

BASIC RESEARCH

Correlation between TIMP-1 expression and liver fibrosis in two rat liver fibrosis models

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TIMP-1 level could reflect the severity of liver fibrosis, while in CCL₄-induced model, the correlation between the serum TIMP-1 level and the severity of hepatic fibrosis was not statistically significant.

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Key words: TIMP-1; Liver fibrosis; Models, rat; Immune-induced, CCL₄-induced; Serum; Tissue of liver

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Abstract

AIM: To evaluate serum TIMP-1 level and the correlation between TIMP-1 expression and liver fibrosis in immune-induced and CCL₄-induced liver fibrosis models in rats.

METHODS: Immune-induced and CCL₄-induced liver fibrosis models were established by dexamethasone (0.01 mg) and CCL₄ respectively. Serum TIMP-1 level was detected with ELISA, while histopathological grade of liver biopsy was evaluated. Spearman rank-correlation test was used to analyse the difference of the correlation between the TIMP-1 expression and hepatic fibrosis in the two fibrosis models. Furthermore, *in situ* hybridization was used to determine the expression difference of TIMP-1 mRNA in the two models.

RESULTS: Positive correlation existed between serum TIMP-1 level of immune induced group and the histopathological stages of fibrosis liver of corresponding rats (Spearman rank-correlation test, $r_s = 0.812$, $P < 0.05$), and the positive *in situ* hybridization signal of TIMP-1 mRNA was strong. In CCL₄-induced liver fibrosis model, the correlation between the serum TIMP-1 level and the severity of hepatic fibrosis was not statistically significant (Spearman rank-correlation test, $r_s = 0.229$, $P > 0.05$). And compared with immune-induced model, the positive *in situ* hybridization signal of TIMP-1 mRNA was weaker, while the expression variation was higher in hepatic fibrosis of the same severity.

CONCLUSION: The correlations between TIMP-1 expression and liver fibrosis in two rat liver fibrosis models are different. In immune-induced model, serum

INTRODUCTION

Hepatic fibrosis is a pathological process with the net deposition of extracellular matrix (ECM) proteins. The change of ECM is mainly regulated by matrix metalloproteinases (MMPs), which are a family of proteolytic enzymes that are capable of degrading the ECM^[1-3]. The activity of MMPs is tightly regulated by the amount of active protein and the concentration of specific inhibitors, called tissue inhibitors of metalloproteinases (TIMPs)^[4,5]. Extensive studies have identified that TIMPs play a key role in the progression of fibrosis. TIMP-1 is the first discovered tissue inhibitor of TIMPs in liver which can inhibit most interstitial collagenases and MMP-9. Murawaki *et al* revealed that serum level of TIMP-1 could reflect the change of liver TIMP-1 in patients with chronic liver disease and liver TIMP-1 concentration increases with progression of the liver disease^[6-9]. So, TIMP-1 has been considered as a useful diagnostic index of hepatic fibrosis^[10-12]. Our previous studies^[13-15] also concentrated on the establishment of the new hepatic fibrosis diagnostic index. Interestingly, in animal experiments we found the expression of TIMP-1 in liver tissue has remarkable difference in different rat liver fibrosis models^[13,14]. In the present experiment, the serum TIMP-1 level of experimental rats of liver fibrosis model was detected with ELISA and association with histopathological grading of fibrosis liver was analyzed. Furthermore, *in situ* hybridization was used to analyze the expression difference of TIMP-1 mRNA between the two models.

MATERIALS AND METHODS

Animals

One hundred and twenty adult female Wistar rats (provided by Experimental Animal Center of Fourth Military Medical University, Xi'an, China) weighing 150-180 g, fed with common stuff and water, were employed in the study (Approval for this project was obtained from the Institutional Review Board at Fourth Military Medical University). Immune-induced rat liver fibrosis model was established according to the method of Wang *et al.*^[16]. Dexamethasone (0.01 mg) was injected into the rats through coccygeal vein in one hour after the first and second injection of human serum albumin (HAS). For CCL₄ group, 55 rats were injected with 400 mL/L CCL₄ in peanut oil subcutaneously at a dose of 3 mL/kg twice (Monday and Thursday) weekly, totally 10 wk^[17]. Ten normal rats were served as control group.

