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Research Paper

Deficiency in Duox2 activity alleviates ileitis in *GPx1*- and *GPx2*-knockout mice without affecting apoptosis incidence in the crypt epithelium

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ABSTRACT

Mice deficient in glutathione peroxidase (GPx)-1 and -2 (*GPx1*$^{-/-}$*GPx2*$^{-/-}$ double knockout or DKO mice) develop very-early-onset (VEO) ileocolitis, suggesting that lack of defense against reactive oxygen species (ROS) renders susceptibility to intestinal inflammation. Two members of ROS-generating NADPH oxidase family, NOX1 and DUOX2, are highly inducible in the intestinal epithelium. Previously, we reported that *Nox1* deficiency ameliorated the pathology in DKO mice (*Nox1*-TKO). The role of Duox2 in ileocolitis of the DKO mice is evaluated here in *Duoxa*-TKO mice by breeding DKO mice with *Duoxa*$^{-/-}$ mice (*Duoxa*-TKO), which do not have Duox2 activity. Similar to *Nox1*-TKO mice, *Duoxa*-TKO mice no longer have growth retardation, shortened intestine, exfoliation of crypt epithelium, crypt abscesses and depletion of goblet cells manifested in DKO mice by 35 days of age. Unlike *Nox1*-TKO mice, *Duoxa*-TKO mice still have rampant crypt apoptosis, elevated proliferation, partial loss of Paneth cells and diminished crypt density. Treating DKO mice with NOX inhibitors (di-2-thienyliodonium/DTI and thioridazine/THZ) and an antioxidant (mitoquinone/MitoQ) significantly reduced gut pathology. Furthermore, in the inflamed human colon, DUOX protein expression is highly elevated in the apical, lateral and perinuclear membrane along the whole length of gland. Taken together, we conclude that exfoliation of crypt epithelium, but not crypt apoptosis, is a major contributor to inflammation. Both Nox1 and Duox2 induce exfoliation of crypt epithelium, but only Nox1 induces apoptosis. NOX1 and DUOX2 may be potential therapeutic targets for treating ileocolitis in human patients suffering inflammatory bowel disease (IBD).

1. Introduction

Elevation in reactive oxygen species (ROS) is well recognized as an essential factor in the pathogenesis of GI mucosal diseases, including peptic ulcers, inflammatory bowel disease (IBD), and GI cancers [1]. Among the well-known sources of ROS are the mitochondrial-respiratory chain and the respiratory burst produced by the NADPH oxidase-2 (NOX2) enzyme complex present in the phagocytes (e.g. neutrophils and monocytes).

Two other members of the NADPH oxidase family, NOX1 and DUOX2, are expressed in the epithelium of the lower GI tract [2,3]. The NOX1 complex produces superoxide whereas the DUOX2 complex produces H_2O_2. Their ileum and colon expression is induced from low-basal levels in response to bacterial colonization of germ-free (B6 and

Abbreviations: 129, 129S1/SvimJ; B6, C57BL6/J; DKO, double knockout of *GPx1* and *GPx2* genes; DUOX 2, dual oxidase-2; Duoxa, Duox activator; Duoxa-TKO, triple-knockout mice with disrupted *Duoxa*, *GPx1* and *GPx2* genes; DSS, dextran sodium sulfate; DTI, di-2-thienyliodonium; GFP, green fluorescent protein; GI, gastrointestinal; GPx1/2, glutathione peroxidase-1 and -2; H & E, hematoxylin and eosin; Hmox1, heme oxygenase-1; IBD, inflammatory bowel disease; IHC, immunohistochemistry; IQR, interquartile range; Lyz1, lysozyme 1; MitoQ, mitoquinone; Mmp, metalloproteinase; Non-DKO, mice having a wild-type allele of *GPx1* or *GPx2*; MPO, myeloperoxidase; NCF1, neutrophil cytosolic factor 1, aka p47phox; NOX, NADPH oxidase; NOX2, gp91phox, aka Cybb or cytochrom b-245 beta polypeptide; Nox1-TKO, triple-knockout mice with disrupted *Nox1*, *GPx1* and *GPx2* genes; nsSNP, non-synonymous single-nucleotide polymorphism; ROS, reactive oxygen species; SD, standard deviation; T4, L-thyroxine; THZ, thioridazine; TKO, triple knockout; TNFR1, tumor-necrosis-factor receptor 1; TUNEL, terminal-deoxynucleotidyl-transferase-mediated dUTP nick end labeling; VEO, very-early-onset; WT, wild-type

