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Research Article

SIRT2 - JAK1 Interaction Decreases IL-6 Induced Inflammatory Response in Cancer Cells

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Abstract

Interleukin-6 (IL-6) is a pro-inflammatory cytokine critical in immunoregulation. Aberrant IL-6 signaling has been implicated in various diseases including inflammation-associated cancer. Sirtuin-2 (SIRT2) is a cytoplasmic deacetylase and is generally considered a tumor suppressor. SIRT2 gene removed mouse develops tumors in many organs, primarily in the liver and breast tissues as the mouse ages. The purpose of the study was to investigate the roles of SIRT2 on IL-6 induced inflammatory response in mouse embryonic fibroblasts (MEFs) with SIRT2 gene removed and HeLa cervical cancer cell lines that overexpress SIRT2 gene. Immunoprecipitation and immunoblotting techniques were utilized in the study. SIRT2 interacts with JAK1, which is one of the downstream proteins of IL-6. We also found that overexpression of SIRT2 in HeLa cervical cancer cells decreases IL-6 induced inflammatory response by decreasing the activity of JAK1. These results suggest that SIRT2 has a protective role against chronic inflammatory diseases including inflammation-associated cancer.

Keywords: *Sirtuin, Inflammation, STAT3, Cervical cancer, HeLa cells, Deacetylation*

SIRT2 - JAK1 Etkileşimi Kanser Hücrelerindeki IL-6'in Neden Olduğu İnflamatuar Tepkiyi Azaltır

Özet

İnterlökin-6 (IL-6), bağışıklık sisteminin düzenlenmesinde kritik bir pro-inflamatuar sitokindir. Anormal IL-6 sinyalinin, inflamatuara bağlı kanser de dahil olmak üzere çeşitli hastalıklarda rol oynadığı gösterilmiştir. Sirtuin-2 (SIRT2), sitoplazmik bir diasetilaz olup, genellikle tümör baskılayıcı olarak kabul edilir. SIRT2 geni çıkarılmış fare yaşlandıkça, başlica karaciğer ve meme dokusunda, ve pek çok organında tümörler gelişir. Bu çalışmanın amacı, SIRT2 geni çıkarılmış embriyonik fare fibroblastlarda (MEF'ler) ve SIRT2 genini aşırı eksprese eden HeLa servikal kanser hücrelerinde SIRT2'nin, IL-6 tarafından tetiklenen inflamatuar yanıtı üzerindeki rollerini araştırmaktı. Bu çalışmada immunolojik çökeltme ve immüno-inkube teknikleri kullanılmıştır. SIRT2, IL-6'nın aşağı tarafındaki takım proteinlerinden biri olan JAK1 ile etkileşime girer. HeLa servikal kanser hücrelerinde, SIRT2'nin aşırı ekspresyonu, JAK1'in aktivitesini azaltarak, IL-6'in neden olduğu inflamatuar yanıtı azalır. Bu sonuçlar, SIRT2 proteinin, kronik inflamatuar hastalıklara ve inflamatuara-bağlı kansere karşı koruyucu bir rol oynadığını önermektedir.

Anahtar sözcükler: *Sirtuin, İnflamasyon, STAT3, serviks kanseri, HeLa hücreleri, Diasetilasyon*

INTRODUCTION

Interleukin 6 (IL-6) is an immune response protein which is secreted primarily by T-cells and macrophages to fight with infection during trauma, burns, and other tissue damages. IL-6 is an important mediator for setting the body temperature up and acute-phase response [1]. IL-6 interacts with IL-6 receptor, and binding of IL-6 to its receptor triggers the interaction between IL-6 receptor and the signal-transducing membrane protein gp130. This dimerization induces the activation of the tyrosine kinases of the Janus family (JAK1, JAK2, and Tyk2). Activated JAK1 phosphorylates the transcription factor Signal Transducer and Activator of Transcription 3 (STAT3) proteins at tyrosine 705 and triggers their homodimerization. Dimerized STAT3 complexes translocate into the nucleus where they bind enhancer elements of IL-6 inducible genes, such as c-Myc, which plays many cellular processes [2,3]. IL-6 activates the inflammatory process in many diseases, such as diabetes, obesity, cancer, and atherosclerosis [4-6]. In the blood of metastatic cancer patients at advanced stages, higher levels of IL-6 were detected, suggesting that IL-6 enhances

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tumor cell survival [7,8]. Therefore, anti-IL-6 agents have been sought as therapeutics for many cancer types.

