*Full Paper***Oral Administration of Curcumin Suppresses Production of Matrix Metalloproteinase (MMP)-1 and MMP-3 to Ameliorate Collagen-Induced Arthritis: Inhibition of the PKC δ /JNK/c-Jun Pathway**

Se Hwan Mun¹, Hyuk Soon Kim¹, Jie Wan Kim¹, Na Young Ko¹, Do Kyun Kim¹, Beob Yi Lee¹, Bokyung Kim¹, Hyung Sik Won², Hwa-Sup Shin², Jeung-Whan Han³, Hoi Young Lee⁴, Young Mi Kim⁵, and Wahn Soo Choi^{1,*}

¹College of Medicine and ²College of Biomedical & Health Science, Konkuk University, Chungju 380-701, Korea

³College of Pharmacy, Sungkyunkwan University, Suwon 440-746, Korea

⁴College of Medicine, Konyang University, Daejeon 302-718, Korea

⁵College of Pharmacy, Duksung Women's University, Seoul 132-714, Korea

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Abstract. We investigated whether oral administration of curcumin suppressed type II collagen-induced arthritis (CIA) in mice and its effect and mechanism on matrix metalloproteinase (MMP)-1 and MMP-3 production in CIA mice, RA fibroblast-like synoviocytes (FLS), and chondrocytes. CIA in mice was suppressed by oral administration of curcumin in a dose-dependent manner. Macroscopic observations were confirmed by histological examinations. Histological changes including infiltration of immune cells, synovial hyperplasia, cartilage destruction, and bone erosion in the hind paw sections were extensively suppressed by curcumin. The histological scores were consistent with clinical arthritis indexes. Production of MMP-1 and MMP-3 were inhibited by curcumin in CIA hind paw sections and tumor necrosis factor (TNF)- α -stimulated FLS and chondrocytes in a dose-dependent manner. As for the mechanism, curcumin inhibited activating phosphorylation of protein kinase C δ (PKC δ) in CIA, FLS, and chondrocytes. Curcumin also suppressed the JNK and c-Jun activation in those cells. This study suggests that the suppression of MMP-1 and MMP-3 production by curcumin in CIA is mediated through the inhibition of PKC δ and the JNK/c-Jun signaling pathway.

Keywords: curcumin, matrix metalloproteinase (MMP)-1, MMP-3, protein kinase C δ (PKC δ), collagen-induced arthritis

Introduction

Accumulating evidence suggests that curcumin (Fig. 1A), which is a yellowish component of dietary curry, is potentially valuable for the treatment of some maladies, including cancer, diabetes, cardiovascular diseases, and Alzheimer's disease (1, 2). Curcumin is currently in human clinical trials for psoriasis, Alzheimer's disease, and several different cancers (2). Although it was recently reported that the parenteral administration of turmeric extract suppresses

streptococcal cell wall-induced arthritis in rats (3), the effect of orally administered curcumin on type II collagen-induced arthritis (CIA) in mice and its mechanism of action on the production of matrix metalloproteinase (MMP)-1 and MMP-3 remain to be further investigated.

Fibroblast-like synoviocytes (FLS) have a pivotal role in the induction of Rheumatoid arthritis (RA) (4, 5). FLS secrete collagenases, stromelysin, and various cytokines including interleukin (IL)-6, IL-8 (6), and IL-32 (7, 8) to mediate matrix degradation and inflammation in RA. Among them, tumor necrosis factor (TNF)- α and IL-1 β play critical roles in these events (9). These cytokines in the synovium are also potent stimulators for the de novo production of MMPs, which are responsible for cartilage

*Corresponding author. wahnchoi@kku.ac.kr

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matrix degradation (9, 10). Therefore, a promising avenue of RA research is the elucidation of specific signaling molecules and development of their inhibitors to modulate the production of MMP-1, MMP-3, and other inflammatory cytokines in RA. Transcription factors, activator protein-1 (AP-1) and nuclear factor- κ B (NF- κ B), are critical for production of MMPs and inflammatory cytokines in FLS and chondrocytes (11–13). Recently, it was reported that turmeric extract inhibits NF- κ B and NF- κ B-mediated genes in the joints (14), curcumin inhibits NF- κ B in chondrocytes (3), and curcumin inhibits AP-1 and NF- κ B in other types of cells (15–17). However, the effects of curcumin on the upstream signaling pathway to regulate AP-1 and NF- κ B remain to be studied.

