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The targeted delivery of the c-Src peptide complexed with schizophyllan to macrophages inhibits polymicrobial sepsis and ulcerative colitis in mice

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ABSTRACT

Hyper-inflammatory responses triggered by intracellular reactive oxygen species (ROS) can lead to a variety of diseases, including sepsis and colitis. However, the regulators of this process remain poorly defined. In this study, we demonstrate that c-Src is a negative regulator of cellular ROS generation through its binding to p47phox. This molecule also competitively inhibits the NADPH oxidase complex (NOX) assembly. Furthermore, we developed the schizophyllan (SPG)-c-Src SH3 peptide, which is a β-1,3-glucan conjugated c-Src SH3-derived peptide composed of amino acids 91–108 and 121–140 of c-Src. The SPG-SH3 peptide has a significant therapeutic effect on mouse ROS-mediated inflammatory disease models, cecal-ligation–puncture-induced sepsis, and dextran sodium sulfate-induced colitis. It does so by inhibiting the NOX subunit assembly and proinflammatory mediator production. Therefore, the SPG-SH3 peptide is a potential therapeutic agent for ROS-associated lethal inflammatory diseases. Our findings provide clues for the development of new peptide-base drugs that will target p47phox.

1. Introduction

Previous studies have implicated the roles of NADPH oxidase (NOX)-dependent reactive oxygen species (ROS) and oxidant-induced protein or lipid alterations in lethal inflammatory responses such as sepsis and colitis [1–4]. Sepsis is characterized by an overwhelming systemic inflammatory response to a gram-negative bacterial infection [2,3]. Growing evidence suggests that ROS play crucial roles in the activation of toll-like receptor (TLR) 4 and in the pathophysiology of sepsis by regulating immune cell activation and end organ injury [5]. Excess intracellular and extracellular ROS (superoxide and hydrogen peroxide) are able to prime phagocytes (macrophages and neutrophils) to mount an acute hyper-inflammatory response [4,6,7]. Host factors that regulate cellular ROS levels may be important modifiers in sepsis pathogenesis [8,9].

Ulcerative colitis (UC) is a relapsing inflammatory bowel disease (IBD) characterized by chronic and recurrent inflammation of the gastrointestinal tract. The pathogenesis of colitis is complex and involves an interaction between the patient's genetics, immune system and environmental factors [10,11]. One of the major factors in the onset of colitis is an inappropriate mucosal immune response toward intestinal microbiota [12]. Furthermore, excessive ROS production has been observed in the inflamed mucosa of IBD patients [4,12–14]. These highly cytotoxic molecules can contribute to tissue damage in IBD [10,14] and non-phagocytes (intestinal epithelial cells), which are recruited into the colonic mucosa of IBD patients, may release these cytotoxic molecules [4,12]. However, the molecular pathways that control ROS production via NOX enzymes in primary intestinal epithelial cells during acute and chronic inflammation are poorly understood.

The NOX complex is composed of two transmembrane proteins: flavocytochrome b components (gp91phox/NOX2 and p22phox)

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and four cytosolic proteins (p47phox, p67phox, p40phox, and Rac1/2) in phagocytes [4,15]. In order for the complex to be activated in intact cells, the phosphorylation of p47phox on several serine residues is essential [3,15]. Upon activation, the cytosolic components translocate to the transmembrane catalytic protein NOX2, resulting in the functional NOX complex. ROS have been implicated in multiple physiological and pathological processes as second messengers in cell signaling [3] [16]. Numerous studies have demonstrated that NOX-dependent ROS generation plays a role in modulating TLR4 signaling, inflammatory response [2], and disease pathogenesis [9,17]. In particular, colonic epithelial cells abundantly express NOX1, which is the homolog most closely related to NOX2 with regard to structure and function [12,14]. This enzyme comes into close contact with normal and pathogenic bacteria; it may play an important role in local innate immune and inflammatory responses in the gut [11,18]. Several studies have demonstrated that NOX expression and ROS production in intestinal epithelial cell cultures can be stimulated *in vitro* by bacteria, bacterial products, and pro-inflammatory cytokines such as interleukin-18 (IL-18), interferon gamma and tumor necrosis factor-alpha (TNF-α) [2,4,12,14]. The activation of NOX involves its interaction with p22phox, and binding to regulatory partners, including: NOX organizer 1 (NOXO1), the p47phox homolog; NOX activator 1 (NOXA1), the p67phox homolog; and Rac1 GTPase [12,14]. In the colon, NOXO1 and NOXA1 transcripts are also abundantly expressed [12,18]. It is not clear whether the dysregulation of NOX expression and activity is linked to pathologies such as sepsis and IBD.

Schizophyllan (SPG) from soluble β-glucan from the *Schizophyllum commune* is a polysaccharide member of the β-(1-3)-glucan family. It forms a triple helix in neutral solution. When an alkaline solution of SPG is neutralized, it denatures, adopts a single chain formation and reverts to its original triple helix through hydrophobic interactions and hydrogen bonds. During this physicochemical interaction, two main-chain glucoses of β-(1-3)-glucans and one oligonucleotide (ODN) base or peptide form a stoichiometric complex. By exploiting this complex, an SPG-based drug delivery system was designed to deliver functional ODNs to targeted cells [19–22].