Specimen preparation

From the first Sunday in the process of model induction (for immune induced group, from the first coccygeal vein injection of HSA), 5 rats from each group were selected randomly on Sunday, until all rats were involved. Rats taken out from immune induced group on the first Sunday were numbered IW1A to IW1E (Immune-induced rat liver fibrosis model wk 1st A-E), while the rats from CCL₄ induced group were numbered CW1A to CW1E (CCL₄-induced rat liver fibrosis model wk 1st A-E). All rats were immediately sacrificed under narcosis after taken out. Following decapitation 2 mL blood was collected from each rat. Liver was quickly excised, and rinsed in ice-cold saline. Ten rats in control group were treated in the same way after 10 wks' feeding. Blood specimen was centrifuged, and the serum was obtained, and stored at -70°C. Livers were cut into 1 mm × 1 mm × 1 mm blocks. Part of it was fixed in 40 g/L formaldehyde, and part was fixed in 25 g/L glutaraldehyde. All liver specimens were stored at -70°C. Specimens were treated all together after collected.

Pathologic observation and histopathological grading of fibrotic liver tissue

Liver sections were processed together for routine hematoxylin-eosin (HE) stain. Pathological diagnosis of each liver specimen was assessed and graded from 0 to VI by three pathologists in a blinded manner (fibrotic stage was determined when more than two pathologists reached the same diagnosis; if inconsistent results were reached among the three pathologists, the fourth determined the final diagnosis) according to the criteria described by Wang *et al.*^[18] (Table 1).

ELISA detection of serum TIMP-1

Enzyme-linked immunosorbent assay (ELISA) was used to evaluate the serum TIMP-2 level^[19,20]. Mouse TIMP-2 monoclonal antibody was purchased from American Maxim Company (MAB-0283) and test was performed according to the protocol described previously^[21]. The absorbance of serum TIMP-2 was determined at 450 nm with an ELISA reader (EL × 800, Bio-Tek Instruments, Inc, USA).

Table 1 Scoring system assessing the degree of fibrosis

Degree of fibrosis	Score
No fibrosis	0
Slight fibrosis expanding to some portal areas and central veins	I
Marked fibrosis expansion, but without portal to portal bridging	II
Fibrosis expanding to most portal areas with occasional portal to portal bridging	III
Pseudolobules formed and partly replacing the normal architecture of the liver lobules	IV
Occasional pseudolobules formed (incomplete cirrhosis)	V
Congested with pseudolobules, and between pseudolobules wide hyperplastic collagen fiber existed (complete cirrhosis)	VI

In situ hybridization of TIMP-1 mRNA in tissue of fibrosis liver

Paraffin sections were hybridized with digoxigenin-labelled cRNA probes as described by Wang *et al.*^[22]. The cRNA probe for TIMP-1 and the *in situ* hybridization kit were purchased from Boshide Biological Technology Limited Company, Wuhan, China (No MK1549). Expression of TIMP-2 mRNA was assessed quantitatively by image pattern analysis with gray scale scanning. Control was performed with normal liver tissue.

Specimen preparation for transmission electron microscopy

Specimens were routinely prepared for transmission electron microscopy (TEM) observation and examined under a JEOL JEM-100CXII electron microscope.

Statistical analysis

Spearman rank-correlation test, LSD-*t* and Student's *t* test were used to analyze the results. *P* < 0.05 was taken as significant.

RESULTS

Comparison of histopathology between immune induced and CCL₄ induced liver fibrosis groups

Distinct difference of histopathological changes presented between the liver fibrosis of immune group and CCL₄ group. The liver fibrosis in immune group rat was characterized by portal fibrosis with marked ductular proliferation extending to fibrous septa (Figure 1A, specimen from IW10E). Pseudolobules formed and partly replaced the normal architecture of the liver lobules, but the fibrotic septum was small and sparse. In CCL₄ group, prominent fatty degeneration and necrosis were found and the normal architecture of the liver lobules was markedly destroyed, most of which were replaced by pseudolobules (Figure 1B, specimen from CW10A). Compared with immune induced group, fibrotic septum in CCL₄ group was wide and compact.

