A Role for Neuronal Alpha-Synuclein in Gastrointestinal Immunity

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Abstract
Background: Alpha-synuclein (αS) is a nerve cell protein associated with Parkinson disease (PD). Accumulation of αS within the enteric nervous system (ENS) and its traffic from the gut to the brain are implicated in the pathogenesis and progression of PD. αS has no known function in humans and the reason for its accumulation within the ENS is unknown. Several recent studies conducted in rodents have linked αS to immune cell activation in the central nervous system. We hypothesized that αS in the ENS might play a role in the innate immune defenses of the human gastrointestinal (GI) tract.

Methods: We immunostained endoscopic biopsies for αS from children with documented gastric and duodenal inflammation and intestinal allograft recipients who contracted norovirus. To determine whether αS exhibited immune-modulatory activity, we examined whether human αS induced leukocyte migration and dendritic cell maturation.

Findings: We showed that the expression of αS in the enteric neurites of the upper GI tract of pediatric patients positively correlated with the degree of acute and chronic inflammation in the intestinal wall. In intestinal allograft subjects who were closely monitored for infection, expression of αS was induced during norovirus infection. We also demonstrated that both monomeric and oligomeric αS have potent chemoattractant activity, causing the migration of neutrophils and monocytes dependent on the presence of the integrin subunit, CD11b, and that both forms of αS stimulate dendritic cell maturation.

Interpretation: These findings strongly suggest that αS is expressed within the human ENS to direct intestinal inflammation and implicates common GI infections in the pathogenesis of PD.

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Introduction

Alpha-synuclein (αS) is a neuronal protein that has excited widespread interest because of its role in the pathogenesis of Parkinson disease (PD) and the related synucleinopathies, Lewy body dementia, and multiple system atrophy. Of particular interest over the past decade or so has been the scientific debate spurred by the work of Braak, who has shown through his studies of pathological specimens from autopsied individuals at various stages of PD that the accumulation of αS actually begins in the enteric nervous system (ENS) [1]. Utilizing the vagus as an escalator, αS produced in the ENS traffics from the gut to the brain, spreading to centers within the central nervous system (CNS) that are ultimately destroyed. αS has no known function. Because it physically associates with vesicular structures, such as synaptic vesicles, it is described as a protein that is somehow involved in neurotransmitter release [2]. Why it would accumulate in the ENS is entirely mysterious. Two studies conducted in rodents suggest that microbes in the gastrointestinal (GI) tract accelerate αS aggregation, one through nucleating a prion-like reaction [3], the other suggesting that bacterial metabolites promote microglial activation [4]. Without a clear understanding of the function of αS and why it would accumulate in a nerve cell, we cannot unravel the pathophysiology of diseases such as PD. Recently, human αS was shown to chemoattract rodent microglia suggesting that αS could directly promote neuroinflammatory damage within the CNS [5]. In addition, αS has also recently been shown to be protective in mice infected with neurotropic RNA viruses, such as West Nile virus (WNV) and Venezuelan equine encephalitis [6], suggesting that it can play a beneficial role in immune defense. Based on these studies we hypothesized that αS in the ENS might play a role in the innate immune defenses of the GI tract.

Methods

Patients

Biopsies were obtained as part of standard clinical practice. Protocols were approved by the institutional review board at each participating center. We retrieved endoscopic biopsy specimens from 42 children (mean age 12.4 years) with upper GI distress with the pathological diagnoses of duodenitis, gastritis, Helicobacter pylori, or reactive gastropathy at 1 academic center over a 9-year period (see online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000477990). A second population included endoscopic biopsies obtained from 14 pediatric (mean age 3.4 years) and 2 adult intestinal transplant recipients (mean age 40 years) who developed a documented norovirus infection (n = 16; see online suppl. Table 2). In this immunosuppressed population, viral infections occur frequently, and biopsies of the allograft are obtained prospectively at specific times following transplantation and when clinically indicated.

Immunohistochemistry and Scoring

The method of tissue preparation followed published procedures [7, 8]. Serial sections were cut at 4 μm thickness. The primary antibodies used were: αS (LB 509, Abcam ab27766), PGF9.5 (Agilent Z5116), and CD68 (Abcam ab955). The secondary antibody was Envision Plus polymer anti-mouse conjugated to HRP (Agilent K4001). HRP was visualized using DAB chromogen (Agilent K3468) following the manufacturer’s protocol.

