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# Profiles of 70-kDa Stress Protein, βAPP, Proliferating Cell Nuclear Antigen, and Glial Fibrillary Acidic Protein in the Gerbil with Focal Infarction and Combined Focal Infarction and Global Ischemia : Relation with Ischemic Tolerance*

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## 성숙 Gerbil뇌에서 유발한 국소 경색의 70-kDa Stress Protein, βAPP, Proliferating Cell Nuclear Antigen, and Glial Fibrillary Acidic Protein 발현 양상

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**목 적** : 본 연구는 성숙 gerbil 뇌에서 대퇴정맥을 통한 자성체 주입과 두개골에 부착한 자석을 이용하여 유발한 국소 경색에서 70-kDa stress protein(hsp70), βAPP, proliferating cell nuclear antigen (PCNA), glial fibrillary acidic protein(GFAP)의 발현을 관찰하여 경색의 시간에 따른 조직 변화와의 관계를 보고 또한 이런 단백이 반복 허혈손상에 의한 보호 작용과 관련이 있는지를 보기 위하여 시행하였다.

**방 법** : 국소 경색의 6시간, 1일, 2일, 3일, 7일 병변은 성숙 gerbil에서 측두부의 두개골 표면에 자석을 접착고정하고 산화철 입자의 생리식염수 현탁액을 대퇴정맥에 주입하여 유발한 국소 경색에서 관찰하였고, 8일, 10일, 14일 병변은 국소 허혈을 일으킨 후 1일, 3일, 7일이 지난 다음에 양측 경동맥을 동시에 결찰하여 5분간 혈류를 차단시킨 다음 7일 후에 검사를 시행한 gerbil에서 관찰하였다. H-E염색에 의한 조직 소견과 면역조직화학 염색법에 의한 각 단백의 발현을 평가하였다.

**결 과** : 국소 허혈을 일으킨 후 1일에 병변의 중심부에 있는 괴사된 신경세포와 경계영역에 있는 변성되는 신경세포는 hsp70과 βAPP에 중등도의 양성 반응을 보이기 시작했다. 경색의 중심부에서 2일에 나타나기 시작하여 3일에서 8일까지 많은 수가 관찰된 hsp70과 βAPP에 강한 양성 반응을 보이는 탐식세포는 10일에는 수가 감소하였다. PCNA 양성인 세포는 6시간에 보이기 시작하여 경색의 주변부에서 1~3일에 많이 증가하였고 탐식세포와 별세포도 양성 반응을 보였다. GFAP 양성인 별세포는 1일에 경색의 주변에서 보였고 시간이 지날수록 점점 증가하였다.

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**결 론** : 국소 경색 병변에서 hsp70과 βAPP, GFAP가 1일부터 7일까지 지속적으로 증가된 소견은 같은 연구방법에 의해 시행한 이전의 실험에서 1일과 3일에 관찰되었던 반복 허혈손상에 의한 보호 작용의 시간에 따른 변화와 일치하지 않으므로 이런 보호 작용에 다른 요소가 함께 관여하는 것을 알 수 있었다.

**중심 단어** : hsp70 · βAPP · PCNA · GFAP · Ischemic · Encephalopathy · Infarction.

## Introduction

The cerebrovascular diseases are clinically very important because of high incidence and prevalence of diseases as well as many complications. The hypoxic-ischemic injuries of central nervous system are induced in the experimental animals by transient global ischemia (TGI) with reperfusion or by transient or permanent occlusion of blood vessels. The TGI showed typical delayed neuronal damage in cornus ammonis (CA) 1 of the hippocampus and selective vulnerability involving different anatomical locations. The selective neuronal necrosis with time sequential changes and glial cell reaction proportional to neuronal damages constituted the main events in TGI. In contrast, the infarction is characterized by cell death in the center and transitional penumbra region between necrosis and surrounding normal tissue. There have been many efforts to understand mechanisms and factors of early events in these lesions to prevent eventual tissue damages[1-4].