Histopathological grading

All rats in immune induced group survived the experiment. In tissue specimens of IW1A-E, only slight hepatocyte swelling could be observed, whereas no obvious damage

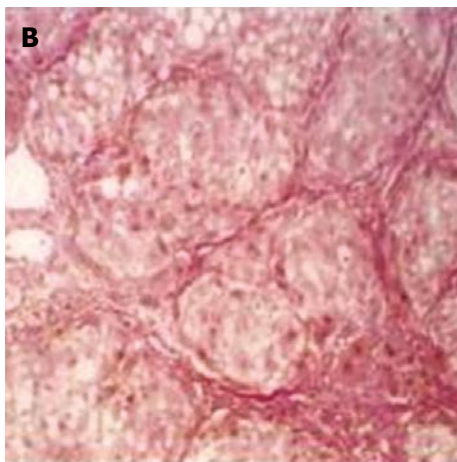
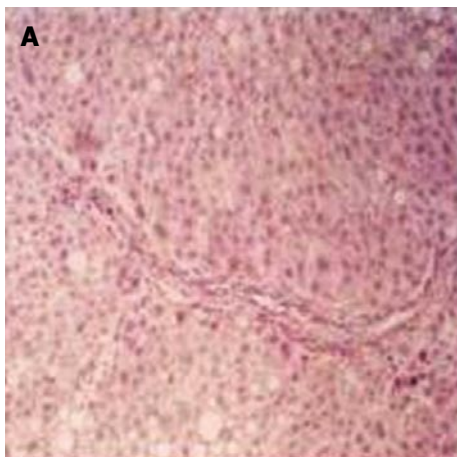


Figure 1 Light microscope observation of rat liver fibrosis (HE ×100). **A:** immune-induced liver fibrosis; **B:** CCl₄-induced liver fibrosis.

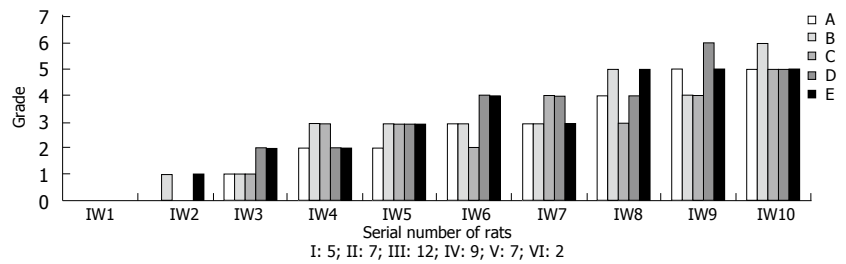


Figure 2 Histopathological grade of fibrosis of immune induced group.

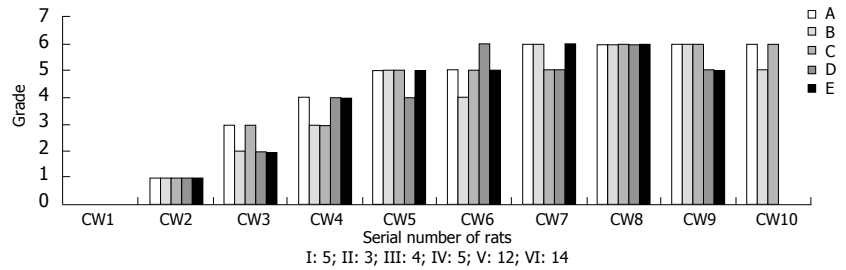


Figure 3 Histopathological grade of fibrosis of CCL₄ induced group.

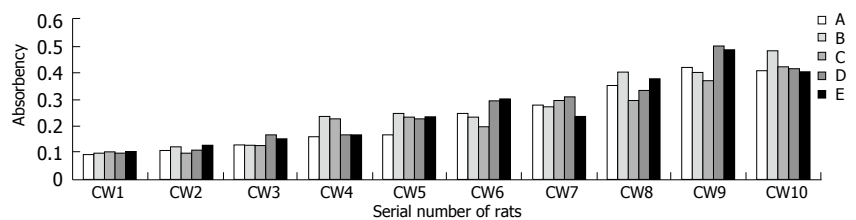


Figure 4 ELISA results of serum TIMP-1 of immune induced group.