In this study, we report for the first time that oral administration of curcumin significantly suppresses CIA and production of MMP-1 and MMP-3, most likely by inhibiting protein kinase C δ (PKC δ) and JNK/c-Jun in CIA mice, FLS, and chondrocytes.

Materials and Methods

Reagents

Curcumin was purchased from Sigma-Aldrich (St. Louis, MO, USA); indomethacin and rottlerin, from Calbiochem (La Jolla, CA, USA); recombinant human TNF- α , streptavidin-horseradish peroxidase (HRP), and TMB enzyme substrate, from Biosource (Camarillo, CA, USA); antibodies against phosphorylated forms of PKC α /II (Thr638/641), PKC δ (Ser643), and PKC ζ /I (Thr410/403), from Cell Signaling Technology (Beverly, MA, USA); antibodies against MMP-1 and MMP-3, from R&D Systems (Minneapolis, MN, USA); goat anti-rabbit HRP-conjugated antibody, from Santa Cruz Biotechnology (Santa Cruz, CA, USA); bovine type II collagen and Freund's complete adjuvant, from Chondrex (Redmond, WA, USA). Cell culture media and other culture reagents were from Gibco RBL (Gland Island, NY, USA).

Induction of collagen-induced arthritis in mice

DBA/1J mice (male, 5–6-week-old, 10 mice per group) (Japan Charles River Breeding Laboratories, Kanagawa) were housed at the specific pathogen-free housing facility of Konkuk University (Chungju, Korea). After a 1-week acclimation period, the animal study was done in accordance with the institutional guidelines. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Konkuk University. Mice were immunized intradermally at the tail with 100 μ g of type II collagen emulsified with an equal volume of Freund's complete adjuvant and were intra-

peritoneally boosted with type II collagen (100 μ g in 0.05 M acetic acid) 23 days after the initial immunization (17). Curcumin (4–100 mg/kg), indomethacin (1 mg/kg), or vehicle (5% Arabic gum) was orally administered once a day from day 23 after the first immunization with collagen. Non-immunized mice were used as a normal control.

Estimation of clinical arthritis indexes in CIA

The clinical severity of arthritis in all four paws of mice was evaluated in a triple-blind fashion by a previous published scoring system (18). Briefly, 0 = normal; 1 = mild, apparent swelling limited to individual digits; 2 = moderate, redness, and swelling of the ankle; 3 = redness and swelling of the paw including digits; and 4 = maximally inflamed limb with involvement of multiple joints. The arthritis score for each mouse was the sum of four paws, with the highest score of 16 for each mouse.

Histological analysis

The mice were euthanized on day 38 after the first immunization with collagen. The right hind paws were fixed in 4% paraformaldehyde for 3 days, decalcified in 10% EDTA for 30 days at 4°C, dehydrated in a graded ethanol series (70%–100%), washed with xylene twice for 2 h each, and then finally embedded in paraffin. Serial 5- μ m-thick paraffin sections were stained with hematoxylin and eosin (H&E). Histopathological changes in joints were scored using the parameters described in a previous report (19). Three pathologists who were kept unaware of the source of the tissues independently evaluated each section on a 3-point scale: 0 = normal, 1 = infiltration of inflammatory cells, 2 = synovial hyperplasia and pannus formation, and 3 = bone erosion and destruction.

Immunohistochemistry

The above-described paraffin-embedded sections were deparaffinized with xylene and ethanol. After hydrating them in PBS, endogenous peroxidase was depleted with 0.3% H₂O₂ for 10 min. The sections were blocked with 10% normal horse serum for 1 h and then incubated overnight at 4°C with the specific antibodies or isotype controls. After washing with PBS three times, they were incubated with biotinylated secondary antibody, and the signal was amplified with HRP-conjugated streptavidin using a Vectastain Elite ABC kit (Vector, Burlingame, CA, USA). All sections were visualized with diaminobenzidine (DAB) and counterstained with methyl green.

