Original Article
Jagged-1 attenuates LPS-induced apoptosis and ROS in rat intestinal epithelial cells

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Abstract: Ulcerative colitis (UC) is a chronic, non-specific inflammatory disease that occurs in the colonic mucosa. This study investigated the role of the Notch pathway in affecting the pathogenesis of UC and regulating intestinal epithelial cell proliferation and apoptosis. Caspase-3 activity was measured and flow cytometry was used to detect reactive oxygen species (ROS) content and Ki-67 expression. Flow cytometry was applied to detect apoptosis, proliferation, and ROS content. Under LPS stimulation conditions, the IEC-6 cells were divided into 3 groups, including control, 5 and 10 μg/mL Jagged-1 protein pretreatment. The mRNA and protein expressions of Jagged-1, Notch1, Hes1, and OLFM4 in colon tissues were detected by real-time quantitative PCR (qRT-PCR) and Western blot. The ROS production, Ki-67 expression, and caspase-3 activity were significantly increased, and Jagged-1, Notch1, Hes1, and OLFM4 mRNA and protein levels were obviously elevated in the colon tissue of UC model rats compared with control. LPS treatment apparently up-regulated Jagged-1, Notch1, and OLFM4 expression in IEC-6 cells, resulting in marked enhancement in apoptosis and ROS generation, and reduction of proliferation. Administration of Jagged-1 before LPS stimulation further upregulated the expressions of Notch1 and OLFM4 in IEC cells, weakened apoptosis and ROS production, and alleviated the inhibitory effect of LPS on IEC-6 cell proliferation. UC lesions can activate the Notch signaling pathway in colon tissue, which may play a role in emergency repair. Upregulation of the Notch signaling pathway significantly reduced inflammatory stimuli-induced apoptosis and ROS generation in intestinal epithelial cells, resulting in increased cell proliferation.

Keywords: Notch, ulcerative colitis, proliferation, apoptosis

Introduction

Inflammatory bowel disease (IBD) is a nonspecific, chronic inflammatory bowel disease with unclear causes and includes ulcerative colitis (UC) and Crohn’s disease (CD) [1, 2]. IBD is requires long-term treatment, has unpleasant symptoms, and has a high rate of relapse. At present, IBD is considered to be an intestinal disease caused by genetic susceptibility factors, an imbalance of intestinal mucosal immune function, impaired barrier function, and altered intestinal microflora [3, 4]. UC is a clinically refractory disease characterized by persistent damage to the colonic mucosa epithelium, with the main symptoms being abdominal pain, diarrhea, hematochezia, and tenesmus. Intestinal and rectal mucosal inflammation and chronic ulceration are the main pathological features of UC [5, 6].

Normal physiological proliferation, apoptosis, differentiation, migration, and tight junctions of intestinal epithelial cells are essential to maintain the integrity of the intestinal mucosa and constitute a biological barrier [7, 8]. More and more research shows that [9-11] intestinal epithelial cell apoptosis plays an important role in the pathogenesis of UC. In the course of UC, a large amount of inflammatory cell infiltration, the release of inflammatory factors, and the production of oxygen free radicals can enhance intestinal epithelial cell apoptosis, leading to a break in the balance between apoptosis and the proliferation of colonic epithelial cells. Excessive UC causes a loss of colonic epithelium and abnormal function [12, 13].

The Notch signaling pathway was originally found in Drosophila. Subsequent studies found that Notch signaling pathways are widespread...
Jagged-1 attenuates UC in many mammals [14, 15]. The Notch signaling pathway mainly expresses in embryonic stem cells, hematopoietic stem cells, lymphocytes, intestinal epithelial cells, and various tumor cells. It is a relatively conserved signaling pathway in the evolution of the transmembrane receptor protein family. Its regulatory function is involved in proliferation, apoptosis, and the differentiation of cells [16, 17]. This study investigated the role of the Notch pathway in affecting the pathogenesis of UC and in regulating intestinal epithelial cell proliferation and apoptosis.

