for inflammatory cell infiltration, tissue damage, and crypt structure was determined in a blinded manner, ranging from 0 (no change) to 9 (severe).

Intestinal permeability assay – Mice administered with 2% DSS for 0, 3, and 6 days were fasted for 4 hours and gavaged with 4-kDa fluorescein isothiocyanate (FITC)–dextran. Four hours after gavage, blood was collected by cardiac puncture, and serum was separated. The concentration of FITC-dextran in the serum was determined by measuring fluorescence at 535nm/485nm using spectrophotofluorometry and then calculated according to a standard curve plotted by serially diluted FITC-dextran.

Immunofluorescence analysis – The colon was fixed for 6 h at 4°C with 4% paraformaldehyde in PBS, transferred to a series of sucrose solutions, embedded in optical cutting temperature compound, and rapidly frozen in liquid nitrogen. Frozen sections (5 μm) were prepared using a cryostat and then stained with primary antibodies (Abs) and fluorescent dye–labeled secondary Abs.

Immunoblot analysis – The colon was washed with ice-cold PBS and then homogenized in lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% sodium dodecyl sulfate). The lysates were heated at 95°C for 5 min and centrifuged at 17,500×g for 15 min at room temperature, and the resulting supernatants were subjected to immunoblot analysis.

RESULTS AND DISCUSSION

To investigate the role of Csk in the regulation of the intestinal epithelial barrier, we gave Csk CKO and control mice drinking water with 2% DSS, a chemical disrupting the epithelium, for 7 days, followed by 2 days of regular drinking water. After DSS treatment, Csk CKO mice showed greater body weight loss, colon shortening, disease activity index and histological score than control mice. Microscopic examination also revealed that transmural inflammation, epithelial erosion, immune cell infiltration, and crypt disappearance were marked in the colon of Csk CKO mice. Moreover, Csk CKO mice showed significantly increased intestinal permeability than control mice on day 6 of DSS treatment. Collectively, these results suggested that Csk in IECs protects against DSS-induced colonic injury and acute colitis.

Disruption of the intestinal epithelial barrier is thought to participate in the development of DSS-induced colitis. The maintenance of the barrier function of the intestinal epithelium requires the proper regulation of IEC proliferation and death. We thus examined the proliferative activity and apoptosis of colonic epithelial cells in Csk CKO mice following DSS administration. We found that Csk CKO mice treated with or without DSS manifested the increased proliferative activity of IECs in the colon. Consistently, the number of apoptotic cells in the colon of the DSS-treated mutant mice was higher than that for DSS-treated control mice, whereas it did not differ between two genotypes at steady condition.

Tight junction and adherens junction proteins, such as occludin, claudin-1, claudin-2, ZO-1, E-cadherin, and β-catenin are thought to be key players in the maintenance of the intestinal epithelial barrier function. We thus determined the expression levels of these junctional proteins after 0 or 6 days of DSS treatment. Immunoblot analysis showed that the expression levels of occludin, claudin-1, claudin-2, ZO-1, E-cadherin, and β-catenin in the colon were similar between Csk CKO and control mice at baseline. DSS treatment resulted in the marked reduction in the abundance of occludin in the colon of Csk CKO mice. The expression of claudin-2 was slightly, but statistically insignificantly, decreased only in DSS-treated mutant mice. In addition, the amounts of claudin-1, ZO-1, E-cadherin, and β-catenin in the colon of Csk CKO and control mice were not significantly changed by DSS treatment. These results thus indicated that Csk likely participated in the development of DSS-induced colitis through regulating the expression of occludin protein in colonic IECs.

CONCLUSION

IEC-specific Csk deficiency in mice resulted in the increased susceptibility to DSS-induced colitis. In addition, following DSS treatment, intestinal permeability in Csk CKO mice was significantly increased compared with that in control mice, suggestive of the impairment of the intestinal epithelial barrier by Csk deficiency. Our results thus suggest that Csk in IECs contributes to protection against the development of DSS-induced colitis through the maintenance of the intestinal epithelial barrier.

神戸大学大学院医学(系)研究科（博士課程）

論文審査の結果の要旨

受付番号	甲 第２８１７号	氏　名	SUN CHUNXIAO
論文題目 Title of Dissertation	Role of Csk in intestinal epithelial barrier function and protection against colitis 腸上皮バリア機能と大腸炎発症抑制における Csk の役割		
審査委員 Examiner	主　査 Chief Examiner 横崎 宏 副　査 Vice-examiner 鈴木 聡 副　査 Vice-examiner 児玉 裕三		