Histological scoring was performed on 2 sections per block for each stain by 2 pathologists blinded to the diagnoses. Mucosa from each biopsy was assessed by HE stain for acute (polymorphonuclear) and chronic (mononuclear) inflammation (1, minimal inflammatory change; 2, moderate inflammatory change; 3, marked inflammatory change), and for FGF 9.5-positive neurite density (1, minimal; 2, moderate; 3, high), neurite αS presence and intensity (1, slight; 2, moderate; 3, high), and CD68-positive cell number (1, rare cells; 2, low cell number; 3, moderate cell number; 4, high cell number). The scoring of each pathologist was averaged.

Chemotaxis and Dendritic Cell Maturation

Chemotaxis assays were performed according to published procedures [9]. Neutrophils and monocytes were isolated from the blood of healthy human donors as described [9]. Recombinant human αS (lot No. 20162050, endotoxin contamination 18 × 6 EU/mg) was from Proteos Inc. via the Michael J. Fox Foundation. The αS aggregates were made following a protocol provided by Proteos. N-terminal peptides were synthesized by solid-phase chemistry and purified to >98% purity by high-performance liquid chromatography. Male CD11b−/− (B6.129S4-Itgam tm1Myd /J) and wild-type C57B6 control mice were from The Jackson Laboratory (Bar Harbor, ME, USA). Anti-human CD11b antibody (clone M1/70, ultra-pure rat IgG) was from BioLegend (San Diego, CA, USA). Dendritic cell maturation studies were conducted as reported [10].

Results

To determine if αS expression in the ENS is associated with intestinal inflammation, we examined upper GI endoscopic biopsies taken over a 9-year period at 1 hospital center from 42 children who exhibited pathological evidence of acute and/or chronic mucosal inflammation, as determined by the presence of neutrophil or mononuclear cell infiltrates, respectively. The population included subjects with H. pylori gastritis, a common cause of upper GI distress in children. The upper GI tract was selected because numerous reports have demonstrated that expression of αS in the human ENS in the proximal portion of the GI tract is minimal except in the setting of PD [8, 11, 12]. For example, αS staining in the ENS of the gastric
The mucosa of subjects over the age of 70 years was noted in about 80% of evaluable biopsies from individuals with PD, but in less than 5% of an aged-matched control population [8]. We selected a pediatric population to avoid the bias of an adult population with "pre-clinical PD" [1].

The biopsies were immunostained for αS (Fig. 1). Since the extent of αS staining depends both on its intraneuronal concentration as well as on the number of neurites present within the tissue specimen, serial sections were also immunostained for the neural protein PGP 9.5. In several specimens, we confirmed the neuronal localization of αS by immunofluorescent colocalization of αS and PGP 9.5, a neuronal marker (Fig. 1, lower panels). In general, the biopsies, being superficial, included the submucosal plexus but spared deeper structures. Because many of the gastric biopsies had insufficient neural tissue to evaluate, we focused our analyses on duodenal specimens.

In all biopsies examined from this pediatric population, immunostained αS was visible in neuronal processes within the lamina propria (Fig. 1). The intensity and extent of αS was assessed for each specimen and graded on a scale of 1–3 by 2 pathologists uninformed of the diagnosis. Similarly, the degree of acute and chronic inflammation was graded by the density of neutrophils and mononuclear cells in the biopsy specimens (e.g., minimal, 1; moderate, 2; intense, 3) visible on the HE-stained speci-
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The number of CD68-positive cells within the lamina propria (principally macrophages and dendritic cells) varied in proportion to the degree of inflammation (Fig. 1). From the assessment of the specimens from the 42 children, the degree of inflammation within each biopsy correlated positively with the intensity and extent of αS staining, both for acute ($p < 0.0002$) and chronic inflammation ($p < 0.001$; Fig. 2). Of all of the cases, 23/42 (55%) had a confirmed upper GI tract infection: *H. pylori* in 20/23 (87%), *H. heilmannii* in 1/23 (5%), and *Candida* in 2/23 (9%). The remaining had chronic upper GI discomfort (pain, nausea, vomiting) associated with mucosal inflammation of unknown etiology, which could include some of the most common infections, such as viruses that are normally not evaluated in this clinical setting. The magnitude of αS expression did not differ significantly between subgroups that had a diagnosed bacterial or fungal infection (e.g., *H. pylori*) and those that did not.