Isolation of fibroblast-like synoviocytes

Informed consent was obtained from all patients, and

the experimental protocol was approved by the Asan Medical Center Institutional Review Board. FLS were isolated from the synovial tissues obtained from RA patients according to the previously described protocol with minor modifications (8, 20). Briefly, synovial tissues were washed thoroughly with RPMI 1640, minced, and digested for 90 min at 37°C in RPMI 1640 containing 1 mg/ml of collagenase. The digested tissue was filtered with a 70- μ m cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA) and the resulting cell suspension was centrifuged at $250 \times g$ for 10 min. The cell pellets were suspended in RPMI 1640, washed 3 times with the same media, and cultured in α -minimum essential medium (MEM) containing 10% fetal bovine serum.

Cell stimulation and immunoblotting

After RA FLS or SW1353 (a human chondrosarcoma cell line) cells (2×10^5 cells/well) were incubated for 2 days, they were washed, followed by medium replacement with 1% L-glutamine and 1% antibiotics in MEM. The cells were pretreated with or without curcumin for 30 min and then stimulated with 20 ng/ml TNF- α . The cells were washed twice with ice-cold PBS and lysed in 50 μ l ice-cold lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 60 mM octyl β -glucoside, 10 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM nitrophenyl-phosphate, 0.7 μ g/ml pepstatin, and a protease-inhibitor cocktail tablet). The cell lysates were denatured by boiling at 95°C for 5 min in 2 $\times$ Laemmli buffer (21). The proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were then transferred to PVDF membranes using standard techniques. Subsequent to blocking in TBS-T buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) containing 5% skimmed milk powder or bovine serum albumin, the membrane was incubated with individual specific antibodies. The immunoreactive proteins were detected by using HRP-coupled secondary antibodies and enhanced chemoluminescence, according to the manufacturer's protocols (Amersham Biosciences, Piscataway, NJ, USA).

Statistical analyses

The data are presented as the mean $\pm$ S.E.M. from three or more independent experiments. Statistical analyses were performed by one-way ANOVA and Dunnett's test. All statistical calculations (* $P < 0.05$ and ** $P < 0.01$) were performed using SigmaStat software (Systat Software, Inc., Point Richmond, CA, USA).

Results

Effects of curcumin on CIA in mice

CIA, a typical animal model for RA, was successfully induced by injection of Type II collagen as described in the "Materials and Methods" section (Fig. 1B). The clinical arthritis indexes were evaluated for 15 days after booster immunizations with type II collagen. Arthritis was suppressed by oral administration of curcumin (4 – 100 mg/kg) in a dose-dependent manner. The reduction of clinical indexes was evident at the dose of 4 mg/kg and was substantially blocked at the dose of 100 mg/kg (Fig. 1C). These macroscopic observations were further confirmed by histological examinations of the hind paw sections from animals (Fig. 2A). Infiltration of immune cells, synovial hyperplasia, cartilage destruction, and bone erosion were evident in the vehicle-treatment. In contrast, only minor damage was exhibited in the curcumin-treated and indomethacin-treated mice (Fig. 2A), and histological scores were significantly suppressed by curcumin in this model (Fig. 2B).

Effect of curcumin on production of MMP-1 and MMP-3 in CIA, FLS, and chondrocytes

MMP-1 and MMP-3 are critical factors in the degradation of joint cartilages (10, 22). We tested whether curcumin inhibits the production of MMP-1 and MMP-3 in the joint sections from CIA mice. As shown in Fig. 3, the expression of MMP-1 and MMP-3 in cartilages (CA) and synovial tissues (ST) was prominently increased in the vehicle-treatment group, and they were suppressed by the treatment of curcumin and indomethacin. Although multiple cell types reside in rheumatoid joints, it has been suggested that secretion of MMPs from FLS and chondrocytes are largely responsible for cartilage destruction and surface cartilage erosion, which leads to joint space narrowing (4, 5). Therefore, we next tested the effect of curcumin on the production of MMP-1 and MMP-3 in FLS and chondrocytes. We tried to isolate both types of cells from the CIA mouse joints. Unfortunately, we failed to obtain enough cells because of the small amount of those cells in the mouse joints. As another option, we used primary FLS isolated from RA patients and the human chondrosarcoma cell line SW1353 as a type of chondrocyte. As shown in Fig. 4, MMP-1 and MMP-3 were secreted from FLS and SW1353 stimulated with TNF- α , and they were significantly suppressed by curcumin in a dose-dependent manner. These results were consistent with the immuno-histochemistry results from CIA mice (Fig. 3).