The ischemic tolerance (IT) can be induced in the brain by subjecting the brain to a brief period of cerebral ischemia (preconditioning, PC) prior to subsequent ischemic challenge[4-12]. The IT has been confirmed in various animal models of forebrain ischemia as well as in the penumbral region in focal ischemia models. In addition to hypoxic or ischemic PC, the metabolic and physical stresses can also induce cross-tolerance to cerebral ischemia, although the protection by cross-tolerance is relatively weak. It has been elucidated that IT protects neurons and at the same time, it preserved brain function[8]. The mechanism of tolerance induction and factors of early events in these lesions to prevent eventual tissue damage has been extensively studied in experimental animals as well as in clinical conditions[5-9], but still is not fully understood. The brain may be protected from ischemia by using multiple mechanisms that are available for cellular survival. The cellular defence function against ischemia may be enhanced by the mechanisms inherent to neurons[5-7]. These cascades of events may strengthen the influence of survival factors or may inhibit apoptosis or cell death, which was demonstrated by triggering of many gene expression in neurons by a brief period of ischemia[13]. In addition, cellular stress response and synthesis of stress proteins may lead to an increased capacity for health maintenance inside the cell. The expression of various subfamilies of heat shock proteins and beta-amyloid precursor protein (βAPP) has been quite extensively studied in TGI and infarction as well as ischemic tolerance[14-26].

We have established a focal PC paradigm that produces significant IT of the brain to subsequent bilateral CCA occlusion for 5 minutes[5]. The PC using iron particle infusion into right femoral vein induces maximum protective effects of right hippocampal CA1 neurons at 1 and 3 days of reperfusion. The PC lesion induced by this method was a permanent occlusive focal infarction similar to embolic infarcts in clinical conditions.

This study was undertaken to elucidate the time sequential changes of 70-KD stress protein (hsp70) and βAPP in focal infarction as well as in combined focal infarction and subsequent TGI and to see the possible role of these factors in IT induced in this PC paradigm. In addition, the changes of two factors involving the repair of the ischemic encephalopathy, proliferating cell nuclear antigen (PCNA) and glial fibrillary acidic protein (GFAP), were observed.

## Material and Methods

### 1. Production of focal infarction and combined focal infarction and global ischemia

The Mongolian gerbils (*Meriones unguiculatus*) of both sexes weighing 50−70gm were used for the present study. Each gerbil was allowed free access to food and

water before and after surgery. The focal infarction was induced in 19 gerbils as described previously[2]. Under the ketamine (10mg/ml) anesthesia (intraperitoneal injection, 0.09ml/kg), incision of the scalp skin was made and a round magnetic of 3mm thickness and 7mm diameter (surface magnetic power of 2,000−2,400 Gauss) was attached on right parietal bone by the glue. The right femoral vein was exposed and the iron particles ($\gamma$-$Fe_2O_3$, 0.2×0.02 $\mu$m) mixed in saline solution were infused through the rubber catheter (0.67mg/100gm of body weight). Two to 5 gerbils were examined after 6h and 1, 2, 3, or 7 days (4, 4, 4, 5, and 2 gerbils, respectively). Ten gerbils with sham operated control were included and two gerbils were examined at each scheduled time. The rectal temperature of the gerbils was maintained at 36℃ using a heating pad and a heating lamp during the procedure.

For the combined focal infraction and global ischemia, the unilateral focal infarctions were induced as previousely described, which were followed by the subsequent global ischemia for 5 minutes after 1, 3, or 7 days. For the global ischemia, both common carotid arteries (CCAs) of each gerbil were exposed through a midline incision in the neck and were occluded using the miniature Mayfield aneurysm clips. For the evaluation of various postischemic periods, the clips were released after 5 minutes' ischemia and the restoration of carotid blood flow was visually verified and the skin incision was sutured. The histopathological examination was performed at 7 days after the global ischemia and the cases showing well defined tolerance of preconditioning ischemic lesion were selected for the immunostain from each time points (6, 5, and 4 gerbils, respectively).

### 2. Tissue preparation and immunohistochemical method

At the scheduled time, each gerbil was anesthesized and the brain was fixed with transcardiac infusion of 4% paraformaldehyde following perfusion with isotonic saline to remove blood from the cerebral vasculature. The brains were removed and fixed in the same solution for a further 24 hours. The coronal sections of the supratentorial portion of each brain were taken and embedded in paraffin.