presented. Histopathological stages of the five specimens were all determined to be stage 0. Among the specimens of IW2-5, obvious hyperplasia of connective tissues could be detected, but most of the histopathological stages were not above III. Among rats of immune induced group taken out after fifth week, with induction time extending, more livers were determined to be stage IV and V fibrosis. However, specimens determined to be stage VI were few (Only two fibrosis liver specimens of rats taken out at tenth week were determined to be stage VI) (Figure 2). Seven rats in CCL₄ induced group died in the process of experiment (5 rats died before wk 5 and 2 died at wk 7), which livers were immediately excised and frozen at -70°C. Histopathological observation of the died rats showed severe liver damages existed in the liver such as inflammation, necrosis, excessive collagen deposition and inflammatory cells infiltration, *etc.* Obvious damage already existed in the liver tissue of the five rats of CW1, but no fiber hyperplasia presented. Histopathological stages were stage 0 according to the criteria described above. Specimens of CW2 presented obvious hepatocyte degeneration and necrosis, and vacuole degeneration and inflammatory cell infiltration also could be observed, but fiber hyperplasia was not predominant. The histopathological stages were determined to be stage I. From wk 3, normal architecture of the liver was rather

lost, replaced with volumes of connective tissue. Hereafter, with the passage of time, fibrotic separations increased between the portal areas or between the portal area and the central vein, leading to the increasing formation of pseudolobules, and histopathologically stage IV or V was determined. In most of the rats taken out after wk 7, liver tissues were congested with pseudolobules and between the pseudolobules large numbers of collagens deposited, which were classified as classic stage VI (Figure 3).

ELISA reading of serum TIMP-1

In theory, reading (absorbency) of ELISA reader correlates with serum TIMP-1 level, which could properly reflect the quantity of serum TIMP-1^[23,24].

Results of serum TIMP-1 level of immune induced group and CCL₄ induced group are presented in Figures 4 and 5 respectively. Data of control group accorded with normal distribution (mean = 0.105, 95% percentile = 0.137).

Positive correlation existed between serum TIMP-1 level of immune induced group and the histopathological stages of fibrosis liver of corresponding rats (Spearman rank-correlation test, *r* = 0.812, *P* < 0.05), indicating serum TIMP-1 level could be considered as an index reflecting the degree of liver fibrosis.

No clear correlation between the serum TIMP-1 level

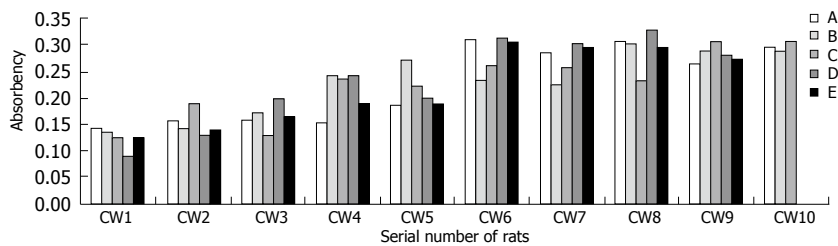


Figure 5 ELISA results of serum TIMP-1 of CCl₄ group.

Table 2 Results of image pattern analysis of expression of TIMP-1 mRNA

Group	n	TIMP-1 mRNA
Immune group ^a	7	421.90 ± 19.00
CCL ₄ group ^c	7	188.80 ± 62.80
Control group	10	52.75 ± 4.25

^a $P < 0.05$ compared with CCL₄ group, $t = 9.398$; ^c $P < 0.05$ compared with control group, $t = 3.414$.

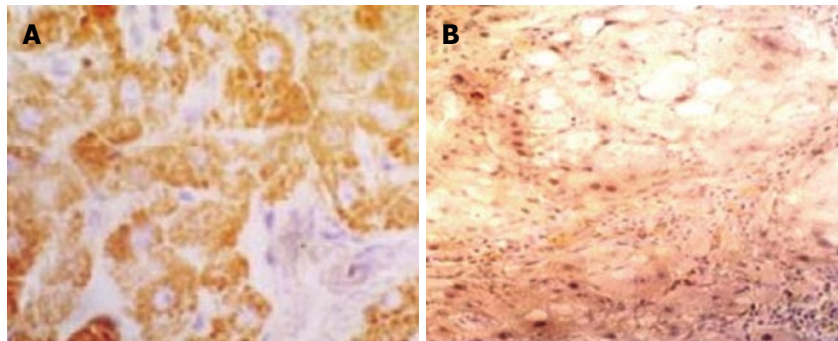


Figure 6 Expression of TIMP-1 mRNA in tissue of rat fibrosis liver. A: Immune-induced liver fibrosis ($\times 400$); B: CCL₄-induced liver fibrosis ($\times 200$).