Materials and methods

Main reagents and materials

Healthy adult male Sprague-Dawley rats (6 weeks, body weight 240±25 g) were purchased from Shanghai Bangyao. Rat intestinal epithelial cells IEC-6 were purchased from Beinao. DMEM medium, fetal bovine serum (FBS), penicillin-streptomycin, and type I collagenase were purchased from Gibco (USA). Rabbit anti-rat Jagged-1, Notch1, Hes1, OLFM4, and β-actin polyclonal antibodies, and PE anti-Cytokeratin 20 (CK20) were purchased from Abacm (USA). FITC labeled Ki-67 flow antibody was purchased from Biolegend (USA). HRP-conjugated Goat anti-Rabbit IgG (H+L) secondary antibody, lipid peroxidation product malondialdehyde (MDA), superoxide dismutase (SOD), and Caspase-3 activity detection kit were purchased from Beyotime (China). Hyaluronidase and LPS were purchased from Sigma. A PrimeScript™ RT reagent kit was purchased from Takara (Dalian, China). DCFH-DA fluorescent dye was purchased from MedChem Express (USA). An Annexin V/FITC Apoptosis Detection Kit was purchased from DOJINDO (Japan). Dextran Sulfate Sodium (DSS) was purchased from MP Biomedicals (USA). Recombinant Jagged 1 Protein was purchased from R&D Systems Inc. (USA).

UC modeling

Twenty SD rats were randomly divided into a UC model group and a control group with 10 in each group. After one week of conventional adaptive feeding, rats in the UC model group were given 5% DSS in water for 7 days. The rats in the control group were given free drinking saline for 7 days. The activities, changes in hair, stool characteristics, bloody stools, and body weight changes of the rats were observed and recorded. Symptoms such as diarrhea, loose stools, fecal occult blood, or a gross bloody stool appearance were considered a successful UC model establishment.

Disease activity index (DAI) assessment

DAI was assessed based on changes in body weight, stool characteristics, and fecal occult blood in rats: percentage weight loss, weight unchanged for 0 points, 1% to 5% for 1 point, 5% to 10% for 2 points, 10% to 15% for 3 points, and > 15% for 4 points; stool consistency, normal for 0, loose stools for 2 points, and diarrhea for 4 points; stool bleeding, normal for 0 points, occult blood positive for 2 points, and gross bloody stool for 4 points. According to the three conditions for comprehensive scoring, the total score of the three results is divided by 3 to obtain the DAI score.

Colonic tissue observation

After 7 days of modeling, the rats were sacrificed and the colon tissue was longitudinally dissected on a filter paper. The tissue was washed with physiological saline, fixed with paraformaldehyde, paraffin-embedded, and sliced at a thickness of 5 μm. After hematoxylin-eosin (HE) staining, the pathological changes of the colon tissue were observed under an optical microscope (CX23, Olympus, Japan).

Ki-67 expression detection

The rat colon tissue was isolated, longitudinally dissected, and the contents were washed with physiological saline. After morcellation, the tissue was digested with digest solution containing 0.1% collagenase I and hyaluronidase for 45 minutes. Then the digestive fluid was filtered and centrifuged at 400 g for 5 min. The cells were resuspended in a stain buffer, and the cell concentration was adjusted to 1 × 10⁴/100 μL. 10 μL of PE labeled CK20 and 10 μL of FITC-labeled Ki-67 were added for double labeling to detect Ki-67 expression in CK20 positive epithelial cells.

Caspase-3 activity detection

According to the instructions of the kit, the pNA standard product is diluted in a concentration gradient to prepare standard products with...
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Table 1. DAI, MDA, and SOD detection results

<table>
<thead>
<tr>
<th>Index</th>
<th>Control (n=10)</th>
<th>UC (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/mg protein)</td>
<td>22.41±5.21</td>
<td>53.92±6.17*</td>
</tr>
<tr>
<td>SOD (U/mg protein)</td>
<td>69.53±8.33</td>
<td>32.68±3.46*</td>
</tr>
<tr>
<td>DAI</td>
<td>0.61±0.15</td>
<td>8.22±1.73*</td>
</tr>
</tbody>
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*P < 0.05, compared with control.

