

# Thrombolysis of deep vein thrombosis and inhibiting chemotaxis of macrophage by MCP-1 blockage

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**Abstract.** – **OBJECTIVE:** Deep vein thrombosis (DVT) is one common vascular complication after trauma or surgery. Macrophage plays an important role in recanalization of thrombosis and monocyte chemotactic protein 1 (MCP-1) has a potent chemotactic role for macrophage. This study investigated the role of MCP-1 and macrophage in DVT thrombolysis.

**MATERIALS AND METHODS:** DVT mice model was established for evaluating thrombosis grades, and divided into DVT, DVT + MCP-1 recombinant protein, and DVT + MCP-1 neutralizing antibody groups. MCP-1 mRNA and protein expression, weight/length ratio of thrombosis were tested at 1, 5, 9 and 15 day after DVT. F4/80 protein expression in thrombosis on day 9 was measured to reflect infiltration of macrophage.

**RESULTS:** DVT model mice had thrombosis grade at  $2.47 \pm 0.22$  whilst no thrombosis occurred in sham group. DVT group had gradually increased MCP-1 mRNA and protein expression, which reached the peak at day 9, followed by decreased expression. Thrombosis weight/length ratio showed decreasing trends. MCP-1 protein injection significantly elevated MCP-1 expression, decreased thrombosis weight/length ratio, and elevated macrophage infiltration. Injection of MCP-1 antibody remarkably decreased MCP-1 expression, elevated thrombosis weight/length ratio and macrophage infiltration.

**CONCLUSIONS:** MCP-1 up-regulation participates in macrophage chemotaxis and thrombolysis after DVT formation. The blockade of MCP-1 weakens its thrombolysis effects.

*Key Words:*

Deep vein thrombosis, Macrophage, Monocyte chemotactic protein-1.

## Introduction

Deep vein thrombosis (DVT) is one vascular disorder caused by abnormal blood coagulation after trauma or surgery<sup>1</sup>. DVT can lead to blockade of major systemic veins mainly in

the hind limb region. The dysfunction of blood circulation can cause severe limb edema. The detachment of thrombosis can migrate to brain or lung to induce cerebral thromboembolism (CTE) or pulmonary thromboembolism (PTE), which severely threatens patient's life. Currently, DVT can be diagnosed based on clinical manifestation, body signs and imaging/lab results. However, due to slow and insidious onset, about 70-80% of DVT patients had no clinical symptoms at early stage, causing frequent misdiagnosis in clinics<sup>2</sup>. Thrombolysis and re-absorption are still major treatment approach. Non-surgical method, including anti-coagulation or thrombolysis, all depend on chronic natural lytic mechanism of our body, accomplishing recanalization of thrombosis<sup>3</sup>. Assays for vein thrombosis tissues in both humans and animals found that activation and recanalization of thrombosis mainly occur in regions with macrophage chemotaxis and prominent aggregation<sup>4</sup>. A recent study<sup>5</sup> also showed the important role of monocyte macrophage in facilitating thrombosis recanalization. Monocyte chemotactic protein 1 (MCP-1), also named as small-induced cytokine A2, belongs to chemokine C-C motif family. C-C chemokine receptor type2 (CCR2) is the major effector of MCP-1, which can also exert various immune cell chemotaxis via binding with CCR2. Macrophage is one major effector cell of MCP-1, which has potent chemotaxis of macrophage<sup>6</sup>. A previous study showed positive correlation between MCP-1 expression in DVT model rats thrombosis tissues, and infiltrated macrophage and shrinkage of thrombosis<sup>7</sup>, indicating potential roles of MCP-1 up-regulation in facilitating macrophage chemotaxis, aggregation and thrombolysis. This study thus established DVT mouse model, on which the role of MCP-1 and macrophage in DVT thrombolysis was investigated.

## Materials and Methods

### **Experimental Animal**

Specific pathogen free (SPF) grade male C57BL/6 mice were purchased from Silaike Experimental Animal Corp (Shanghai, China), with 6-8 weeks age and 18-22 g body weight. Rats were used for all experiments and all procedures were approved by the Animal Ethics Committee of Qingdao Hiser Medical Center, Qingdao, China.