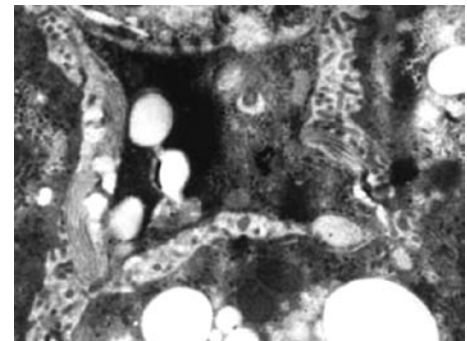


Figure 7 Activated HSC in immune induced rat liver fibrosis ($\times 10000$).

of CCL₄ induced group and the histopathological stages of corresponding rats (Spearman rank-correlation test, $r_s = 0.229$, $P > 0.05$), indicating serum TIMP-1 level of CCL₄ group could not reflect the degree of liver fibrosis. Even so, the serum TIMP-1 level of CCL₄ group was obviously higher than that of control group (LSD- t , $P < 0.001$).

Expression of TIMP-1 mRNA in tissue of fibrosis liver

Seven specimens from immune group and CCL₄ group respectively, which were all determined as stage V histopathologically (for CCL₄ group, the seven specimens were randomly selected from the 12 stage V specimens), were used to compare the difference of TIMP-1 mRNA expression. Ten specimens of control group were detected at the same time. In the fibrosis liver of immune group, expression of TIMP-1 mRNA was detected in myofibroblasts, fibroblasts and vascular endothelial cells, especially predominant in the portal areas and fibrotic septum. The positive signals of TIMP-1 mRNA located in cytoplasm as brown particles, but not found in nucleus (Figure 6A). For CCL₄ group, myofibroblasts, fibroblasts in the portal area and vascular endothelial cells expressed TIMP-1 mRNA. However, compared with immune group, the signals were weaker (Figure 6B).

Image pattern analysis of *in situ* hybridization revealed that among immune, CCL₄ and control groups, expression of TIMP-1 mRNA of immune group was highest ($t = 9.398$, $P < 0.05$); while between CCL₄ group and control group, expression of TIMP-1 mRNA of CCL₄ group was obviously higher ($t = 3.414$, $P < 0.05$) (Table 2). The standard deviation of data of CCL₄ group was distinctly higher than immune group ($62.80 > 19.00$), indicating greater variation existed in the specimens of CCL₄ group

even with the same histopathological stage.

Transmission electron microscope examinations

In liver fibrosis of immune group, large quantities of activated hepatic stellate cells (HSC) were detected. In the cytoplasm of activated HSCs, there were abundant rough endoplasmic reticulum, but fatty droplets were obviously decreased (Figure 7). Around the activated HSCs, in Disse space, between hepatocytes, and surrounding areas of bile duct, dense deposition of collagens could be found. Portal areas extended, where great number of myofibroblasts existed. In denatured hepatocytes, many swelling mitochondria and areas of fatty drops existed. For CCL₄ group, severe collagen deposition could be observed, but with fewer activated HSCs.

DISCUSSION

Hepatic fibrosis is a consequence of different chronic liver diseases caused by hepatitis viruses, drugs, alcohol, parasite, and autoimmune mechanism. Nowadays there are several kinds of animal model of liver fibrosis for the study of the mechanisms of liver fibrosis, among which CCL₄-induced rat liver fibrosis model and immune-induced rat liver fibrosis model were extensively investigated and applied^[25]. Fibrosis caused by different pathogens has different pathogenesis processes. Therefore, not every fibrosis model is universally suitable. Which model should be applied in a given study is of great importance. But now, there is still no clear guideline for this.