concentrations of 200 μM, 100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM, and 0 μM. The absorbance was measured at 405 nm to make a standard curve. The tissue was smashed to prepare a homogenate. Then a caspase lysis buffer on ice was added to the homogenate for 20 min and centrifuged at 12000 g and 4°C for 10-15 min. Then the supernatant was taken to a new 1.5 ml centrifuge tube and quantified by the BCA kit. A 65 μl Assay buffer, a 25 μl lysate supernatant, and a 10 μl Ac-DEVD-pNA (2 mM) were added to a 96-well plate and incubated for 2 hours at 37°C. When the color change was obvious, the plate was measured at 405 nm on a microplate reader (Awareness, USA). The relative enzyme activity was calculated based on A405 in the experimental group/A405 in the control group × 100%.

MDA and SOD detection

A rat colon homogenate was prepared and quantified using the BCA method. MDA and SOD contents were tested in accordance with the instructions to assess oxidative stress conditions and antioxidant capacity.

IEC-6 cell culture and treatment

IEC-6 cells were cultured in DMEM containing 10% FBS and 1% penicillin, and maintained in a 37°C and 5% CO₂ cell incubator. The medium was changed every 3 days. The cells were passaged at 1:3 and used for experiments in a logarithmic phase.

LPS treatment: IEC-6 cells were divided into 2 groups. The cells in the control were routinely cultured. The LPS group was supplemented with LPS in the culture medium to a final concentration of 100 μg/mL. Cells were harvested after 48 hours incubation for detection.

Jagged-1 treatment: Under LPS stimulation conditions, the cells were divided into 3 groups, including the control, the 5 μg/mL Jagged-1 protein pretreatment group, and the 10 μg/mL Jagged-1 protein pretreatment group. Cells were harvested after 48 hours incubation for detection.

Flow cytometry detection of cell apoptosis

The cells were washed twice in PBS and digested by 0.125% trypsin. After being centrifuged at 300 g for 5 min, the cells were resuspended and added to a 100 μL binding solution. Then added to the cells was 5 μL of Annexin V-FITC and 5 μL of PI Solution in sequence, and the solution was incubated in darkness for 15 minutes. After supplementation with 400 μL of binding solution, the cells were tested on a Beckman CytoFLEX flow cytometer.

Flow cytometry detection of ROS

ROS detection in rat colon tissue: The rat tissue was collected and cut into pieces. The tissue was digested with a 0.1% collagenase I and hyaluronidase-containing digestive solution for 45 min. After centrifugation at 250 g for 5 min, the cells were incubated in 0.1% DCFH-DA at 37°C in darkness for 30 min. After being washed twice in PBS and resuspended in 500 μL PBS, the tissues were tested on a Beckman CytoFLEX flow cytometer to measure the ROS content.

In vitro IEC-6 intracellular ROS assay: Cells were washed twice in PBS and digested with 0.25% trypsin. After being centrifuged at 250 g for 5 min, the cells were incubated in 0.1% DCFH-DA probe at 37°C for 30 min. Then after their resuspension in 500 μL PBS, the cells were tested using a Beckman CytoFLEX flow cytometer.

Flow cytometry detection of cell proliferation

The IEC-6 cells were resuspended in a DMEM complete medium with 10% FBS and incubated with 10 μM EdU for 2 h. Then the cells were further cultured for 48 h. After being digested by trypsin, the cells were fixed in 100 μL fixative at room temperature for 15 min. After centrifugation in PBS, the cells were treated with a 100 μL permeabilization solution at room temperature for 15 min. Next, 500 μL of detection reaction solution was added to the cells and they were incubated at room temperature for 30 min in darkness. After suspending, the cells were tested using a Beckman CytoFLEX flow cytometer to measure their proliferation.
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A PrimeScript™ RT reagent Kit was used to reverse transcribe RNA to cDNA for the q-PCR reaction. The qPCR reaction system contained 5.0 μL 2 × SYBR green mixture, 0.5 μL forward primer (5 μM), 0.5 μL reverse primer (5 μM), 1.0 μL cDNA, and ddH2O. The reverse transcription conditions were 50°C for 15 minutes and 85°C for 5 minutes. The qPCR reaction conditions were pre-denatured at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 minute on the Bio-Rad CFX96 Real-Time PCR Detection System.

