Red Ginseng Water Extract Aggravates Inflammation in Sebocytes and Outer Root Sheath Cells After Treatment With Lipopolysaccharide and Mice With Cutibacterium Acnes-induced Inflammatory Nodules

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Research Article

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Abstract

Ginseng has been known in Korea as a health-supportive herbal medicine from time immemorial. Red ginseng is one of processed ginseng that is produced from white ginseng through steaming and drying. Many protective functions of red ginseng have been reported from various groups in many diseases. In this study, we first investigated whether red ginseng water extract (RG) aggravates inflammation in human sebocytes and outer root sheath (ORS) cells after treatment with lipopolysaccharide (LPS) and mice with Cutibacterium (C.) acnes strain (ATCC 1182)-induced inflammatory nodules. Sebocytes and ORS cells were isolated and cultured from the human scalp. The RG augmented LPS mediated inflammation by increased the mRNA and protein expression of inflammatory cytokines in sebocytes and ORS cells. In addition, RG also showed the increased protein expression of p-NFκB, p-c-jun and p-JNK in the LPS-treated sebocytes and ORS cells. Furthermore, RG upregulated the LPS-induced production of sebum in sebocytes. In addition, RG inhibited improvement of inflammatory nodules and showed the increased expression of inflammatory biomarkers in inflammatory nodules of Cutibacterium acnes injected mice. Collectively, our data strongly suggest that RG is one of the aggravating factors of acne vulgaris. It would be better to stop taking RG in patients with inflammatory acne.

Introduction

Acne is an inflammatory follicular disease commonly occurring in face, chest and back during an adolescent period. It is caused by various factors such as excessive sebum production, Cutibacterium (C.) acnes proliferation, hyperkeratosis of follicular infundibulum and inflammation (1). In addition, it can be aggravated or developed by stress, hormone and nutrition. It is controlled by a variety of therapeutic options, such as topical and oral medicine and surgical devices. Nevertheless, other therapeutic options are still needed for the complete and safe treatment of acne. Complementary and alternative medicines are one of the therapeutic options of acne.

In general, ginseng improves general health including immunity and memory, and increases anti-inflammatory and antioxidant effects (2-7). The beneficial effects of ginseng come from ginsenosides, main ingredients of ginseng. Recently, pharmacological effects of non-ginsenosides have been reported. Polyacetylene, one of the non-ginsenosides, has been found in red ginseng, and has anti-inflammatory and anti-bacterial effects. Red ginseng can be produced by repeated steaming and air-drying of fresh ginseng.

This study was conducted to investigate the effect of red ginseng water extract (RG) on the acne-related cultured cells and animal model mouse. Cultured human sebocytes and outer root sheath (ORS) cells of hair follicle were used for in vitro test. HR-1 mice with C. acnes-induced inflammatory nodules were used for in vivo test.

Materials And Methods
Sebocyte and outer root sheath cell culture

The occipital non-balding scalp specimens were obtained from patients undergoing hair transplantation surgery. The study was conducted according to the Declaration of Helsinki Principles. Informed written consent was obtained from the patient. The Medical Ethical Committee of the Kyungpook National University Hospital (Daegu, Korea) approved all of the described studies (IRB No. KNU 2018-0155). And this study was carried out in compliance with the ARRIVE guidelines. Primary sebocyte cultures were prepared from occipital hair follicle sebaceous glands. The sebaceous glands were isolated from hair follicles under a binocular microscope and transferred to Biocoat collagen type I-coated tissue culture dishes (CORNING, Kennebunk, USA). The explants were maintained in Dulbecco's modified Eagle medium (DMEM; Hyclone Laboratories, Logan, UT, USA) at 37°C in a humidified atmosphere of 5% CO2. Explants were left for a period of 4 days, and the medium was then changed to Epilife (MEPI500CA; Gibco BRL, Grand Island, NY, USA). The medium was changed every 3 days. After cell outgrowth became sub-confluent, cells were harvested with 0.25% trypsin/10 mM EDTA in Hank's balanced salt solution (HBSS) and sub-cultured.

ORS cells were isolated from hair shafts of hair follicles. ORS cells were isolated and cultured as previously described (20). ORS Cells were used from the second passage for the experiments in this study.

Preparation of red ginseng

Red ginseng preparation RGE (Rg1 + Rb1 + Rg3 = 5.5 mg/g) was purchased from the Daedong Korea Ginseng Cooperation.

MTT assay

Primary human sebocytes and ORS cells were plated in 96-well collagen-coated plates (CORNING, 5,000 cells per well) for 24 h. The day after seeding, sebocytes and ORS cells were incubated for 3 days without supplement medium in various concentrations of RG. MTT solution (3-[4,5]dimethylthiazol-2,5-diphenyltetrazolium bromide) was then added at 70 μg per well for 3 hours. The formazan product was dissolved with DMSO, and optical density was measured at 570 nm.

RT-PCR and Real time PCR analysis

Cells were treated with 0.1% DMSO (control), 5-μg/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) or 50-μg/mL RG for 6 h and 24 h. The dose of RG was determined by MTT assay. The methods of total RNA isolation, RT-PCR and Real time PCR as previously described (20). The primers used in the study are shown in supplement Table 1.