The immunohistochemical reaction was accomplished using peroxidase-antiperoxidase method as described with some modification. Briefly, each deparaffinized 5-$\mu$m coronal section was reacted with primary antisera for 60min before reaction with the peroxidase-antiperoxidase complex by LSAB kit from DAKO (Santa Babara, CA, USA). The primary antisera used in this study were hsp70 (1 : 20 ; monoclonal ; StressGen, Victoria, Canada), $\beta$APP (1 : 10 ; monoclonal ; Zymed, San Francisco, USA), GFAP (1 : 100 ; monoclonal ; Dako, Glostrup, Denmark), and PCNA (PC10) (1 : 100 ; polyclonal ; Dako, Glostrup, Denmark). The peroxidase reaction was carried by incubation with link antibody and streptavidin for 20min, respectively, and subsequently with AEC (3-aminoethyl 9-carbasol). The sections were counterstained with Meyer's hematoxylin to visualize cell nuclei. For comparison, the adjacent sections were stained with H-E. A coronal section was incubated with nonimmunized serum from the same species used to raise each primary antiserum. No control section showed a positive reaction. The grading scale for immunoreactivity (IR) was as follows : −, none detectable ; 1+, weak positive ; 2+, moderate IR ; 3+, intense IR.

## Results

### 1. Immunohistochemical findings in sham operated control animals

The sham operated control gerbils showed hsp70 IR only in ependymal cells lining the ventricles and widely distributed neurons with 1+ $\beta$APP IR in cerebral cortex, basal ganglia and all fields of hippocampus. The IR products were granular and concentrated in periphery of neuronal perikarya. The control gerbils showed rare GFAP positive (2+) astrocytes in corpus callosum and subependymal area and rare PCNA postive (2+) cells in subependymal plate. The immunohistochemical findings in the control gerbils remained unchanged during all examined time.

### 2. Changes in focal infarction

The time sequential changes of single or multiple infarcts involving frontoparietal cortex, hippocampus, and basal ganglia were compatible with previously reported

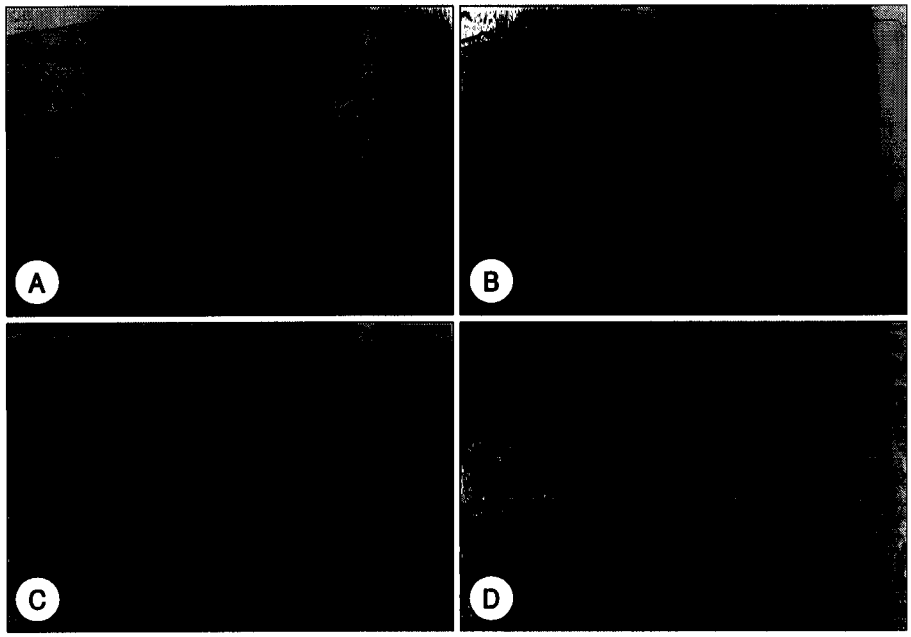


**Fig. 1.** A : Ghost neurons in the center of the infarct and degenerating neurons at penumbra region showed 2+ hsp70 IR at 1 day (peroxidase-antiperoxidase, ×33). B : The cortical neurons surrounding the infarct showed 2+ hsp70 IR at 1 day (×40). C : The macrophages in the center of the infarct showed 3+ hsp70 IR at 8 days (×100). D : Thick edematous neuronal process like structures in the necrotic center showed 3+ APP IR at 8 days (×66).

findings[2]. Fifteen out of 19 gerbils showed single or multiple infarcts on right hemisphere and four showed additional small infarcts involving left hemisphere. At 6h after infusion of iron particles, the infarcts appeared as a localized pale area and vacuolization of neuropil with eosinophilic neurons and at 1 day, the ghost neurons as well as vascular wall necrosis with hemorrhage were noted in the center of the infarcts. The macrophages started to show up at 2 days and increased with fragmentation of tissue and early cystic changes at 3 days. The macrophages were increased in numbers with relatively prominent blood vessels at 7 days.