**Western blot**

Cells and tissues were lysed by RIPA. 40 μg proteins were separated by 10% SDS-PAGE gel and 4% concentrated gel. Then the protein was transferred to PVDF membrane at 300 mA for 100 min. Next, the membrane was blocked with 5% skim milk at room temperature for 60 minutes and incubated in a primary antibody (Jagged-1, Notch1, Hes1, OL-FM4, and β-actin at 1:2000, 1:2000, 1:1000, 1:10000, 1:10000, respectively) at 4°C overnight. After that, the membrane was incubated with HRP-conjugated Goat anti-Rabbit IgG (H+L) secondary antibody (1:15000) at room temperature for 60 min and finally detected by ECL chemiluminescence.

**Statistical analysis**

All data analyses were performed on SPSS 18.0 software. Measurement data were expressed as the mean ± standard deviation and compared by t-test or one-way ANOVA. P < 0.05 was considered as statistically significant.

**Results**

Two rats in the UC model group exhibited a hair disorder, apathy, loose stools, and bloody stools on the first day of modeling. On the second day, five rats showed signs of dietary loss, loose stools, and bloody stools. After 3 days, all 10 rats suffered from loss of appetite, hair loss, loose stools, and blood in the stool, indicating that all the models were successful. There was

![Figure 1. Colon tissue cell apoptosis and proliferation changes in UC rat. A. Spectrophotometric detection of Caspase-3 activity; B. flow cytometry detection of Ki-67 expression in colon epithelial cells. *P < 0.05, compared with control.](image-url)
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no significant abnormality in body weight, diet, mental status, stool, hair, or activity in the control group. Compared with the control group (0.61±0.15), the DAI score in the model group was significantly increased (8.22±1.73) (Table 1). There was necrotic shedding of the colonic epithelium, formation of ulcer erosions, incomplete mucosal structure, unclear glandular structure, disordered arrangement, goblet cell decrease, submucosal abscesses, and inflammatory cell infiltration in the model group. The rats of the control group exhibited complete colonic mucosa, ordered arrangement of epithelial cells, abundant goblet cells, clear glandular structure, and no obvious inflammatory cell infiltration.

Spectrophotometric results showed that compared with the control group, the caspase-3 activity in the colon tissue of the UC model group was significantly increased, indicating obviously enhanced apoptosis (Figure 1A). It was found that the MDA content in colon tissue of the UC rats was apparently higher, while the SOD enzyme activity was markedly lower than that in the control group, indicating significant degree of oxidative stress (Table 1). Ki-67 flow cytometry demonstrated that the expression of Ki-67 in the intestinal epithelial cells of UC rats was obviously elevated, indicating that the body initiated an emergency response mechanism to increase the proliferation of colonic epithelial cells and thus to repair damage in the UC rats (Figure 1B).

**Notch signaling pathway enhanced in the colon tissue of UC rats**

qRT-PCR revealed that the expressions of Jagged-1, Notch1, Hes1, and OLFM4 mRNA in the colons of the UC model rats were significantly higher than those in the control group (Figure 2A). Western blot showed that the expressions of Jagged-1, Notch1, Hes1, and the OLFM4 protein in the UC model group were apparently elevated compared with the control group (Figure 2B).

**LPS stimulus induced IEC-6 cell apoptosis and enhanced Notch signaling pathway**

DCFH-DA staining showed that the ROS content in the IEC-6 cells in the LPS-treated group was significantly higher than that in the control group (Figure 3A). Flow cytometry revealed that the apoptosis rate of the IEC-6 cells in the LPS-treated group was obviously increased compared with the control group (Figure 3B). Edu staining showed that LPS treatment apparently reduced the number of Edu-positive cells (Figure 3C). qRT-PCR demonstrated that the expressions of Jagged-1, Notch1, and OLFM4 mRNA were markedly elevated by LPS treatment, and there was no significant impact on the expression of Hes1 mRNA (Figure 3D). Western blot found that compared with the control group, the expressions of Jagged-1, Notch1, and the OLFM4 protein in the IEC-6 cells of the LPS-treated group were apparently enhanced, and there was no significant effect on the Hes1 protein expression (Figure 3E).