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129 strain) mice [4,5]. *NOX1* and *DUOX2* genes are moderately and highly induced, respectively, in the ileum and colon of IBD patients compared to healthy controls [6–8]. The inflammation-associated induction of Nox1 gene expression is also exhibited in the ileum of B6- and 129-strained GPx1/2-DKO (DKO here after) [9]. Duox2 mRNA was elevated in the ileum of 129, but not B6, DKO ileum, due to WT B6 ileum having a high level of Duox2 mRNA [9]. The fact that DKO mice without Nox1 gene expression (*Nox1-GPx1/2*-TKO or *Nox1*-TKO) have ameliorated ileocolitis raises the question whether DUOX2 contributes to gut inflammation in the DKO mice. To study the impact of DUOX2 in the intestine of the DKO mice, we bred Duoxa-KO mice with DKO mice to create *Duoxa-GPx1/2*-TKO (*Duoxa*-TKO) mice. *Duoxa*-KO disabled *Duoxa1* and *Duoxa2* gene expression; the Duoxa1 and Duoxa2 are accessory proteins required for Duox1 and Duox2 enzymatic activity [10]. In the intestine, DUOX1 is barely expressed [5]. Therefore, the major impact of the Duoxa-KO is suppressing Duox2 activity in the intestine.

The histopathological features of DKO mice include elevated levels of apoptosis and anoikis (exfoliation and subsequent apoptosis) in the crypt epithelium, depletion of mature Paneth and goblet cells, as well as crypt abscesses. All of these morphological features have been associated with inflammation in other studies. The high levels of apoptosis in crypt epithelium found in the intestines of IBD patients may permit leakage of bacterial products into the circulation [11,12]. Exfoliation of intestinal epithelium is linked to increased bi-directional permeability and is associated with relapse in IBD [13]. Paneth cells are essential for limiting translocation of pathogenic bacteria across the intestinal barrier [14]. It is unclear whether any of these morphological changes is a dominating factor for inflammation.

Because some NOX inhibitors have efficacy in ameliorating ROS-associated injury, we also examined the value of monotherapy with NOX inhibitors or antioxidants in the DKO mice to test their efficacy in alleviating gut inflammation [15]. The NOX inhibitors studied were DTI (an iodonium-class flavin dehydrogenase inhibitor) [21], celastrol [18,22,23], ebselen [19], GKT137831 (a pyrazolopyridine dione analog) [24] and THZ (an N-substituted phenothiazine) [25]. The antioxidants tested were MitoQ (a mitochondria-targeted antioxidant), which had been shown to alleviate DSS-induced colitis [26], as well as caffeic acid [27] and deferoxamine mesylate [28]. The latter two compounds also have iron-chelating activity. The Nox1 inhibitors tested were selected based on their bioavailability in animals [15–20]. The best small molecule inhibitors identified will be used as scaffolds for further modification to be developed into more specific Nox1 inhibitors.

In this study, we demonstrated that Duox2 also contributes to the ileocolitis phenotype of DKO mice. However, unlike *Nox1*-TKO mice which no longer have pathology, the *Duoxa*-TKO mice still exhibit abundant apoptosis in crypt epithelium, elevated cell proliferation, partial loss of Paneth cells and decreased crypt density. Because *Duoxa*-TKO mice were healthy, we conclude that elevated levels of exfoliation in the crypt compartment, but not apoptosis in the crypt epithelium is an indicator for inflammation. In the inflamed human tissue, DUOX is also expressed in the crypt epithelium. Our drug treatment study in the DKO model suggests that targeting of Nox1 and Duox2 may be a strategy to prevent or treat ileocolitis in IBD patients.