To pursue the relationship between infection and the induction of αS, we examined intestinal biopsies taken as part of the normal standard of care of intestinal allograft recipients. Because this is an immunosuppressed population, allograft recipients are monitored closely for bacterial, fungal, and viral infections through prospective examination of endoscopically obtained tissue specimens as well as blood, urine, and stool. We identified 14 children and 2 adults who received an intestinal transplant and had contracted a norovirus infection after the surgery, definitively diagnosed by PCR. Biopsies were examined that had been taken before, during, and after the infection. In most duodenal biopsies sampled during the infection, a robust expression of αS was seen (Fig. 3; online suppl. Tables 2A, B). In 9 of the 16 cases, biopsies had been taken shortly before the norovirus infection (1–6 months). In 4 of these 9 subjects (44%) αS was not observed in tissue from either the native or the transplanted duodenum prior to the onset of the norovirus infection, consistent with the hypothesis that the expression of αS was induced during the norovirus infection (Fig. 3; online suppl. Table 2A, B). Tissues taken between 2 weeks and 6 months following clinical resolution of the infection still exhibited the presence of αS but generally at lower levels than observed during the period of active infection (Fig. 3; online suppl. Table 2A, B).

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**Fig. 3.** Representative biopsies from intestinal transplant recipients with documented norovirus infection stained for αS (×20). **a** “Case 16, Pre” (native duodenum): a biopsy taken 1 month prior to the norovirus infection shows minimal expression of αS. **b** “Case 16, During” (native duodenum): a biopsy taken at the time of norovirus infection shows a robust expression of αS in neurites within the lamina propria. **c** “Case 3, During” (allograft jejunum): a biopsy taken at the time of norovirus infection shows areas with high expression of αS. **d** “Case 3, Post” (allograft jejunum): a biopsy taken 4 months after the initial norovirus infection shows continued high expression of αS. The patient still tested PCR positive for norovirus. Images 1 and 2 are enlargements of the outlined areas.
Our observation that the increased expression of αS within the duodenum was associated with an increased tissue density of inflammatory cells within the lamina propria prompted us to examine whether αS exhibited chemotactic activity. Indeed, both monomeric and aggregated recombinant human αS were chemotactic at nanomolar concentrations towards human neutrophils and monocytes exhibiting the classical bell-shaped concentration curve characteristic of chemoattractants (Fig. 4 a–d). Furthermore, the N-acetylated peptide, corresponding to the first 21 amino acids of αS; 1-25, a peptide corresponding to the first 25 amino acids. a Human neutrophils.

**Fig. 4.** αS is a chemoattractant dependent on CD11b. Assays were conducted as described in the Methods section. The average of 3 independent assays for each sample is presented. The concentration of the αS aggregate is in terms of the monomer. A positive control, FMLP, is noted in black. NAc 1-21, the N-acetylated peptide corresponding to the first 21 amino acids of αS; 1-25, a peptide corresponding to the first 25 amino acids.

b Human monocytes. c, d Representative photos of the cellular response. e Neutrophils from CD11b−/− or wild-type mice (0.5 × 10⁶/mL), assayed as described in the Methods section. f Human neutrophils incubated in the presence of anti-CD11b antibody or the control (50 μg/mL each). CM, control media (negative control); HPF, high-powered field.

We next asked whether αS could activate dendritic cells. Human monocyte-derived dendritic cells were exposed for 2 days to αS monomer, aggregate, and NAc 1-21 peptide, and then analyzed by flow cytometry to measure the extent of phenotypic maturation, using CD80, CD83, CD86, HLA-ABC, and HLA-DR as determinants (Fig. 5; online suppl. Fig. S1). While both monomer and aggregate...
gate promoted dendritic cell maturation, the N-terminal peptide did not, demonstrating that the chemotactic activity of αS and the segment involved in dendritic cell maturation reside on different portions of the molecule. Maturation of dendritic cells by the monomer was not inhibited by pretreatment of the cells with a blocking antibody directed at TLR4 (online suppl. Fig. S2), demonstrating that the cellular response was independent of the small amount of endotoxin present in the recombinant αS preparation, and that αS does not engage the TLR4 receptor to effect maturation.