**Notch signaling pathway enhanced in the colon tissue of UC rats**

qRT-PCR showed that the expressions of Notch1 and OLFM4 mRNA in IEC-6 cells were further increased after treatment with recombinant Jagged-1 protein on the basis of LPS stimulation, but its impact on Jagged-1 and Hes1 mRNA was not obvious (Figure 4A). Western blot showed that the expressions of Notch1 and the OLFM4 protein in IEC-6 cells were further elevated after treatment with recombinant Jagged-1 protein without affecting the expression of Jagged-1 and Hes1 protein (Figure 4B). Flow cytometry revealed that treatment with the Jagged-1 protein attenuated the inhibitory effect of LPS on IEC-6 cells, enhanced cell pro-
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Discussion

During the pathogenesis of UC, immune cells in the lymphocytes and lamina propria of the intestinal epithelial layer can produce a large number of harmful factors such as inflammatory mediators and oxidative stress products. These harmful factors can upregulate the expression of the apoptosis-initiating protein caspase to induce intestinal epithelial cell apoptosis [18-20]. They also can induce first apoptosis signal receptor (Fas) expression via the Fas/FasL pathway (FasL) directly to cause intestinal epithelial cell apoptosis [21]. Zeissig et al. [22] showed that the use of TNF-α antibody treatment can specifically neutralize the pro-apoptotic effects of TNF-α, prevent epithelial cell apoptosis, and decline the permeability of the intestinal mucosa in intestinal tissues of...
IBD patients, suggesting that alleviating the apoptosis of intestinal epithelial cells may be one of the strategies for the treatment of IBD.

The Notch signaling pathway plays an important role in the regulation of cell proliferation, apoptosis, and differentiation [23, 24]. It is a key factor in the regeneration of the intestinal mucosal epithelium and a crucial pathway for maintaining the antibacterial activity of intestinal epithelial cells [25]. After the Notch ligand binds to the receptor, under the action of tumor necrosis factor alpha converting enzyme (TACE), the Notch receptor is digested outside the cell membrane, releasing the extracellular portion linked to the Notch ligand. Then, under the action of γ-secretory enzymes, the intracellular domain is digested to form a soluble Notch intracellular domain (NICD) that is transferred to the nucleus, which binds with the transcription repressor recombination signal binding protein for the Ig-κ-J region (RBP-J) to form a transcriptional regulatory complex that regulates the expression of the target gene, ulti-
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mately affecting cell differentiation, proliferation, apoptosis, and other biological processes. Among them, the increased expression of Hes1 can differentiate the intestinal epithelial cells towards the absorptive cell line and reduce the differentiation to secretory intestinal epithelial cells by inhibiting Hath1 expression, thus playing a decisive role in the fate of intestinal epithelial cells [26]. Olfactomedin-4 (OLF-M-4) is one of the important target genes to promote the proliferation of intestinal epithelial cells. It was found that [27] the Notch signaling pathway can promote intestinal cell proliferation by promoting the expression of the OLFM-4 gene. This study investigated the role of the Notch pathway in affecting the pathogenesis of UC and regulating intestinal epithelial cell proliferation and apoptosis.