Over the past 15 years, substantial progress has been made in understanding the role of TIMPs (TIMP-1 and TIMP-2) in the regulation of hepatic fibrosis. Hepatic

fibrosis is a pathological process with the net deposition of ECM proteins^[26]. The change of ECM is mainly regulated by MMPs. MMPs are a family of proteolytic enzymes that are capable of degrading the ECM and the activity of MMPs is tightly regulated by the amount of active protein and the concentration of TIMPs^[27]. It has been identified that TIMP-1 plays a predominant role in regulating fibrosis by inhibiting the activity of MMP-1, MMP-3, MMP-9, and TIMP-1 is far stronger than TIMP-2^[28]. So, TIMP-1 has been considered as an important factor in the progression of liver fibrosis and the expression level could be used as a new index of fibrosis severity.

In our previous studies^[13,14], we found that the expression of antigen and mRNA of TIMP-1 in liver tissue of CCL₄-induced fibrosis rat was weaker compared with immune-induced fibrosis. Although higher than normal rat liver, the expression of TIMP-1 of CCL₄-induced liver fibrosis was not consistent with the severity of hepatic fibrosis. In the current study, the fibrosis in CCL₄-induced rat liver fibrosis model developed fast and almost all the histopathological grading of the rats which survived the experiment in this group belonged to grade V, and VI. Fibrous tissue in the fibrosis liver of this model was presented as wide and compact fibrotic septum, and most of the normal architecture of the liver lobules were destroyed and replaced by pseudolobules. But, *in situ* hybridization and immunohistochemical staining showed mRNAs and antigens of TIMP-1 expressed weakly, not proportional to the severity of the fibrosis. While, in immune-induced rat liver fibrosis model, even the fibrotic septum were small and sparse and histopathological grading was lower, the expression of mRNAs and antigens of TIMP-1 was higher compared with CCL₄-induced rat liver fibrosis model. We used Spearman rank-correlation test to analyse the correlation between the serum levels of TIMP-1 and the histopathological stages of corresponding rat, and found that no clear correlation existed between the serum levels of TIMP-1 and the histopathological stages of corresponding CCL₄-induced rat. While, serum levels of TIMP-1 of immune-induced fibrosis liver could reflect the severity of liver fibrosis. In the process of experiment, we failed to detect clear pathologic progression in the inducing process of CCL₄, because the pathological changes developed very fast. We also analyzed the results of *in situ* hybridization of TIMP-1 mRNA in CCL₄-induced fibrosis liver, which indicates that even with same histopathological stage, the TIMP-1 mRNA expression varied greatly, which was distinct from immune-induced rat liver fibrosis.

Currently studies on fibrosis are mostly focused on chronic hepatitis caused by hepatitis virus infection, which is a chronic progressive process. Our study revealed that the fibrosis induced by CCL₄ progressed fast, and autopsies of the fibrosis livers presented hepatocyte vacuole degeneration and necrosis at the early period of CCL₄ induction, indicating the subsequent ECM deposition might be the recovery process, during which connective tissue replaced the damaged architecture. The nosogenesis of CCL₄ induced fibrosis may contribute to this change^[29]. The hepatotoxicity of CCL₄ results from its metabolites, the free radicals CCL₃, which could damage the hepatocyte

by causing lipid peroxidation and by binding covalently to cell structures, leading to flaky hepatocyte vacuolar degeneration and necrosis. This is of obvious difference from the known fibrosis mechanism of viral hepatitis (HBV and HCV), which has been considered as a consequence of immunologic injury caused by the virus infection.

The fibrosis of immune-induced rat model is caused by type III allergy (immune complex type)^[30]. The severity of the fibrosis is not so much high as CCL₄-induced rat liver fibrosis, while the progression is similar to human viral hepatitis. Immune complex in serum exerts its hepatotoxicity through activating HSCs that can express TIMP-1, which enhances the deposition of ECM through inhibiting the activity of MMPs^[31]. Under TEM, larger numbers of activated HSCs supported the above hypothesis.

In conclusion, the pathological changes of immune-induced rat liver fibrosis model is a gradual process and serum TIMP-1 level can reflect the severity of fibrosis. CCL₄-induced rat liver fibrosis model develops fast in comparison with immune-induced rat liver fibrosis model and no significant correlation exists between the serum TIMP-1 level and the severity of fibrosis, both indicating that the model is unsuitable for experimental observation. Taken together of the difference of the two models, we think immune-induced rat liver fibrosis model is more suitable for the study of human chronic viral hepatitis.

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