At 6h, the hsp70 IR was slightly increased (1+) unilaterally on right hemisphere (neurons in cortex, striatum, and all subfields of hippocampus) in cases with infarcts on right hemisphere. Those four cases with bilateral infarcts showed additional focally increased IR on left side, which were dependent on size and location of the infarcts. The βAPP IR was similar with control at 6h.

The findings of hsp70 and βAPP IR were similar thereafter. At 1 day, the ghost neurons in the center of infarcts and degenerating neurons at the penumbra region showed 2+ IR (Fig. 1A). The cortical neurons surrounding the infarcts and the neurons in hippocampus and basal ganglia also showed postitive reaction (2+), which were continued up to 7 days (Fig. 1B). The macrophages started to show 2+ IR at 2 days and the positive reaction was increased at 3 and 7 days (3+).

The GFAP positive cells were noted in surrounding areas of infarcts, starting at 1 day and increased progressively up to 7 days (Fig. 2A). The PCNA positive cells were rarely noted at 6h and markedly increased in numbers in surrounding areas of infarcts at 1 to 3 days (Fig. 2D). At 3 days, the PCNA positive macrophages and astrocytes were also present in necrotic center and surrounding areas (Fig. 2E).

### 3. Changes in combined focal infarction and global ischemia

All gerbils showed single or multifocal infarcts involving right frontoparietal cortex, hippocampus, and basal ganglia and the infarcts were variable in size and