Sirtuin (SIRT) deacetylase enzymes play important roles in the regulation of stress response, metabolism, aging, gene silencing, and cancer. SIRT2 is a cytoplasmic member of SIRT family. SIRT2 gene removed mouse does not display any major phenotype; however, as mice age, they display increased incidence of tumor formation especially in the liver and breast tissues; and at a lesser extent in the lung, pancreas, stomach, intestine and other organs [9]. Previous studies supports the idea of SIRT2 enzyme may have some anti-aging, anti-tumorigenic and anti-inflammatory functions [10-13]. SIRT2 has been shown to deacetylate numerous substrates and the mechanisms how SIRT2 decreases inflammation and inflammation-associated disorders has been poorly understood up to date. Here, the role of SIRT2 on the regulation of immune response was investigated.

MATERIAL and METHODS

Cell Lines

SIRT2 wild type (WT) and knockout (KO) MEF cells were maintained in a 37°C incubator with 5% CO_2 and 6% oxygen in Dulbecco's Modified Eagle's medium (DMEM) containing 15% fetal bovine serum (FBS) as described previously [9,14]. HeLa cells were maintained in 37°C incubator with 5% CO_2 and 21% oxygen in DMEM containing 10% FBS.

Immunoprecipitation (IP) of SIRT2 and JAK1

HeLa cells were lysed with IP buffer (10 mM HEPES, pH 7.9, 180 mM KCl, 1.5 mM $MgCl_2$, 0.1% NP-40, 1 mM EDTA, 0.1 mM PMSF), including protease and phosphatase inhibitors. Total cell extracts were incubated with anti-SIRT2 (Sigma, St. Louis, MO) or anti-JAK1 (BD Biosciences, San Jose, CA) antibody for 16 h at 4°C, followed by the incubation with magnetic beads (Dynabeads; Life Technologies Carlsbad, CA) for 2 h. After washing three times with IP buffer, bound proteins were extracted by boiling the samples in the loading buffer for 5 min, and isolated proteins were resolved via 10% SDS PAGE.

Western Blotting

Western blotting on nitrocellulose membrane was performed using iBlot transfer system (Life Technologies). PVDF membranes were incubated with anti-STAT3 (Cell Signaling, Danvers, MA), anti-P-STAT3 (Cell Signaling), anti-SIRT2 (Sigma), anti-a-tubulin (Cell Signaling) primary antibodies for 16 h at 4°C.

Immunofluorescence

Cells seeded on glass coverslips were fixed in 4% paraformaldehyde in PBS and then blocked with 1% BSA in PBS. Cells were incubated with anti-JAK1 (Cell Signaling) or anti-SIRT2 (Sigma) antibodies in PBS followed by incubation with goat-rabbit IgG conjugated with Alexa Fluor 488 and 594 secondary antibodies (Life Technologies) in PBS. Cells were washed in PBS, stained with the nuclear marker DAPI, mounted, and imaged on a fluorescence microscope.

RESULTS

SIRT2 Interacts with JAK1 Protein

To determine if there is a physical interaction between SIRT2 and JAK1 proteins, immunoprecipitation (IP) technique with anti-JAK1 and anti-SIRT2 antibodies along with magnetic beads were used. After IP of SIRT2, samples were subsequently immunoblotted with anti-JAK1 antibody *(Fig. 1)*. Presence of JAK1 protein in the immunoprecipitated SIRT2 protein complexes showed a physical interaction between SIRT2 and JAK1. In addition, immunofluorescence was used to determine whether these two proteins colocalized in cancer cells. Immunofluorescence staining showed that these proteins colocalized mainly in the cytoplasm *(Fig. 2)*.