（要旨は１，０００字～２，０００字程度）

腸管上皮は吸収上皮、杯細胞、パネート細胞、内分泌細胞など様々な細胞により構成されるが、いずれの細胞も陰窩底部に存在する腸管上皮幹細胞に由来し、持続的に更新されている。これら腸管上皮細胞は腸管粘膜の統合性とバリア機能の維持に重要な役割を果たす。腸管上皮細胞の機能不全は腸上皮バリアの崩壊を介して腸炎や癌腫などの胃腸管疾患の原因となると考えられている。しかしながら、腸上皮バリアの維持機構の分子メカニズムは十分解明されていないのが現状である。COOH-terminal Src kinase (Csk)は、c-Src, Fyn, c-Yesなど細胞の増殖、生存および細胞間接着に重要な役割を果たすSrcファミリーキナーゼを負に調節するチロシンキナーゼである。申請者の所属する研究室では腸上皮特異的 Csk 欠損マウス（Csk CKO マウス）が、c-Src, Fyn, c-Yes の活性上昇を伴う腸上皮の増殖活性と細胞更新の促進と杯細胞数の増加がみられる一方で、腸管幹細胞とパネート細胞の有意な減少が認められることを報告している。これらは、生理的状態における腸管上皮の恒常性維持にCsk が重要な役割を果たしていることを示唆したが、腸管上皮のバリア機能や腸炎におけるCsk の役割については不明である。そこで本研究では Csk CKO マウスと野生型マウスにおけるデキストラン硫酸ナトリウム（DSS）誘発腸炎を比較することにより腸炎発生におけるCsk の役割を解析した。

用いた方法は以下のごとくである。DSS 腸炎は 8 から 10 週齢雄マウスに 2% (wt/vol) DSS を 7 日間飲水投与後 2 日間通常飲水にて誘発した。マウスの体重、便の性状、便潜血は毎日スコア 0（正常）から 12（高度）までの段階評価で記録した。組織学的腸炎の評価は 4% パラフォルムアルデヒド含有 PBS 固定パラフィン包埋 5μm 切片をヘマトキシリン・エオシン染色したものを検鏡し、炎症細胞浸潤、組織破壊および陰窩形状をスコア 0（無変化）から 9（強い変化）までの段階で評価した。腸管透過性アッセイでは、DSS 投与後 0, 3, 6 日目のマウスを 4 時間絶食後 4-kDa FITC 標識デキストランを強制摂食させ、4 時間後に心臓穿刺で採取した血液より血清を分離しそこに含まれる FITC 標識デキストラン濃度を計測した。腸管粘膜における Ki-67, cleaved caspase-3, β-catenin の可視化には蛍光抗体法を、occludin, claudin-1, claudin-3, ZO-1, E-cadherin タンパク発現はイムノブロット法を用いて解析した。

得られた結果は以下のごとくである。

1. DSS 摂取後 Csk CKO マウスには対照に比較して、強い体重減少、大腸短縮が見られ、組織学的にも全層性炎症、上皮びらんおよび陰窩消失が顕著であった。
2. DSS 処理後 6 日における腸管透過性は Csk CKO マウスにおいて対照に比較して有意に亢進していた。
3. Csk CKO マウス大腸上皮の増殖活性およびアポトーシスは DSS 処理にかかわらず対照に比較して亢進していた。

4．大腸上皮のアポトーシス数はDSS処理においてCsk CKOマウスが対照に比較して高かったが、DSS未処理では両群間に差は見られなかった。

5．DSS処理0および6日目の大腸上皮における細胞間結合タンパク発現を解析したところ、0日目（未処理）でのoccludin, claudin-1, claudin-2, ZO-1, E-cadherinおよびβ-cateninの発現レベルはCsk CKOマウスと対照でほぼ同様であったが、DSS処理によりoccludinの発現レベルがCsk CKOマウスにおいて有意に減少し、claudin-2も減少傾向を示した。一方、両群において claudin-1, ZO-1, E-cadherin およびβ-cateninの発現レベルはDSS処理で有意の変化を示さなかった。

以上、申請者らはCsk CKOマウスではDSS誘発大腸炎に対する感受性が高まり、その背景にはDSS処理による腸管透過性がCsk CKOマウスで有意に亢進しており、腸管上皮バリア機能不全が存在する可能性を明らかにし、腸管上皮においてCskは腸管バリア機能の維持を介してDSS誘発腸炎発生に予防的に作用していることが示唆された。

本研究は腸管粘膜統合性維持におけるCskの役割を腸管上皮特異的コンディショナルノックアウトマウスを用いたDSS誘発腸炎系により解析し、Cskが細胞接着分子の発現を調節することにより上皮バリア機能の恒常性を維持し DSS 誘発腸炎抵抗性に寄与している可能性を明らかにしたものであり、上皮統合性維持不全の観点から腸疾患の病態発生を考察する上で重要な知見を得たものとして価値ある業績であると認める。よって、本研究者は博士（医学）の学位を得る資格があると認める。