### **Major Reagents and Materials**

Recombinant MCP-1 protein, neutralizing antibody MCP-1 was purchased from R&D System (Minneapolis, MN, USA). Rabbit anti-mouse MCP-1 polyclonal antibody (Catalogue No. sc-358917; 1:3000), rabbit anti-mouse  $\beta$ -actin polyclonal antibody (Catalogue No. sc-7210; 1:3000), Alexa Flour 488 labeled secondary antibody (Catalogue No. sc-3891; 1:2000) were purchased from Abcam (Cambridge, MA, USA). PCR primer was synthesized by Sangon Bio. Co. (Shanghai, China); Trizol was purchased from Invitrogen (Invitrogen/Life Technologies, Carlsbad, CA, USA). Reverse transcription kit and SYBR Green dyes were purchased from Toyobo (Co. Ltd., Osaka, Japan). Horse radish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody was purchased from Boster Co. Ltd. (Catalogue No. BA1055; 1:1000; Wuhan, China). RIPA lysis buffer and BCA protein quantification kit were purchased from Beyotime (Beijing, China). DAPI was purchased from Sigma-Aldrich (St. Louis, MO, USA).

### **DVT Model Generation and Animal Grouping**

Mice were fed in a facility with 22-25°C and 50-60% relative humidity, with food and water ad libitum. Animal handling and experimental protocols all followed experimental animal welfare codes. After 7-day acclimation, mice were randomly divided into Sham and DVT model group. In Sham group, mice were anesthetized with 3% pentobarbital, and were made a 2.0 cm incision along abdominal midline. Inferior vena cava was separated and returned to abdominal cavity, followed by suture and normal feeding. In DVT group, mice were anesthetized and sterilized. The abdominal cavity was opened to separate inferior vena cava under the crossing of left renal vein. 5-0 suture was placed along inferior vena cava, followed by vessel ligation using 4-0 suture. After removing 5-0 suture, two

lateral branching veins upper of iliac veins that crossing of inferior vena cava were separated and ligated by 5-0 suture. Vessels were returned and abdominal cavity was closed, followed by normal feeding. DVT model mice were further divided into three groups: control, MCP-1 and MCP-1 blockade groups. Control group received 500  $\mu$ L saline Sigma-Aldrich (St. Louis, MO, USA) into abdominal cavity 1 h before DVT model preparation (five times with every 24 h). MCP-1 group received 500  $\mu$ L recombinant mice MCP-1 protein into abdominal cavity (five times for every 24 h). MCP-1 blockade group received 500  $\mu$ L MCP-1 monoclonal antibody (Catalogue No. sc-358917; 1:3000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) injection into abdominal cavity 1 h before DVT model preparation (five times for every 24 h).

### **Thrombosis Weight and Grading**

1, 5, 9 and 15 days after DVT model preparation, mice were anesthetized to remove thrombosis at surgical site. Thrombosis was weighted and measured. Thrombosis ratio = weight/length of vessels ( $\mu$ g/cm). Thrombosis can be classified into grade 0 (no thrombosis formation), grade I (less than 50% blockade), grade II (> 50% blockade but incomplete embolization, and grade III (complete blockade of vessels).

### **Immunofluorescence for Macrophage Infiltration**

Thrombosis in the inferior vena cava was removed for preparing frozen section. Immunofluorescence was used to test F4/80 expression to reflect macrophage infiltration. In brief, tissues were fixed in 4% paraformaldehyde Sigma-Aldrich (St. Louis, MO, MI, USA) and were rinsed in phosphate buffered saline (PBS) for three times. After treated with 0.1% Triton X-100 Sigma-Aldrich (St. Louis, MO, USA), tissues were blocked with 1% bovine serum albumin (BSA) Sigma-Aldrich (St. Louis, MO, USA), and were cultured with anti-F4/80 monoclonal antibody (Catalogue No. sc-71086; 1: 200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. After phosphate buffered saline (PBS) rinsing for three times, Alexa Flour 488 labeled secondary antibody (Catalogue No. sc-3891; 1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added for 60 min room temperature incubation. With DAPI nuclear staining Sigma-Aldrich (St. Louis, MO, USA), cover-slips were mounted for observation under fluorescent microscopy.

