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ORIGINAL RESEARCH

NOD-Like Receptor Protein 3 Inflammasome Priming and Activation in Barrett's Epithelial Cells

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SUMMARY

Microbial products activate Toll-like receptors and inflammasomes. Gram-negative bacteria (which dominate the microbiome of reflux esophagitis) produce lipopolysaccharide, a Toll-like receptor-4 ligand. To explore this system's contribution to esophageal inflammation, we studied lipopolysaccharide effects on inflammasome priming and activation in esophageal cells.

BACKGROUND & AIMS: Microbial molecular products incite intestinal inflammation by activating Toll-like receptors (TLRs) and inflammasomes of the innate immune system. This system's contribution to esophageal inflammation is not known. Gram-negative bacteria, which dominate the esophageal microbiome in reflux esophagitis, produce lipopolysaccharide (LPS), a TLR4 ligand. TLR4 signaling produces pro-interleukin (IL)1β, pro-IL18, and NOD-like receptor protein 3 (NLRP3), which prime the NLRP3 inflammasome. Subsequent NLRP3 inflammasome activation cleaves caspase-1, inducing secretion of proinflammatory cytokines and pyroptosis (inflammatory cell death). We explored LPS effects on NLRP3 inflammasome priming and activation in esophageal cells.

METHODS: We exposed esophageal squamous and Barrett's epithelial cells to LPS and measured the following: (1) TLR4, pro-IL1β, pro-IL18, and NLRP3 expression; (2) caspase-1 activity; (3) tumor necrosis factor-α, IL8, IL1β, and IL18 secretion; (4) lactate dehydrogenase (LDH) release (a pyroptosis marker); and (5) mitochondrial reactive oxygen species (ROS). As inhibitors, we used acetyl-Tyr-Val-Ala-Asp-CHO for caspase-1, small interfering RNA for NLRP3, and (2-(2,2,6,6,-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride for mitochondrial ROS.

RESULTS: Squamous and Barrett's cells expressed similar levels of TLR4, but LPS induced TLR4 signaling that increased tumor necrosis factor-α and IL8 secretion only in Barrett's cells. Barrett's cells treated with LPS showed increased expression of pro-IL18, pro-IL1β, and NLRP3, and increased mitochondrial ROS levels, caspase-1 activity, IL1β and IL18 secretion, and LDH release. Acetyl-Tyr-Val-Ala-Asp-CHO, NLRP3 small interfering RNA, and Mito-TEMPO all blocked LPS-induced IL1β and IL18 secretion and LDH release.

CONCLUSIONS: In Barrett's cells, LPS both primes and activates the NLRP3 inflammasome, causing secretion of proinflammatory cytokines and pyroptosis. By triggering molecular events promoting inflammation, the esophageal microbiome might contribute to inflammation-mediated carcinogenesis in Barrett's esophagus. *(Cell Mol Gastroenterol Hepatol 2016;2:439–453; http://dx.doi.org/10.1016/j.jcmgh.2016.03.006)*

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Gastroesophageal reflux disease (GERD) causes esophageal inflammation (reflux esophagitis) that damages the esophageal squamous epithelium and enables its replacement by a metaplastic, intestinal-type columnar epithelium (Barrett's esophagus).[1] GERD-induced chronic inflammation in Barrett's esophagus is thought to contribute to the development of esophageal adenocarcinoma, a tumor whose frequency has increased at an alarming rate in

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Abbreviations used in this paper: **Ac-YVAD-CHO, acetyl-Tyr-Val-Ala-Asp-CHO; AIM, absent in melanoma; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; ATP, adenosine triphosphate; DAMP, damage-associated molecular pattern; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GERD, gastroesophageal reflux disease; IL, interleukin; LPS, lipopolysaccharide; LDH, lactate dehydrogenase; mRNA, messenger RNA; NF-κB, nuclear factor-κB; NLRP3, NOD-like receptor protein 3; NOD, nucleotide-binding domain, leucine-rich repeat containing proteins; PAMP, pathogen-associated molecular pattern; PBS, phosphate-buffered saline; pro, protein; PRRs, pattern-recognition receptors; qPCR, quantitative reverse-transcription polymerase chain reaction; ROS, reactive oxygen species; RT-PCR, reverse-transcription polymerase chain reaction; siRNA, small interfering RNA; TLR, Toll-like receptor; TNF, tumor necrosis factor.**

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Western countries.[2,3] A number of proinflammatory cytokines have been found in biopsy specimens of inflamed Barrett's metaplasia, with especially high levels of interleukin (IL)1β.[4] Mice genetically engineered to express high levels of IL1β in the esophagus develop esophagitis with a Barrett's-like metaplasia that progresses to adenocarcinoma, suggesting that IL1β might play a key role in Barrett's-associated tumorigenesis.[5] GERD can cause the esophagus to secrete proinflammatory cytokines,[6] but there also is reason to believe that the esophageal microbiome might contribute to esophageal inflammation with production of IL1β.