**Discussion**

In the present study, we have demonstrated that the expression of αS in neurites and neuronal cell bodies within the gastrointestinal wall of children is associated with mucosal inflammation. Furthermore, we have shown that αS is both a potent chemoattractant for neutrophils and monocytes, and a maturation factor for dendritic cells. Since it is known that αS is secreted from enteric nerves upon stimulation [14], it is reasonable to propose that it is the induction and secretion of αS from the ENS that promotes mucosal inflammation rather than the induction of αS having been a consequence of inflammation.

Because intestinal transplant recipients are intensively monitored prospectively for signs and symptoms of enteric infection (which can mimic allograft rejection), we succeeded in obtaining biopsies of both native and allograft tissues in 4 individuals prior to the onset of a norovirus infection, who had also remained free of any other clinically symptomatic enteric infection during that period. In these patients, no significant expression of αS could be detected until an active norovirus infection occurred and persisted months after the virus was no longer detected in feces. This observation strongly suggests that...
enteric viral infection is one of the factors that can induce the expression of αS in the human ENS. Interestingly, since the αS response of the grafted (vagotomized) tissue was as robust as that of the native duodenum, it appears that αS can be produced by the ENS without input from higher neural centers.

The discovery that αS is expressed during a GI infection and mobilizes an inflammatory response leads to the conclusion that induction of this protein within the ENS is part of its normal immune defense mechanism. Even the transport of αS, as either monomer or aggregate, from the ENS to the brain appears to occur normally. Human αS injected into the gastric wall of rodents, for example, is taken up by the vagus nerve and transported to the dorsal motor nucleus within the brainstem in a time-dependent manner [15]. By virtue of this mechanism, αS produced in the ENS can be transported cranially, protecting higher centers of the nervous system. Since it is known from genetic studies that individuals with multiple copies of αS invariably develop PD, an increase in the expression of αS is sufficient to cause PD [16, 17]. A comparable increase in expression of αS might also occur as a consequence of repeated acute or chronic GI infections caused by organisms that specifically provoke the expression of αS within the ENS. Potential pathogens would include those known to infect and replicate within the ENS, such as strains of influenza, varicella, and JC virus. Epidemiological studies support an association between chronic H. pylori infection and the risk of developing PD [21, 22]. Strikingly, individuals who have received a full truncal vagotomy (as treatment for peptic ulcer) are at a decreased risk of developing PD [23, 24].

A recent report that individuals with PD have increased intestinal permeability suggests another mechanism, in addition to infection, that might provoke the expression (or accumulation) of αS within the ENS [25, 26], namely the exposure of the ENS to commensal microbes. In another study, orally administered Escherichia coli-producing curli protein, a protein that facilitates bacterial attachment to epithelial cells and subsequent invasion, enhanced αS deposition in plexi in the gut and in the hippocampus and striatum in aged Fischer 344 rats (which spontaneously accumulate αS within their ENS as they age [27]) as compared to rats exposed to mutant bacteria lacking the capacity to produce curli or to rats exposed to vehicle [3].

In a study involving αS-overexpressing mice, short-chain fatty acids, produced by the intestinal microbiome, increased the presence of αS aggregates in basal ganglia and substantia nigra and enhanced the motor deficit, as did fecal transplants from patients with PD [4].

Our report provides support for the hypothesis that αS is a component of the innate immune defensive response of the ENS and might provide insight into the pathophysiology of certain human chronic inflammatory disorders. With respect to PD, the discovery reported here extends the hypothesis of Braak et al. [11] that PD begins in the ENS by proposing that PD results from the excessive response of a normal innate immune component of the ENS.

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Author Contributions

E.S., D.B., D.Y., J.O., G.W., S.S., and E.L. were responsible for the laboratory studies; S.K., T.F., and A.K. managed the transplant population and provided access to that population; E.S. and B.H. evaluated the histology of each patient sample; E.S., D.B., and M.Z. designed the study and drafted the manuscript.

References