**qRT-PCR for Gene Expression**

A total of 30 mg thrombosis tissues was lysed in Trizol (Beyotime Co. Ltd., Beijing, China). After extracting tissue RNA, qRT-PCR was used to test MCP-1 mRNA expression, using primers (MCP-1<sub>F</sub>: 5'-ATTTC CACAC TTCTA TGCCT CCT-3'; MCP-1<sub>R</sub>: 5'-ATCCA GTATG GTCCT GAAGA TCA-3';  $\beta$ -actin<sub>F</sub>: 5'-GTGAC GTTGA CATCC GTAAA GA-3';  $\beta$ -actin<sub>R</sub>: 5'-GCCGG ACTCA TCGTA CTCC-3').

**Western Blot Assay**

RIPA buffer was used to extract tissue proteins, which were quantified by BCA method. 40  $\mu$ g protein sample was loaded, separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to membrane. After blocking in 5% defatted milk powder, primary antibody (MCP-1 at 1:200,  $\beta$ -actin at 1:600) was added for 4°C overnight incubation. Secondary antibody (1:6000 dilution) was added for 60 min culture after three times of PBST washing. Enhanced chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ, USA) substrate was used to develop the membrane, which was exposed and captured images for analysis.

**Statistical Analysis**

SPSS 18.0 (SPSS Inc. Chicago, IL, USA) was used for data input and analysis. Measurement data were presented as mean  $\pm$  standard deviation. Student *t*-test or analysis of variance (ANOVA) was used for comparing measurement data between groups. Statistical significance was defined when  $p < 0.05$ .

**Results****Thrombosis Formation in Vascular Cavity in DVT Model Mice**

Sham group mice had unchanged diameter of inferior vena cava, which had evenly distributed cavity diameter and smooth blood flow, with elastic vascular walls. 24 h after DVT model preparation, thrombosis was formed at surgical site, with tight adhesion to vascular walls, and no signs of blood flow inside. In DVT mice, 24 h thrombosis score was  $2.47 \pm 0.22$ , which was significantly higher than Sham group ( $p < 0.05$ , Table I). Results thus indicated successful generation of mouse DVT model.

**Table I.** 24 h thrombosis grade at 24 h after DVT model preparation.

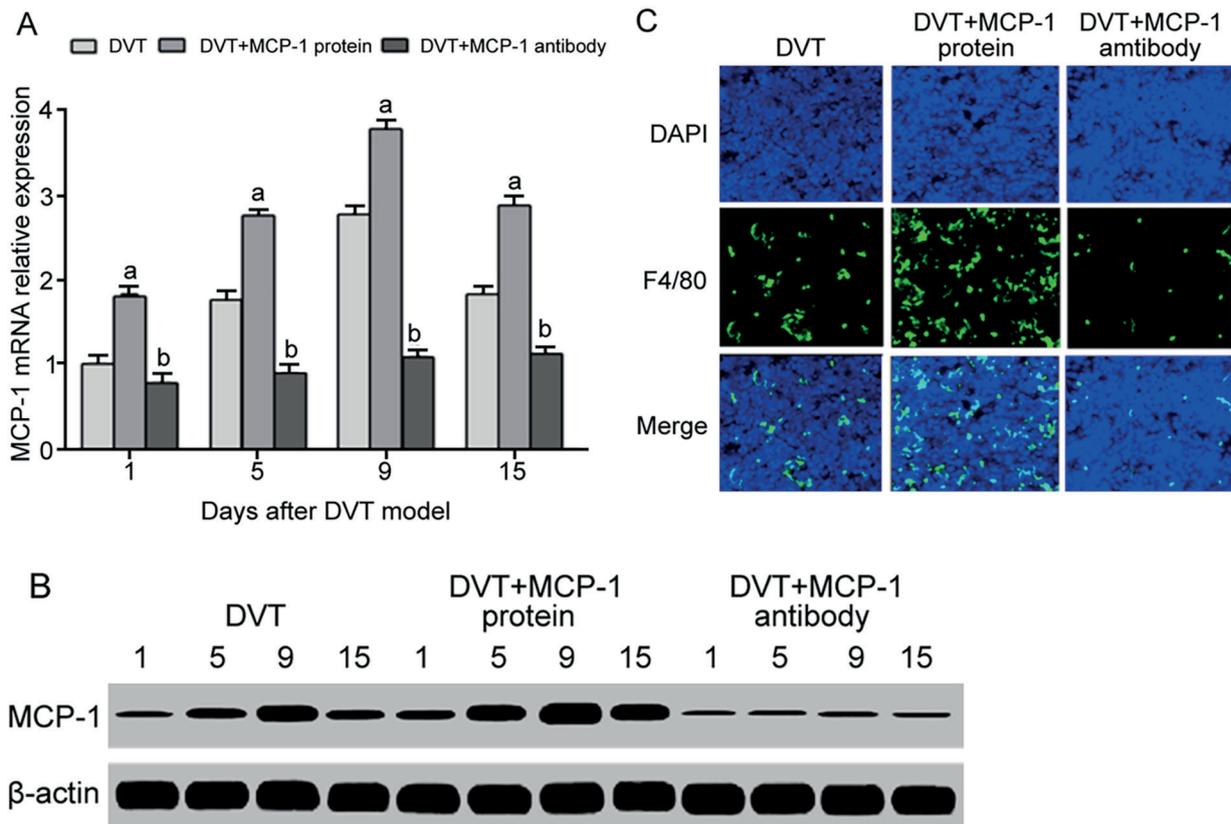
	Thrombosis grade
Sham group	0
DVT group	$2.47 \pm 0.22$