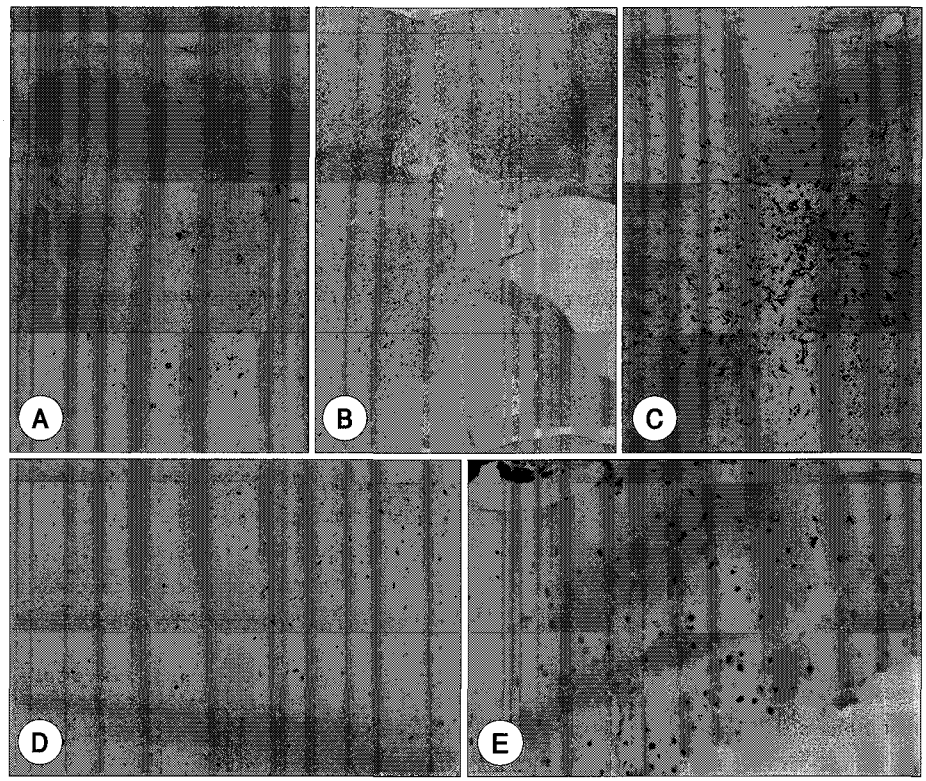


**Fig. 2.** A : The GFAP positive cells were noted in surrounding areas of infarct at 3 days(×40). B : The GFAP IR was strongly positive in surrounding areas of the infarct at 8 days(×13). C : The GFAP IR was extensive in glial scar tissue at 14 days(×40). D : Many PCNA positive cells were noted in surrounding areas of infarct at 2 days(×33). E : The PCNA positive macrophages and astrocytes were noted in necrotic center and surrounding areas(×66).

numbers. The infarcts showed time sequential changes according to the age of the infarcts(8, 10, and 14 days, respectively). The hippocampal neurons of right side showed clear protective effects compared to left side, which showed typical delayed neuronal damages.

The infarcts noted in gerbils with combined 1 day-focal infarct and global ischemia were filled with numerous macrophages and surrounded by glial scar tissue. The macrophages showed strong positive(3+) hsp70 and βAPP IR(Fig. 1C). In addition, thick edematous neuronal process like structures in the necrotic center showed 3+ hsp70 and βAPP IR(Fig. 1D). The reactive astrocytes in surrounding area of infarction as well as the cortical and striatal neurons showed 2+ hsp70 and βAPP IR. The PCNA positive macrophages were occasionally noted. The GFAP IR was strongly positive in surrounding areas of the infarcts(Fig. 2B).

The gerbils with combined 3 day-focal infarct and global ischemia showed decreased numbers of macrophages with hsp70 and βAPP IR and increased GFAP IR in glial scar tissue. The PCNA positive macrophages were occasionally noted in the necrotic area. The hippocampus showed 2+ βAPP IR in reactive astrocytes. The small round cells with PCNA positive nuclei were occasionally noted in the hippocampus.

The infarcts noted in the gerbils with combined 7 day-focal infarct and global ischemia showed considerably less numbers of macrophages and increased glial scar tissue. The hsp70 and βAPP IR was decreased in the macrophages and astrocytes. The neurons in cortex, striatum, thalamus, and hippocampus as well as surviving and degenerating hippocampal neurons showed 1+ βAPP IR, which were similar to sham-operated control gerbils. The GFAP reaction was extensive in glial scar tissue

(Fig. 2C). The PCNA positive cells were scattered in hippocampus.

## Discussion

Since the definition of the IT was the tolerance to subsequent severe ischemic challenge after a brief period of PC ischemia or various sublethal injuries to the brain, most of the experiments was performed under the similar conditions[6-9]. In comparison, the PC lesion in this study induced multifocal ischemic infarction due to iron particle emboli in blood vessels, which showed pan-necrosis of tissue. The histochemical findings in this study showed typical time sequential changes observed in early and subacute stage of infarction : the changes (red neurons, palor of neuropil, and leukocytes margination at blood vessels) were already noted at 6h after the infusion of iron particle and the cellular necrosis was prominent at 3 and 7 days. The necrotic areas were filled with numerous macrophages (Gitter cells), which were decreased in number after 10 days. The changes of infarction at late stage (8, 10, and 14 days) in this study were the results of the combined focal infarction and subsequent global ischemia induced by 5 minutes occlusion of both CCAs. The microscopic findings in the focal infarction areas were similar to the findings observed in the previous study on late stage of the focal infarction[2]. Only noted difference in this study was the protection of the neurons in right side of the hippocampus compared with the neurons in left side of the hippocampus.

The expression of hsp70 and βAPP in hypoxic-ischemic conditions in experimental animals depends on the type (global or focal ischemia) and duration (severity) of hypoxic-ischemic insults. The induction of hsp has been implicated as acute response in hypoxic-ischemic injury. The highly inducible member of 70-kDa family of hsps, hsp70, has been associated with IT in several organs including brain and spinal cord. The sublethal hypoxic-ischemic injury used as PC (10min right MCA occlusion) in one previous study showed hsp70 IR in neurons of the PC ischemic area during 12h to 4 days[14]. They also showed hsp70 positive astrocytes at 6 and 12h. The previous study with aortic balloon occlusion model in rats[15] showed a modest increase in hsp72 protein IR in dorsal horns at 12h reperfusion after 3min of ischemia and a more rubust and wide spread hsp72 protein expression in both dorsal and ventral horn neurons and peak of the expression at 24h reperfusion after 6min of ischemia. In contrast, after 10min of ischemia and 24h of reflow, a significant increase in spinal neuronal hsp72 expression was seen in perinecrotic regions.