Removal of SIRT2 Protein in Mouse Embryonic Fibroblast Cells Increases the Activity of JAK1 in Response to IL-6

JAK1 phosphorylates the transcription factor STAT3 at

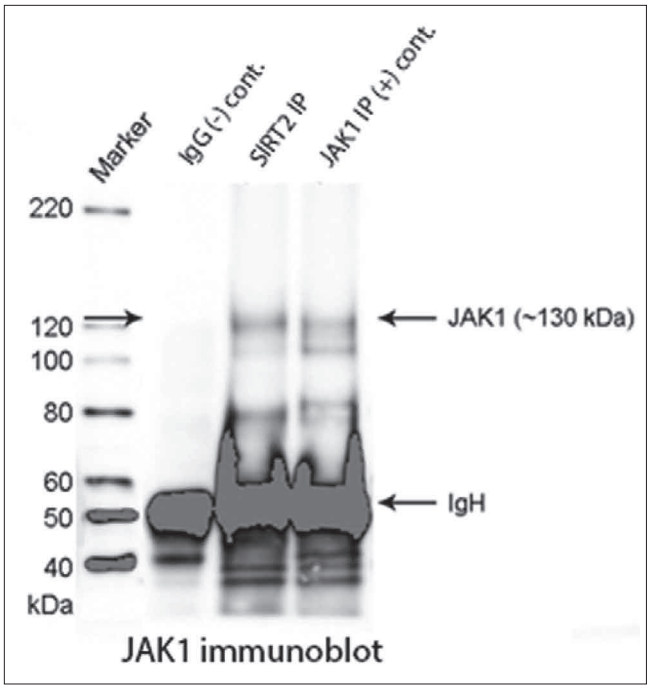

Fig 1. SIRT2 physically interacts with JAK1. Cell extracts were immunoprecipitated with anti-SIRT2, anti-JAK1, and IgG antibodies, and subsequently immunoblotted with anti-JAK1 antibody. IgG was used as a negative control and JAK1 immunoprecipitation was used as a positive control

Şekil 1. SIRT2 ile JAK1 fiziksel etkileşim gösterir. Hücre ekstreleri anti-SIRT2, anti-JAK1, ve IgG antikorları ile çökeltilir, ve daha sonra, anti-JAK1 antikoru ile immun-inkübe edilmiştir. IgG, negatif kontrol ve JAK1 immüno-çökeltme pozitif kontrol olarak kullanıldı