IL1β and IL18 (another proinflammatory cytokine) can be generated through proteolytic cleavage of their precursor proteins (pro-IL1β and pro-IL18) by the cysteine protease caspase 1.[7–9] In addition to activating these proinflammatory cytokines, caspase-1 can induce pyroptosis, a unique form of programmed cell death in which the dying cells release their proinflammatory cytoplasmic contents into the extracellular space, causing further inflammation.[7,10] Thus, caspase-1 can play a major role in promoting inflammation, and caspase-1 can be activated by a cytoplasmic protein complex called an *inflammasome*.[11]

Inflammasomes contain pattern-recognition receptors (PRRs) that recognize certain pathogen-associated molecular patterns (PAMPs) produced by microbes, and certain damage-associated molecular patterns (DAMPs) produced by damaged cells.[12] There are a number of families of PRRs, including the nucleotide-binding domain, leucine-rich repeat containing proteins (NOD; NOD-like receptors [NLRs]), and the absent in melanoma 2 (AIM)-like receptors,[12] and different inflammasomes usually are named for the PRRs they harbor.[13] After the sensing of PAMPs or DAMPs, the PRRs assemble a large macromolecular complex containing the PRR itself and an adapter protein called apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). This complex activates caspase-1 in canonical inflammasomes, and/or murine caspase-11 (caspase-4/-5 in human beings) in noncanonical inflammasomes.[13,14]

Canonical and noncanonical inflammasome activation is a 2-step process. The first step primes the inflammasome by increasing the expression of pro-IL1β and pro-IL18. This priming step in canonical and noncanonical inflammasomes is virtually identical, and is accomplished by activation of Toll-like receptors (TLRs), NOD1 or NOD2, or by signaling through tumor necrosis factor (TNF)-α or IL1.[13,14] In contrast, canonical and noncanonical inflammasomes differ in the second activating step. In canonical inflammasomes, activation involves the sensing of PAMPs or DAMPs by the PRRs, which then activate caspase-1. The activated caspase-1 cleaves the pro-IL1β and pro-IL18 produced during the priming step into mature active forms that are secreted into the extracellular space. Activated caspase-1 also induces pyroptosis, which promotes further inflammation.[13] In noncanonical inflammasomes, the PRRs recognize intracellular lipopolysaccharide (LPS) (a product of gram-negative bacteria) to activate caspase-11.[14] Activated caspase-11 can mediate pyroptosis directly, but the secretion of IL1β and IL18 induced by caspase-11 is an indirect process that requires caspase-1, ASC, and the NOD-like receptor protein 3 (NLRP3) PRR to convert pro-IL1β and pro-IL18 into their active forms.[14,15]

TLRs also are PRRs, and some of the same molecular patterns that activate the PRRs in inflammasomes also activate the TLRs located on the cell surface or in endosomes. In collaboration with inflammasomes, TLRs protect against microbial infection by triggering an innate immune response.[16] However, inappropriate TLR activation can lead to uncontrolled chronic inflammation and promote carcinogenesis.[17,18] Chronic inflammation caused by inappropriate TLR activation can, in some cases, be attributed to disturbances in the local microbiome, which is the collection of microbes and their genes that reside in a biological niche.[19] The human microbiome comprises bacteria, fungi, protozoa, and viruses, although bacteria have been the most well characterized and studied.[19]

In some cell types, LPS produced by gram-negative bacteria has been shown to function as a PAMP that activates TLR4 and primes the inflammasome (step 1). In nonesophageal cells, LPS also has been shown to cause inflammasome activation (step 2).[13,20,21] Gram-negative bacteria dominate the esophageal microbiome of patients with GERD and Barrett's esophagus,[22–24] and one earlier study found that Barrett's epithelial cells express TLR4 that can be activated by LPS.[25] In this study, we have explored the effects of LPS on TLR4 signaling and on priming and activation of the inflammasome in esophageal squamous and Barrett's epithelial cells.

Materials and Methods

Esophageal Squamous and Barrett's Epithelial Cell Primary Cultures, and Barrett's Epithelial Cell Lines

We established primary cultures of esophageal squamous epithelial cells (NES-B3, NES-B10, NES-G2, and NES-G4) using biopsy specimens of esophageal squamous epithelium from 4 patients with GERD, and primary cultures of Barrett's epithelial cells (BAR-12, BAR-15, and BAR-18) using biopsy specimens of Barrett's metaplasia from 3 patients with nondysplastic Barrett's esophagus as previously described.[26] We used 2 non-neoplastic, telomerase-immortalized Barrett's epithelial cell lines (BAR-T, BAR-10T) that were developed in our laboratory[27,28] for the mechanistic studies described later, and we compared the response of the Barrett's cell lines to that of the primary cultures of Barrett's epithelial cells. These studies were approved by the Institutional Review Board of the Dallas VA Medical Center. Primary cultures and BAR-T cell lines were co-cultured with a fibroblast feeder layer and maintained in growth medium as previously described.[26,27] Primary cultures and cell lines were maintained at 37°C in a 5% CO_2 incubator. For individual experiments, primary esophageal cells and BAR-T cell lines were seeded equally into collagen IV–coated wells (BD Biosciences, San Jose, CA), in the absence of fibroblast feeder layers, and maintained in growth medium.

LPS and Adenosine Triphosphate Treatment

In preliminary experiments, we treated telomerase-immortalized, non-neoplastic esophageal squamous[29,30]