Fig. 1. Effects of curcumin on CIA in mice. **A:** Chemical structure of curcumin (1,7-bis-[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione). **B** and **C:** Male DBA/1J mice were intradermally immunized at the tail with 100 μ g of type II collagen. After 23 days, the mice were intraperitoneally boosted with type II collagen (100 μ g in 0.05 M acetic acid). Curcumin (CUR, 4–100 mg/kg), indomethacin (IND, 1 mg/kg), or vehicle (5% Arabic gum) was orally administered once a day after the boosting administration of type II collagen. The arthritis indexes of all four paws of mice were evaluated as described in the “Materials and Methods”. **B:** Representative images of paws: Normal (normal mouse), vehicle (5% Arabic gum), CUR (curcumin, 100 mg/kg), or IND (indomethacin, 1 mg/kg). **C:** Arthritis indexes are presented as the mean $\pm$ S.E.M. from 10 mice per group. * P <0.05 or ** P <0.01 vs. the vehicle group.

Effect of curcumin on activation of PKC isoforms in FLS and chondrocytes

It has been generally accepted that cytokines including TNF- α and IL-1 β stimulate production of MMPs through the activation of mitogen-activated protein kinases (MAPKs), NF- κ B, and AP-1 (23–25). However, the upstream signaling molecules to regulate those molecules remain unclear in FLS and chondrocytes as well as CIA. Recently, several reports suggested that protein kinase C isoforms are associated with the

Fig. 2. Effects of curcumin on histological changes in CIA mice. **A:** Representative histological images (upper panel) and high-power view of the boxed area (lower panel). After scarifications of CIA mice described in Fig. 1, the hind paws joints were fixed in 4% paraformaldehyde for 3 days, decalcified in 10% EDTA for 30 days at 4°C, dehydrated in a graded series of ethanol (from 70% to 100%), washed with xylene twice for 2 h each, and then finally embedded in paraffin. Serial paraffin sections (5- μ m-thick) were stained with hematoxylin and eosin. **B:** Scoring of histopathological changes. The histological scorings were evaluated as described in the “Materials and Methods”. Data are presented as the mean $\pm$ S.E.M. from three independent experiments. ** P <0.01 vs. the vehicle group. CUR: curcumin, IND: indomethacin, T: tibia, TA: talus.

production of MMPs in another type of cells (26, 27). Based on these previous reports, we examined the effects of curcumin on the activation of PKC isoforms in TNF- α -stimulated FLS and chondrocytes. Interestingly, only the activating phosphorylation of PKC δ was significantly inhibited by curcumin in these cells (Fig. 5A). To prove its involvement in CIA, we tested whether the activating phosphorylation of PKC δ was increased in CIA joint sections (cartilage and synovial tissues) and whether its phosphorylation was inhibited by curcumin. As shown in Fig. 5B, the increased staining for the phosphorylated form of PKC δ was suppressed by curcumin in cartilages and synovial tissues. Next, to verify that PKC δ also regulates the

Fig. 3. Effect of curcumin on MMP-1 and MMP-3 expression in cartilages and synovial tissues from CIA mouse joints. The hind paw sections, prepared as described in Fig. 2, were deparaffinized with xylene and ethanol. After hydrating them in PBS, endogenous peroxidase was depleted with 0.3% H_2O_2 for 10 min. The sections were blocked with 10% normal horse serum for 1 h and then incubated overnight at 4°C with the specific antibodies directed to MMP-1 or MMP-3. Then, they were incubated with biotinylated secondary antibody and the signal was amplified with HRP-conjugated streptavidin using a Vectastain Elite ABC kit. All sections were developed with diaminobenzidine and counterstained with methyl green. Representative images are shown from three independent experiments. The stained MMP-1 or MMP-3 in the cartilages and synovial tissues were emphasized with arrows. CA: cartilage, ST: synovial tissue, CUR: curcumin, IND: indomethacin.

production of MMP-1 and MMP-3 in FLS and chondrocytes, we checked whether rottlerin, an inhibitor of $PKC\delta$, suppressed MMP production. The productions of MMP-1 and MMP-3 were inhibited by rottlerin in a dose dependent manner (Fig. 5C).