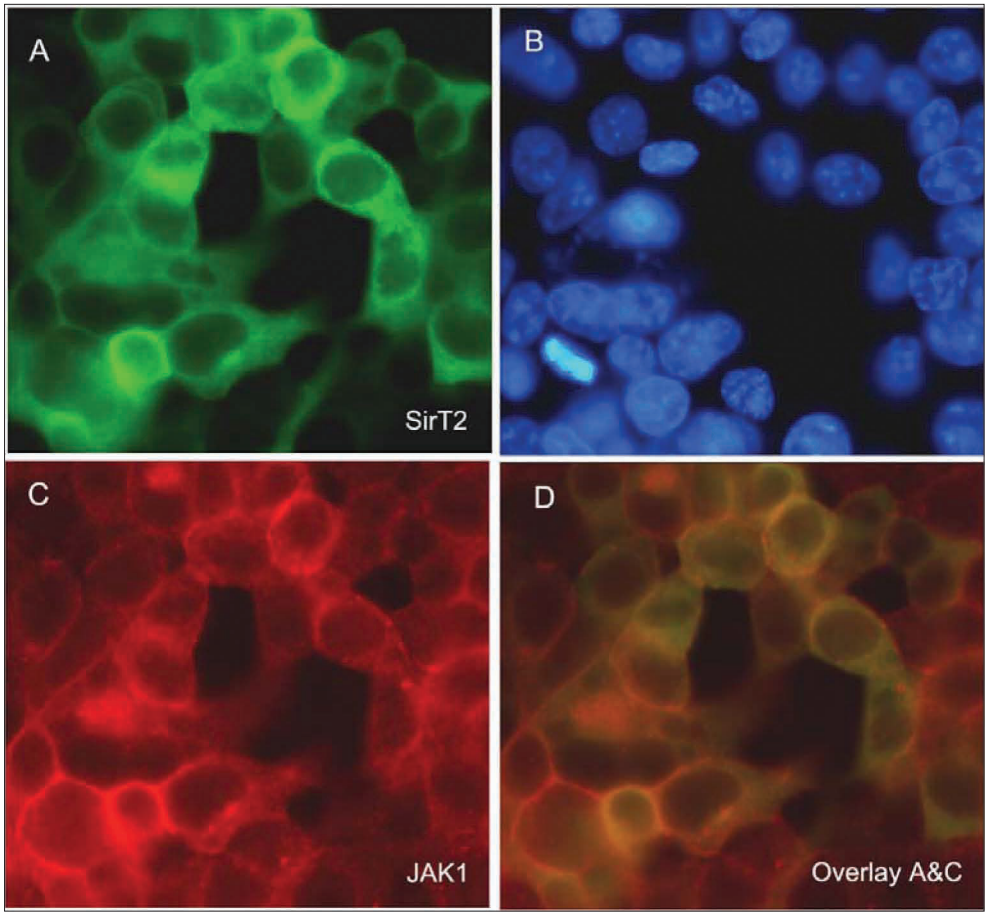

Fig 2. SIRT2 and JAK1 are colocalized in the cytoplasm. HeLa cervical cancer cells were stained with (A) anti-SIRT2 *(green staining)* (B) DAPI nuclear marker *(purple staining)* or (C) anti-JAK1 *(red staining)*, and (D) anti-SIRT2 and anti-JAK1 staining were subsequently merged

Şekil 2. SIRT2 ile JAK1 sitoplazmada birlikte lokalize halde bulunmaktadır. HeLa servikal kanser hücreleri (A) anti-SIRT2 ile boyandı *(yeşil boyama),* (B) DAPI nükleer işaretleyici *(mor boyama),* veya (C) anti-JAK1 *(kırmızı boyama),* ve (D) anti-SIRT2 ve anti-JAK1 boyama sonradan birleştirildi

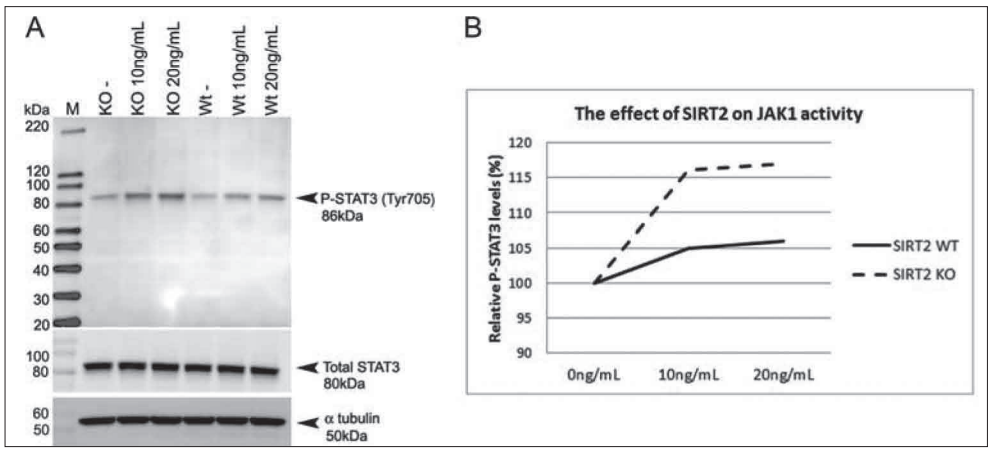

Fig 3. Removal of SIRT2 protein increases the activity of JAK1. (A) WT and SIRT2 KO MEFs were serum starved for 24 h. IL-6 was added into the cell culture media at 10 and 20 ng/mL concentrations and incubated for 30 min. Immediately after IL-6 exposure, cells were harvested, and lysed using cell lysis buffer including 1% NP-40, protease and phosphatase inhibitors. Proteins were resolved using 10% SDS PAGE, and immunoblotted with anti-P-STAT3, total anti-STAT3, and anti-a-tubulin. Images are representative of three independent experiments. (B) The intensity of protein bands in the representative western blot in *Fig. 3A* was quantified using the ImageJ software