**Injection of MCP-1 Protein and MCP-1 Antibody Facilitated or Inhibited Macrophage Infiltration Inside Thrombosis**

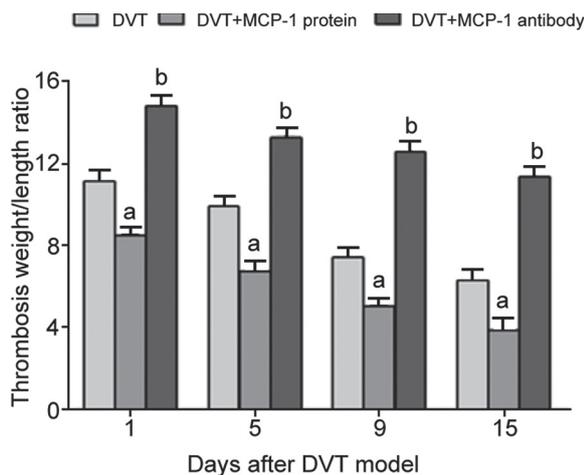
Test results showed gradually increased MCP-1 mRNA and protein expression levels in vein thrombosis of DVT group at 1, 5 and 9 days after surgery. MCP-1 expression level reached the peak at day 9, followed by gradual decrease (Figure 1A and 1B). qRT-PCR results showed that intra-peritoneal injection of MCP-1 recombinant protein and MCP-1 antibody elevated or decreased MCP-1 mRNA expression in thrombosis, respectively (Figure 1A). Western blot results showed that intra-peritoneal injection of MCP-1 recombinant protein and MCP-1 antibody elevated or decreased MCP-1 protein expression in thrombosis, respectively (Figure 1B). Therefore, this study utilized immunofluorescence to detect macrophage infiltration inside thrombosis at day 9. Results showed that compared to DVT group, MCP-1 recombinant protein injection significantly increased F4/80 expression in vein thrombosis, whilst MCP-1 antibody injection significantly decreased F4/80 expression in mouse vein thrombosis. These results showed that injection of MCP-1 recombinant protein and antibody facilitated and suppressed macrophage infiltration inside thrombosis (Figure 1C).

**Injection of MCP-1 Protein and MCP-1 Antibody Facilitated and Suppressed Thrombolysis**

Test for thrombosis in DVT model mice showed significantly lower thrombosis weight/length ratio with elongated time, indicating thrombosis organization (Figure 2). Comparing to DVT model group, injection of MCP-1 recombinant protein decreased thrombosis weight at all time points (Figure 2), whilst injection of MCP-1 neutralizing antibody elevated thrombosis weight at all time points compared to DVT model group. The trend of thrombosis weight decrease with time elapse, was significantly lowered than DVT model group or MCP-1 recombinant protein injection (Figure 2). These results indicated that elevation of MCP-



**Figure 1.** Injection of MCP-1 protein and MCP-1 antibody facilitated or inhibited macrophage infiltration inside thrombosis. **(A)** qRT-PCR for MCP-1 mRNA expression; **(B)** Western blot for MCP-1 protein expression; **(C)** Immunofluorescence for macrophage infiltration. Note: a,  $p < 0.05$  comparing between DVT + MCP-1 protein group and DVT group; b,  $p < 0.05$  comparing between DVT + MCP-1 antibody group and DVT group.