The βAPP is a widely expressed transmembrane protein, of which proposed functions include stabilization of neuronal calcium fluxes, inhibition of clotting cascade and cell-cell or cell-matrix adhesion. The upregulartion of βAPP has been observed in neurons or glial cells after various brain damages, including focal ischemic lesion, ischemia-reperfusion brain injury, and neonatal hypoxic-ischemic brain injury[18-26]. Shi et al[21] showed that brain hypoperfusion enhances APP mRNA expression to 208% and 152% in the penumbra and core ischemic regions, respectively, on the fourth day after MCA occlusion and remained high through seventh day of ischemia. In other report, the βAPP expression was increased by 2h post-injury, peaked, fourfold above control levels, at 24h and gradually declined over the following 4 days in 14-day-old rat pups subjected to unilateral hypoxic-ischemic brain injury[25]. They also showed increased predominantly neuronal expression of βAPP in the human infants within 24h of injury and greatest reaction in those infants dying within 3 days. The TGI in gerbil showed markedly increased APP-like IR in neuronal cell bodies in the subiculum and CA3 of hippocampus, and layers III and V/VI of the cerebral cortex[22].

The positive HSP and βAPP IR in necrotic neurons in center of the infarcts and degenerating neurons in penumbra region at 1 day in this study was compatible with acute response of brain to ischemic injury and similar to previous studies. The most striking finding in this study was the strong HSP and βAPP IR of macrophages in necrotic area of infarction, which was increased in numbers up to 8 days after ferrite infusion and decreased thereafter. The mononuclear phagocytic system in central nervous system including microglia and macrophages have been extensively studied in pathogenesis of various diseases including cerebral ischemia[4][12]. The significance of strong positive IR in this study is not clear, but suggests the possible relationship between progression of ischemic

cellular necrosis and macrophages including these factors.

The thick edematous neuronal process like structures noted in the necrotic center at 8 days after ferrite infusion was similar to APP-positive axonal swelling, dystrophic neurites, and myelinated fiber tracts described in previous studies[18-20]. Those structures were noted in rats subjected to focal cerebral ischemia by permanent occlusion of the MCA at 4 and 7 days post-occlusion along the periphery of the infarct[18]. The dystrophic axons and neurons with accumulated APP were also evident in the ipsilateral neocortex and hippocampus in the rats with repeated reversible occlusions of one MCA as well as in rats subjected to partial forebrain ischemia by occlusion of bilateral CCAs[19]. Yam et al.[20] described increased APP IR in subcortical white matter and myelinated fiber tracts at the margin of ischemic zone at 24h after permanent MCA occlusion in the rat and proposed APP as a sensitive marker of axonal injury. The markedly thickened appearance of these structures in this study with strong βAPP and hsp70 IR suggests similar disruption of axonal transport of these material. Similar structures were not noted in peri-necrotic areas in this study, which could be due to the difference of experimental paradigm and/or examination method.

The presence of diffusely scattered PCNA positive cells in peri-infarct area and PCNA positive macrophages in necrotic area at 1 to 3 days was consistent with temporary vigorous reactive changes of cells in ischemic lesion of the brain. The GFAP positive astrocytes were scattered in surrounding area of infarct at 1 day and progressively increased thereafter. The positive reaction was proportional to the size of infarct or severity of DND noted in hippocampus.

The IT of hippocampal CA1 neurons noted in this PC paradigm was most remarkable at 1 and 3 days in our previous study[5]. Since the neurons in surrounding cortex of infarction, hippocampus, and basal ganglia as well as macrophages in necrotic center showed increased hsp70 and βAPP IR at 1 day through 7 days, these factors could not fully account for the mechanisms of such protective effect. The most prominent positive reaction of PCNA at 1 to 3 days was compatible with proliferative activity of the cells, and further studies are necessary to figure the significance of this finding.

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