Şekil 3. SIRT2 proteininin uzaklastirilmasi JAK1 aktivitesini artırır. (A) WT ve SIRT2 KO MEF'ler 24 saat boyunca serumdan mahrum bırakıldı. IL-6, 10 ve 20 ng/ml konsantrasyonlarda, hücre kültür ortamına ilave edildi ve 30 dakika süre ile inkübe edildi. Hemen IL-6'le maruz kaldıktan sonra, hücreler toplandi ve %1 NP-40, proteaz ve fosfataz inhibitörleri de dahil olmak üzere hücre liziz tamponu kullanılarak lize edildi. Proteinler %10 SDS PAGE kullanılarak çözüldü ve anti-p-STAT3, toplam anti-STAT3 ve anti-a-tubulin ile immun-inkübe edilmistir. Temsili western fotoğraflari, üç bağımsız deneyden elde edilmiştir. (B) *Şekil 3A*'daki temsili western blottaki protein bantlarının yoğunluğu ImageJ programı kullanılarak hesaplanmıştır

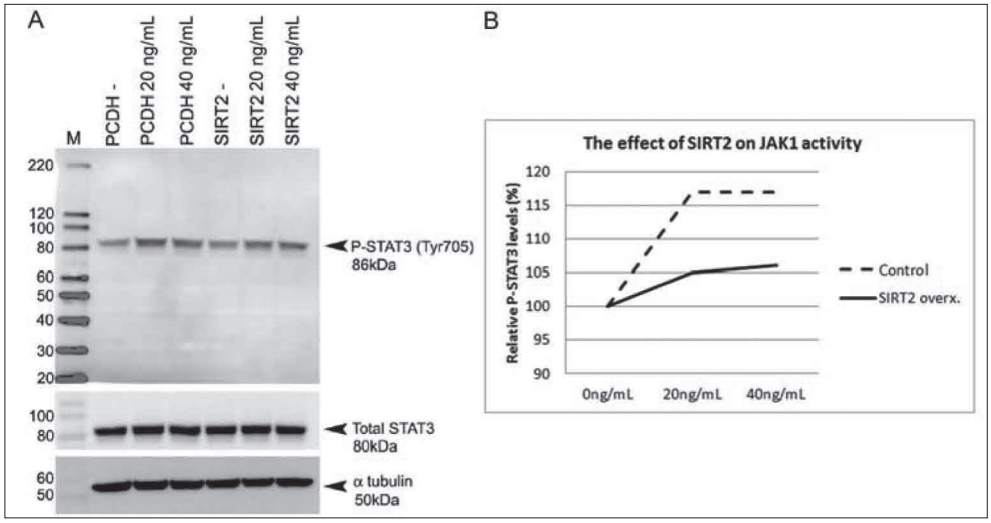

Fig 4. Overexpression of SIRT2 in HeLa cells decreases the IL-6 mediated STAT3 phosphorylation. (A) SIRT2 and control vector (PCDH) overexpressing stable HeLa cells were serum starved for 24 h. IL-6 were added into the cell culture media at 20 and 40 ng/mL concentrations and incubated for 30 min. Cells were harvested, and lysed using lysis buffer. Proteins were resolved using 10% SDS PAGE, and immunoblotted with P-STAT3, total STAT3, and a-tubulin. Images are representative of three independent experiments. (B) The intensity of protein bands in the representative western blot in *Fig. 4A* was quantified using the ImageJ software

Şekil 4. SIRT2 proteininin aşırı eksprese edilmesi JAK1 aktivitesini azaltir. (A) SIRT2 ve kontrol vektörü (PCDH) 24 saat boyunca serumdan mahrum bırakıldı. IL-6, 20 ve 40 ng/ml konsantrasyonlarda, hücre kültür ortamına ilave edildi ve 30 dakika inkübe edildi. Hücreler toplandi ve hücre liziz tamponu kullanılarak lize edildi. Proteinler %10 SDS PAGE kullanılarak çözüldü ve anti-p-STAT3, toplam anti-STAT3 ve anti-a-tubulin ile immun-inkübe edildi. Temsili western fotoğraflari, üç bağımsız deneyden elde edilmiştir. (B) *Şekil 4A*'daki temsili western blottaki protein bantlarının yoğunluğu ImageJ programı kullanılarak hesaplanmıştır

tyrosine 705. Phosphorylation of STAT3 induces formation of STAT3 dimers and leads to translocation to the nucleus. We investigated if the removal of SIRT2 protein changes the activity of JAK1 by measuring the phosphorylation of STAT3 at tyrosine 705 *(Fig. 3)*. In SIRT2 KO MEFs, phosphorylation of STAT3 was higher than that of in WT MEFs in response to IL-6, indicating SIRT2 decreased the kinase activity of JAK1 *(Fig. 3A, 3B)*.