Our study showed that abnormal changes in body weight, fecal traits, and blood in stools were observed, and the DAI score was significantly increased in UC rats, indicating that the UC models were successfully established and could be used for subsequent experiments. It was demonstrated that ROS level, MDA content, SOD enzyme activity, and caspase-3 activity in the colon tissue of the UC model group were significantly higher compared with the control group, indicating obvious oxidative stress in the colon tissue of the rat UC model. In flow-separated colonic epithelial cells, the expression of Ki-67 in the colon epithelial cells of UC model rats was obviously higher than that of the control group, revealing that UC model rats may initiate emergency response mechanisms to increase the proliferation of colon epithelial cells to repair the lesioned colon mucosa. Notch pathway-associated molecular detection showed that the expressions of Jagged-1 and Notch1 mRNA and protein in the lesioned colon tissue increased markedly after UC modelling, indicating that the activity of the Notch pathway was enhanced, which may be from the stress of rat’s body. Compared with the control group, the expression level of the target gene Hes1 in the Notch pathway was significantly elevated, suggesting that the colonic epithelial cells transformed to the absorption type, but not the secretory type. The expression of OLFM-4 in the colon tissue of UC model rats was also significantly higher it was in the control group, which was consistent with Ki-67 expression, indicating the proliferation and repair of colonic epithelial cells. Okamoto et al. [26] reported that Notch activation was detected in colonic mucosa of UC lesions, and Notch played a crucial role in mediating colon epithelial cell proliferation. Dahan et al. [28] indicated that the digestive type of Notch1 level in the intestinal epithelial cells of IBD patients was significantly elevated, revealing that the Notch pathway activity was significantly enhanced. In this study, the expression of the Notch pathway protein in UC lesions was apparently enhanced, which was similar to the results observed by Okamoto [26] and Dahan [28].

LPS is one of the most important factors in various potential stimuli of intestinal epithelial cells, which can cause intestinal epithelial cell damage through various pathways and can seriously affect the function of intestinal epithelial cells. Therefore, we used LPS to stimulate rat intestinal epithelial IEC-6 cells to in turn simulate the inflammatory environment in vivo, and further explored the effects of the Notch signaling pathway on the proliferation and apoptosis of intestinal epithelial cells under an inflammatory environment. We observed that LPS stimulation markedly restrained the proliferation, increased ROS production, and induced the apoptosis of colonic epithelial cells. Consistent with animal experiments, the in vitro inflammatory environment apparently promoted intestinal epithelial cell apoptosis. However, the difference is that although the inflammatory stimulation significantly activated the Notch signaling pathway and upregulated the expressions of Jagged 1, Notch1, and OLFM-4, the proliferation of epithelial cells is obviously suppressed. This may be due to the lack of a repair mechanism in the cultured cells in vitro or the upregulation of the Notch pathway activity is not sufficient to increase the proliferation capacity of colonic epithelial cells. In a study on the relationship between LPS inflammation stimuli and Notch pathway, Tsao et al. [29] also observed that LPS stimulation can upregulate the function of Notch receptor ligand Jagged 1, indicating that the Notch pathway activity may be affected by LPS, which is consistent with our results.

Further results demonstrated that ligand Jagged 1 treatment, on the basis of LPS stimulation, enhanced the expressions of Notch1 and OLFM-4. At this time, the proliferation of IEC-6 cells was observed to be strengthened, accom-
panied by ROS production and a reduction in apoptosis. Chen et al. [23] reported that Notch pathway inhibitor DAPT treatment obviously promoted the apoptosis of intestinal epithelial cell IEC-6 cultured in vitro. Van et al. [30] revealed that the knockdown or blockade of the Notch signaling pathway can significantly restrain the proliferation of intestinal epithelial cells. Obata et al. [27] showed that inhibiting the activity of the Notch signaling pathway markedly reduced the number of intestinal epithelial cells in mice and enhanced cell apoptosis. Okamoto et al. [26] applied a Notch signaling pathway inhibitor to UC mice and observed a large loss of colonic epithelial cells and suppressed epithelial cell proliferation and regeneration. The above studies proved that the activation of the Notch signaling pathway plays a crucial role in promoting the proliferation, reducing apoptosis, and protecting the intestinal mucosa of UC lesions. In contrast, this study, colonic epithelial cells cultured in vitro were used as subjects, and Jagged 1 treatment was directly observed to play a role in activating the Notch pathway and reducing inflammation-induced damage. This study shows that the proper activation of the Notch signaling pathway can reduce the damage of inflammatory stimuli on intestinal epithelial cells, which may have a potential therapeutic value. However, it is unclear whether the intervention of the Notch pathway has the effect of alleviating UC lesions.

Conclusion

UC lesions can activate the Notch signaling pathway in the colon tissue, which may play a role in emergency repair. The upregulation of Notch signaling pathway significantly reduced inflammatory stimuli-induced apoptosis and ROS generation in intestinal epithelial cells, resulting in increased cell proliferation.

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Disclosure of conflict of interest

None.

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