Dectin-1 is a pathogen pattern recognition receptor that binds β-glucans such as SPG. It is the major β-glucan receptor on antigen presenting cells (APCs) (such as macrophages and dendritic cells) and on non-phagocytes (epithelial and endothelial cells) [23]. The complex, therefore, is presumably recognized by Dectin-1 on APCs. After recognizing the complex, these cells take up ODNs or peptides. When β-glucans engage Dectin-1, the immunoreceptor tyrosine-based activation motif-like sequence becomes phosphorylated within its cytoplasmic domain. Subsequently, association of the c-Src or Syk tyrosine kinase induces assembly of a scaffold consisting of the CARD9/BCL10/MALT1 complex-nuclear factor-κB (NF-κB) pathway in dendritic cells and macrophages [24,25]. However, SPG's role in ROS-induced immune responses has not been characterized in detail.

In this report, we report that c-Src is a critical negative regulator of NOX activation through a physical and functional interaction with p47phox. We found that c-Src competitively inhibits p47phox binding to other NOX subunits. In doing so, it efficiently blocks the assembly of the NOX complex. Furthermore, the SH3 peptide, derived from c-Src, conjugates with SPG and blocks the NOX interaction. This interaction suppresses ROS and inflammatory cytokine production. Using *in vivo* models of cecal ligation procedure (CLP)-induced polymicrobial sepsis and dextran sodium sulfate (DSS)-induced ulcerative colitis, we showed that treatment with the SPG/c-Src-SH3 peptide complex, dramatically reduced the mortality involved in controlling the excessive production of ROS and inflammation, as well as pathologic responses in mice. Our findings demonstrate that c-Src plays a role in NOX activation through its direct binding with p47phox. It also prevents excessive inflammatory responses.

2. Materials and methdos

2.1. Cells

The mouse macrophage cell lines RAW264.7 (ATCC TIB-71; American Type Culture Collection) and HEK293T (ATCC-11268) cells were maintained in DMEM (Invitrogen) containing 10% FBS (Invitrogen), sodium pyruvate, nonessential amino acids, penicillin G (100 IU/ml), and streptomycin (100 μg/ml). Human monocytic cell line THP-1 (ATCC TIB-202) cells were grown in RPMI 1640/glutamax supplemented with 10% FBS. Transient transfections were performed with Lipofectamine 3000 (Invitrogen), or calcium phosphate (Clontech), according to the manufacturer's instructions. THP-1 and Raw264.7 stable cell lines were generated using a standard selection protocol with 2 μg/ml of puromycin.

2.2. Reagents

LPS (*Escherichia coli* O111:B4), Zymosan (cell wall preparation of *Saccharomyces cerevisiae*), Curdlan AL from *Alcaligenes faecalis*, Scleroglucan from *Sclerotium rolfsii*, and Laminarin from *Laminaria digitata* were purchased from Invivogen. Schizophyllan from *S. commune* was provided from QueGen Biotech. Succinic anhydride, dimethyl sulfoxide (DMSO), 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC), and N-Hydroxy succinimide (NHS) were all purchased from Sigma–Aldrich Korea. Nanopure water and 25 mM of 2-(*N*-morpholino) ethanesulfonic acid buffer (MES, pH 5.0) were used for synthesis of SPG-SH3 peptide.

2.3. Plasmid construction

The plasmid encoding full-length of the p47phox (Flag-p47phox) and NF-κB luciferase reporter plasmid were previously described [16,25]. Plasmids encoding different regions of p47phox (ΔPX, ΔPX-SH3, ΔPX-AIR), p67phox, p40phox, c-C-Src and c-C-Src (ΔSH3, ΔSH3-SH2) genes were generated by PCR amplification cDNA and subcloning into a pEF-IRES between the *Afl*II and *Xba*I sites. All constructs for transient and stable expression in mammalian cells were derived from the pEF-IRES-Puro expression vector. All constructs were sequenced using an ABI PRISM 377 automatic DNA sequencer to verify 100% correspondence with the original sequence.

2.4. Immunoprecipitation and immunoblot analysis

Immunoprecipitation and immunoblot assays were performed as described previously [16,25]. Antibody binding was visualized by chemiluminescence (ECL; Millipore) and detected by a Vilber chemiluminescence analyzer (Fusion SL 3; Vilber Lourmat).

2.5. Peptides

The c-Src-SH3 peptides were commercially synthesized and purified in acetate salt form to avoid abnormal responses in preclinical studies (Peptron, Korea). The amino acid sequences of the peptides in this study are described in Supplementary Table 1.

2.6. Synthesis of COOH-terminated SPG

The synthesis was conducted following a modified protocol reported elsewhere [26,27]. SPG (5 mg, 0.011 mM) was dissolved in