Overexpression of SIRT2 in HeLa Cells Decreases the IL-6 Mediated STAT3 Phosphorylation

Next, we investigated if the overexpression of SIRT2 decreases JAK1 activity, and consequently reduces the phosphorylation of STAT3 in HeLa cervical cancer cell line. Consistent with the findings in SIRT2 WT and KO MEFs, when SIRT2 was overexpressed, phosphorylation of STAT3 at tyrosine 705 decreased *(Fig. 4A, 4B)*.

In summary, in this study, we showed that SIRT2 enzyme interacted with JAK1 protein. IL-6 mediated inflammation response was higher in SIRT2 gene removed mouse embryonic fibroblasts (MEFs). Moreover, when SIRT2 was overexpressed in cervical cancer cell line (HeLa), it decreased the activity of JAK1 and the phosphorylation of STAT3.

DISCUSSION

IL-6 has been associated with cell proliferation, immunomodulation, inflammation, and tumorigenesis. Activation of IL-6 signaling has been associated with advanced stages of cancer progression, such as in multiple myeloma, non-small cell lung carcinoma, colorectal cancer, renal cell carcinoma, prostate cancer, breast cancer, and cervical cancer. IL-6 which is a growth signal, inhibits apoptosis and enhances angiogenesis [16,17]. Chronic inflammation may lead to cancer development [5]. Inhibition of the activity of IL-6 may be therapeutically beneficial against chronic inflammation and cancer formation [18,19].

SIRTs have been associated with aging and longevity [20]. SIRTs are class III histone deacetylases and ADP-ribosyltransferases. These enzymes have a variety of substrates within the cells and are not restricted only to histones. In mammals, there are 7 SIRTs which are located in different compartments of the cells [21]. SIRT2 removed mouse model suggests that these proteins have various anti-aging, anti carcinogenic, and immune regulatory functions. Since these enzymes include numerous substrates, the anti tumorigenic effects of SIRTs are complex to elucidate. According to our results, JAK1 is one of the substrates of SIRT2. SIRT2 is a mainly cytoplasmic protein; however, in the G2/M transition and during mitosis, it translocates into the nucleus [15]. Like SIRT2, JAK1 is a mainly cytoplasmic protein and localized in the cytoplasmic side of plasma membrane. Consistently, our results also indicated that these proteins colocalized mainly in the cytoplasm.

Deacetylation of JAK1 by SIRT2 may be one of the contributors for the anti-tumorigenic effects of this SIRT protein. SIRT2 require NAD^+ as a co-factor, and its dependence on NAD^+ may provide a molecular link between nutrient/energy availability and the regulation of immune response. Likewise, inflammation is highly associated with aging and metabolism. Metabolic disorders, such as obesity, or aging may be functionally linked to the immunomodulatory roles of SIRT2 [22].

It has been reported that SIRT2 suppresses inflammatory response in arthritis autoimmune disease [12]. SIRT2 deacetylates nuclear factor-kappa B (NF-kB); and accordingly, the expression of NF-kB dependent genes including IL-6 decreases [12]. Consistent to this report, in this study, we found that SIRT2 interacts with JAK1 protein and decreases IL-6 mediated JAK1/STAT3 pathway; consequently, SIRT2 has a protection function against chronic inflammatory diseases and inflammation-associated carcinogenesis.

To conclude, our results indicate that SIRT2 interacts and decreases the activity of JAK1 kinase; and ultimately, it decreases the activity of IL-6 induced JAK/STAT3 signaling cascade, suggesting that SIRT2 have an immune response regulatory function through IL-6 in cancer cells. The activity of SIRT2 can be enhanced by calorie restriction and various chemicals, such as resveratrol [20]. Resveratrol has been reported to have beneficial effects for anti-cancer, anti-viral, anti-aging, and neuroprotective. Calorie restriction, resveratrol or other small molecules to increase the activity of SIRT2 may be beneficial to regulation of the immune response, and consequently, to prevent against chronic inflammatory diseases and inflammation-associated carcinogenesis. A sirtuin-based anti-inflammatory therapy should be further investigated with respect to both clinical and veterinary standpoints.

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