2. Materials and methods

2.1. Mice

We bred DKO mice with *Duoxa*-KO [10] and *Nox1*-KO [29] mice all in the C57BL/6J (B6) background. The *Duoxa*-TKO line was supplemented with L-thyroxine (T4) to maintain the euthyroidism following the described procedure [10]. Some $Duoxa^{+/+}$ and $Duoxa^{+/-}$ mice were treated with vehicle by the same routes (subcutaneously from day 5 to 20, and in drinking water afterwards) to observe the T4 effect on mice. All studies were approved by the City of Hope Institutional Animal Care and Use Committee.

2.2. Disease analysis

Mouse body weight and disease signs (perianal alopecia, wet tail, and diarrhea) were monitored daily from 5 to 35 days of age before euthanasia. B6 DKO mice begin to have ileitis at 27 days of age [9]. The lengths of the small intestine and colon (ileocecal junction to anus) were recorded as an indication of inflammation. Prior analysis of mice showed that GPX2 activity is readily detectable in the ileum (the distal half of the small intestine) [30,31]. Knockout of *Gpx2* alone shows increased crypt apoptosis in the ileum and colon [32], and the ileum is the consensus site of the pathology in the small intestine of DKO mice. Within the ileum, no marked regional variation in pathology is noted in the diseased mice by 35 days of age. In the large intestine, the cecum shows pathology, while the upper colon has mild or no pathology, and the rectum has the strongest pathology [33,34]. For sample collection, 1 cm of the distal ileum (ileocecal junction) and rectum was immersed in RNAlater (Life Technologies) and processed for RNA isolation. Two adjacent 1-cm sections of the ileum and a single 1-cm section at mid-colon were frozen as backup samples. The next 4 cm of the ileum and the remaining cecum and colon were fixed in phosphate-buffered formalin for histological analysis.

Table 1
Ileum 12-Point Pathology Scoring Criteria.

Scores	0	0.5	1	1.5	2	3
Inflammation intensity[a]	0	–[b]	low	–	medium	high
Inflammation foci/field[c]	0	–	0.01–0.20	–	0.21–0.50	> 0.50
Crypt density[d]	≥39.1	–	33.6–39.0	–	27.8–33.5	22–27.7
Apoptosis/ crypt[e]	0–0.1	–	> 0.1	–	–	–
Frac. crypt w/Paneth cells[f]	0.8–1	0.6–0.79	0.34–0.59	0.15–0.33	0–0.14	–

[a] Inflammation intensity score was based on the general impression of the peak inflammatory pathology in the section. For B6 *GPx1/2*-DKO mice, this score usually is at 0–1; the score of 1 indicates isolated, small crypt abscesses. A value of 2 denotes large crypt abscesses and 3 denotes significant erosion/ulceration of the epithelium; the latter observed often in B6;129 and 129 strain *GPx1/2*-DKO mice and very rarely in B6 GPx1/2-DKO mice.

[b] Dash means no score was given for the specific parameter.

[c] The inflammation foci per field was determined by counting total number of crypt abscesses or areas of erosion per 10x field (2 mm, both sides of ileum/colon).

[d] Crypt density was rated by estimating the average number of crypts per 10x field from 4 to 6 of 10x fields (one side only).

[e] Apoptotic cells per crypt were determined by counting H & E stained cells with apoptotic figure from 100 to 200 crypts. This score was revised from our previous 0–3 scale to 0–1 to distinguish normal levels (0–0.1 per crypt) found in control mice from above normal levels (> 0.1) in the DKO mice. Down grading the apoptosis count in the pathology score was done because unlike other parameters, the apoptotic cell count was not correlated to the final pathology score (R^2=0.25) or to any other measures. The colon pathology scoring was altered from previous studies only for the apoptosis category.

[f] The fraction of Ileum crypts with visible mature Paneth cells was averaged from counting 4–6 10X fields (one side only) and based on the visible eosinophilic granules.