Recently, there was a report supporting that the JNK/c-Jun pathway is regulated by $PKC\delta$ in RA FLS (8). In this context, the effect of curcumin on the activation of JNK/c-Jun and other MAP kinases was analyzed in FLS and chondrocytes. Of note, the activation of JNK/c-Jun, not ERK and p-38, was inhibited by curcumin in a dose-dependent manner (Fig. 6).

Discussion

RA is characterized by systemic autoimmune inflammation of synovial tissue and the destruction of cartilage and bone in multiple peripheral joints (28, 29). Although non-steroidal anti-inflammatory drugs are clinically common, they have limited use because of partial efficacy and several side effects including gastrointestinal ulcers and cardiovascular risks (30, 31).

Fig. 4. Effect of curcumin on the secretion of MMP-1 and MMP-3 in RA FLS and chondrocytes. RA FLS and SW1353 were stimulated by 20 ng/ml $TNF-\alpha$ with or without curcumin (CUR, 0–10 μM), or remained not stimulated (NS) for 24 h. MMP-1 and MMP-3 in media were analyzed with an immunoblot analysis using specific antibodies. Relative band density is presented as the mean $\pm$ S.E.M. of the percent of control from three independent experiments (lower panel). * $P < 0.05$, ** $P < 0.01$ vs. the control without curcumin (0).

Fig. 5. Effects of curcumin on PKC δ activation in RA FLS, chondrocytes, and CIA mouse joints. **A:** RA FLS and SW1353 (2×10^5 cells/well) were stimulated by 20 ng/ml TNF- α with or without curcumin (CUR) or remained not stimulated (NS) for 15 min. Then, the cells were lysed in the lysis buffer and subjected to immunoblot analysis with individual specific antibodies. Representative immunoblots (upper panel) and band densities (lower panel) indicated as the mean $\pm$ S.E.M. from three independent experiments are shown. **B:** The joint sections prepared in Fig. 2A were immunostained with the antibody against the phosphorylated form of PKC δ . Representative images are shown from three independent experiments. **C:** RA FLS and SW1353 (2×10^5 cells/well) were stimulated with 20 ng/ml TNF- α for 24 h. The levels of MMP-1 and MMP-3 in the media were measured by immunoblot analysis. Representative images are shown from three independent experiments (upper panel). Relative band densities (lower panel) are indicated as the mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ vs. the control without rottlerin (0). Ro: rottlerin.

Clinical application with TNF- α -neutralizing antibody and IL-1-receptor antagonist exhibits substantial efficacy, but carries the disadvantages of high cost, hypersensitivity to medications, and possibility of serious infections (32, 33). Therefore, further efforts are necessary to develop new drugs with fewer side effects for treatment of RA. Although the exact mechanisms that contribute to RA pathogenesis largely remain unknown, it is well-established that the hyperplasia of

FLS and the infiltration of T cells, B cells, dendritic cells, and mast cells in joint synovial tissues and their production of proinflammatory mediators such as TNF- α and IL-1 are critical in the pathogenesis of RA (34). Among these cells, FLS contributed principally to the pathological process of RA (4, 5). Therefore, the specific agents inhibiting the activation of FLS may have good potential as anti-arthritis drugs.