**Figure 2.** Comparison of thrombosis weight after DVT model preparation. Note: a,  $p < 0.05$  comparing between DVT + MCP-1 protein group and DVT group; b,  $p < 0.05$  comparing between DVT + MCP-1 antibody group and DVT group.

1 content can facilitate thrombosis organization and lysis, whilst blockade of MCP-1 effects significantly retards the process of thrombosis organization and lysis.

## Discussion

Currently, anti-coagulation, thrombolysis and absorbance facilitation methods all depend on gradual organization and recanalization of thrombosis<sup>3</sup>. The lysis, organization and recanalization of DVT, is one natural process involving inflammation and immune response, accompanying with activation, infiltration of inflammatory cells such as macrophage and neutrophils, plus the release of multiple inflammatory factors. It can facilitate lysis and recanalization of vessels via mediating expression of related factors including uridylyl phosphate adenosine (uPA), and matrix metalloproteinases (MMPs)<sup>8</sup>. During

this process, as the major inflammatory cells, macrophage can directly engulf necrotic tissues, synthesize and release protein hydrolase uPA and MMPs, to lyse peripheral matrix and fiber proteins, thus facilitating lysis and recanalization of thrombosis<sup>9</sup>. A previous work<sup>4</sup> found that thrombosis activation and recanalization mainly occurred in those regions with potent macrophage aggregation and chemotaxis, indicating the important role of macrophage in facilitating thrombosis organization and recanalization. In addition to lysis and endocytosis of thrombosis, macrophage also plays a critical role in facilitating vessel recanalization via its angiogenesis effects. Under the stimuli of inflammatory factors, macrophage can stimulate vascular endothelial cell proliferation, migration and facilitation of vascular like structure inside thrombosis via lysing extra-cellular matrix, facilitating vascular endothelial cell migration and releasing angiogenesis factors including IL-1, TNF, VEGF<sup>10</sup> and bFGF<sup>11</sup>. Besides inflammatory stimuli, hypoxia environment inside thrombosis can stimulate macrophage to expression hypoxia-sensitive genes such as hypoxia inducible factor (HIF-1), thus facilitating expression of its downstream target genes including VEGF, SDF-1 and PLGF, as well as angiogenesis<sup>12</sup>. Chemotaxis factors are a group of chemotactic cytokine superfamily with similar molecular structures. It can be further divided into four groups (C, CC, CXC and CX3C4) based on differential distribution of the first two cysteine at highly conserved N-terminus<sup>13</sup>. Chemotactic factors can bind to receptors on target cell membrane to induce phosphorylation of serine/alanine at C-terminus of receptor proteins, to activate downstream cellular signal transduction pathway, thus participating cell motility, invasion, interaction of extracellular matrix, migration of leukocyte subpopulation, and modulating various biological processes including hematological cell differentiation<sup>13</sup>, inflammation<sup>14</sup>, immune cell growth/development<sup>14</sup> and invasion/dispersion of tumor cells<sup>15</sup>. Human MCP-1 gene locates on chromosome 17q11.2-q21.1, and has 1927 base pairs with 3 exons and 2 introns<sup>16</sup>. Human MCP-1 protein includes one precursor protein including 97 amino acids. During post-translational modification and processing, 23 amino acids were digested to form mature MCP-1 protein with 76 amino acids<sup>17</sup>. As the major effector receptor of MCP-1, CCR-2 can bind with MCP-1 to exert biological functions for chemotaxis of mononuclear macrophage, basophil granular cells and