Curcumin is a pluripotent pharmacologic agent, which

Fig. 6. Effect of curcumin on the activation of MAP kinases in RA FLS and chondrocytes. RA FLS and SW1353 (2×10^5 cells/well) were stimulated by 20 ng/ml TNF- α with or without curcumin (CUR) or remained not stimulated (NS) for 15 min. Then, the cells were lysed in the lysis buffer and subjected to immunoblot analysis with individual specific antibodies. Representative immunoblots (upper panel) and band densities for phosphorylated forms of JNK and c-Jun (lower panel) indicated as the mean $\pm$ S.E.M. from three independent experiments are shown. * $P < 0.05$, ** $P < 0.01$ vs. the control without curcumin (0).

likely accounts for its broad biologic activity. For example, curcumin exhibits anti-inflammatory, anti-oxidant, chemopreventive, and anti-cancer activities (2). Accumulating evidence suggests that a variety of activities of curcumin are associated with its effect on multiple signaling pathways. For example, curcumin downregulates transcription factors and inflammatory enzymes such as NF- κ B, AP-1, signal transducer and activator of transcription-3, transcription factor Egr-1,

cyclooxygenase-2, 5-lipoxygenase, and inducible nitric oxide synthase; and it also downregulates growth factor receptors, cell-surface adhesion molecules, and cyclin D1 (2). In contrast, little is known about the upstream signaling pathways to regulate the above molecules. Recently, several reports suggested that the production of inflammatory mediators such as MMPs and IL-32 are regulated by isoforms of PKC (8, 26, 27). In this study, we checked which isoform of PKC is associated with the production of MMP-1 and MMP-3 in FLS and chondrocytes. The results show that only PKC δ was inhibited by curcumin, and the inhibition of PKC δ with rottlerin also suppresses the production of MMP-1 and MMP-3 in FLS and chondrocytes. These observations suggest that curcumin suppresses PKC δ to inhibit secretion of MMP-1 and MMP-3 in FLS and chondrocytes. Furthermore, the activating phosphorylation of PKC δ was inhibited by curcumin in CIA joint sections as determined by immunohistochemistry. Collectively, the results from cell cultures and CIA mice lead us to conclude that the inhibition of MMP-1 and MMP-3 production by curcumin is exerted through the inhibition of PKC δ .

Recently, we reported for the first time that PKC δ regulates the JNK/c-Jun pathway (8), which is critical for producing MMP-1 and MMP-3 (11–13). The suppression of PKC δ using the specific inhibitor rottlerin or siRNA against PKC δ inhibits the activation of JNK and c-Jun activation in FLS (8). Based on our previous and current observations, it is quite plausible that curcumin suppresses the production of MMP-1 and MMP-3 through inhibition of PKC δ and the JNK/c-Jun pathway in CIA, FLS, and chondrocytes.

In addition to the current results to inhibit MMP-1 and MMP-3 production, curcumin has anti-inflammatory and anti-oxidant activity (2). Recently, it was reported that curcumin inhibits the activation of mast cells (34), one of the infiltrating cells during the development of RA (35). Those effects of curcumin may additively or synergistically ameliorate the pathogenic process of RA. There is also evidence that curcumin suppresses cell proliferation in several types of cells (2). Therefore, we can not rule out the possibility that curcumin partially exhibits the anti-arthritis effect through the suppression of RA FLS proliferation in the CIA mice.

The pharmacokinetics data in mice are very limited but indicate that curcumin accumulates in peripheral tissues such as small intestinal mucosa (39–240 μ mol/kg) and colonic mucosa (15–715 μ mol/kg) after dietary or intraperitoneal administration of curcumin (36, 37). However, it is unclear whether the tissue concentration of curcumin after oral administration of 5–50 mg/kg (14–140 μ mol/kg) curcumin are sufficient to suppress the activation of RA FLS, namely,

1–10 μ M curcumin in vitro. Our results clearly demonstrate that curcumin exhibits anti-arthritis effect in the joints in CIA mice, indicating that the concentration of curcumin reaches to the effective level after oral administration. However, the distribution of curcumin into the mouse joints after oral administration remains to be further investigated.

Several clinical applications of curcumin are actively under investigation for a variety of disorders including multiple myeloma, pancreatic cancer, myelodysplastic syndromes, colon cancer, psoriasis, and Alzheimer's disease (2). Promising results have been obtained for post-operative inflammation (38) and pancreatic cancer (39). Furthermore, curcumin has very low toxicity (40, 41). Taken together, it is worthwhile to investigate whether curcumin exhibits anti-arthritis efficacy in patients with RA.

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