dendritic cells, with macrophage as the major effector cell of MCP-1<sup>18</sup>. A previous study showed positive correlation between MCP-1 expression and macrophage infiltration number, shrinkage of thrombosis<sup>7</sup>, indicating possible role of MCP-1 up-regulation in facilitating macrophage chemotaxis, aggregation and thrombolysis. Therefore, this report established a DVT mouse model, on which the role of MCP-1 and macrophage in DVT thrombolysis was investigated. Grading of thrombosis showed that DVT model mice had 24 h thrombosis score at  $2.47 \pm 0.22$ , indicating successful generation of mouse DVT model for further development. Test results showed gradually increased MCP-1 mRNA and protein expression in vein thrombosis at 1, 5 and 9 days after model, with peak level at day 9 followed by gradual decrease, whilst thrombosis weight/length showed decreasing trends, suggesting the involvement of macrophage during thrombolysis. However, other factors such as neutrophil may also be involved in thrombolysis. Expression trend of MCP-1 expression indicated prominent macrophage aggregation at day 9 post-DVT. Therefore, we used immunofluorescence to detect the expression of macrophage marker F4/80 at day 9. Results showed abundant aggregation and infiltration of macrophage inside thrombosis. McGuinness et al<sup>5</sup> found abundant aggregation of mononuclear and macrophage inside thrombosis in spontaneous vein thrombosis patients and animal models, as consistent with our study showing major macrophage aggregation and infiltration. Nosaka et al<sup>19</sup> reported the peak level of neutrophil granular cells aggregation and infiltration at 1 day after thrombosis, followed by gradual decrease. At day 7 after DVT, macrophage aggregation reached maximal level, as consistent with our results showing peak MCP-1 expression, indicating similar phenomena and mechanisms. Wakefield et al<sup>20</sup> found abundant aggregation and infiltration of neutrophil granular cells inside thrombosis. Only until day 4, those aggregated inflammatory cells transform from neutrophil granular cells to monocyte macrophage, whose level reached the peak after several days, indicating the major role of macrophage at late stage of thrombolysis, as similar to our observation. Comparing to DVT group, intraperitoneal injection of MCP-1 recombinant protein significantly elevated MCP-1 mRNA/protein expression in mouse thrombosis, and remarkably increased macrophage aggregation at day 9 post-DVT surgery. Intraperitoneal injection of MCP-1 antibody remarkably

decreased MCP-1 mRNA/protein expression in mouse thrombosis, and remarkably suppressed macrophage aggregation at day 9 post-DVT. From known knowledge, the injection of MCP-1 recombinant protein or antibody should have no effects on MCP-1 mRNA or protein expression level in thrombosis. This study, however, observed significant effects of MCP-1 recombinant protein or antibody on its mRNA/protein expression. Such reasons can be attributed to the effect of aggregated number of thrombosis macrophage by elevating MCP-1 expression or blocking MCP-1 effector, whilst macrophage is one major origin of MCP-1 expression and secretion, thus affecting gene expression via modulating origins of aggregated macrophage inside thrombosis<sup>21</sup>. Quality check of thrombosis showed that elevation of MCP-1 contents facilitated thrombosis organization and lysis, whilst blocking MCP-1 effector significantly retarded such process, and decreased its degree of organization and lysis. Chen et al<sup>7</sup> showed positive correlation between MCP-1 expression and macrophage infiltration or shrinkage of thrombosis in DVT rats. Wakefield et al<sup>22</sup> found that with more macrophage in thrombosis, its lysis and angiogenesis are more potent. Henke et al<sup>23</sup> also found that after DVT formation, activation and chemotaxis of macrophage toward thrombosis site have importantly facilitating role for thrombolysis and *de novo* angiogenesis. Humphries et al<sup>24</sup> demonstrated the macrophage with uPA over-expression had potentiated collagen lysis activity and thrombolysis function, in addition to the fact that over-activation of macrophage post-thrombosis benefited the thrombolysis and vascular cavity recanalization. This study generated a DVT mouse model, on which elevated MCP-1 expression was found to be important for inducing macrophage chemotaxis and thrombosis organization, whilst antibody blockade weakened such effects.

## Conclusions

We observed that MCP-1 up-regulation after DVT formation participates in macrophage chemotaxis and thrombolysis. Antibody blockade of MCP-1 weakens such thrombolysis effects.

## Conflict of Interest

The Authors declare that they have no conflict of interests.

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