ELISA
Analysis of IL-1β, IL-6, IL-8 and TNF-α (R&D Systems, Minneapolis, CA, USA) protein expression using enzyme-linked immunosorbent assay (ELISA) was conducted according to the manufacturer's instructions. For measuring protein levels in conditioned medium of sebocytes and ORS cells from passage 2, they were plated overnight at a density of 30,000 cells/24-well culture dish and were washed three times with PBS. To examine protein induction in cells in response to the RG, the cells were treated with varying concentrations of RG in serum-free medium for 6 h or 24 h, and protein concentrations in the conditioned medium were measured.

**Western blot analysis**

Cells were treated with 0.1% DMSO (control), 5 μg/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) or 50 μg/mL RG for 6 h. NE-PER Nuclear and Cytoplasmic extraction reagents (Pierce, Rockford, IL, USA) were used according to the manufacturer’s protocol to extract nuclear and cytoplasmic proteins. Proteins (10 μg/lane) were separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk in PBS for 1 h. They were probed with rabbit monoclonal antibody against p-NFκB (1:1000 dilution; Cell signalling, Beverly, MA). Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit Ig (Jackson ImmunoResearch, Baltimore, PA) at a 1:7,000 dilution. The bands were visualized using SuperSignal West Femto (Thermo Scientific, Rockford, IL, USA). The membranes were also probed with mouse monoclonal antibody against Lamin B1 (1:500 dilution; Zymed Laboratories, San Francisco, CA, USA) to evaluate the quantity and integrity of the protein samples.

Total cell lysates (10 μg/lane) were separated using 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk in PBS for 1 h. They were probed with rabbit polyclonal antibodies against p-c-jun (1:1000 dilution; Cell Signaling, Beverly, MA, USA) and p-JNK (1:1000 dilution; Cell Signaling). Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit Ig (Jackson ImmunoResearch, Baltimore, PA) was used as the secondary antibody at a 1:7,000 dilution. The bands were visualized as above. The membranes were also probed with mouse monoclonal antibody against Actin (1:5000 dilution; Chemicon, Temecula, CA, USA) to evaluate the quantity and integrity of the protein samples.

**Quantification of lipid production**

The supernatant of RG-treated sebocytes for 24 h was collected in a clean tube and homogenized with 50 μl 0.9% NaCl plus 1% triton ×100 followed by vortex agitation. After incubation at 4 °C for 30 min, the solution was centrifuged at 13,000 rpm for 15 min. The TG-S reaction kit (Asan Pharm. Co., Seoul, Korea) was used for the detection of neutral lipids, according to the manufacturer's protocol.

Oil red O staining kit (Abcam) was used according to the manufacturer's instructions. Briefly, Slides were incubated in propylene glycol for 2 min, and then incubated in Oil red O solution for 30min. Slides were immersed in 85% propylene glycol for 1 min and were washed twice in water and stained hematoxylin for 2min. The slides were washed with water and mounted.
AdipoRed assay reagent (Lonza, Walkerville, MD, USA) was used for Nile red. Slides were incubated in Adipo red solution (1:100 dilution) for 10 min at dark. The slides were washed with water and mounted.

**Immunofluorescence staining for cells**

Cultured human sebocytes and ORS cells were seeded in an eight chamber slide (Nunc Lab-Tek, Roskilde, Denmark) at a density of 50,000 for 24 h and fixed with 4% paraformaldehyde for 10 min. Immunostaining was performed as previously described (20). Antibodies against CK-1-3 (1:100 dilution; Chemicon), CK-15 (1:100 dilution; Chemicon), CK-17 (1:100 dilution; Abcam, Cambridge, UK) and CK-19 (1:100 dilution; Chemicon) were used.

For immunofluorescence staining of p-NFκB and p-c-jun, cells were cultured in EpiLife medium in the presence of 5 μg/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) and 50 μg/mL RG for 6 h. Fixation and blocking procedures were performed as above. Cells were incubated with rabbit polyclonal p-c-jun antibody (1:100 dilution; Cell Signaling) at 4°C overnight, washed three times with PBS and incubated with Alexa Fluor 488-labeled donkey anti-rabbit secondary antibody (Molecular Probes, Eugene, OR, USA). Cells were washed with PBS and counterstained with 4,6 diamidino-2-phenylindole (DAPI) for 10 min.

**Animal study**

*C. acnes* strain (ATCC 1182) was isolated from the pustular lesions of Korean patients with moderate inflammatory acne. Our animal care and treatment protocols were in accordance with the guidelines of the use of Laboratory Animals. Animal experiments and were approved by the Institutional Animal Care and Use Committee of the Kyungpook National University (IRB No. KNU 2018-167). And this study was carried out in compliance with the ARRIVE guidelines. *acnes* from post-log phase cultures were grown on brain–heart infusion agar, harvested, and lyophilized prior to injection. *C. acnes* suspensions were prepared at concentrations of 10^9 colony forming units (CFU)/20 μL. Using a 30-gauge needle, *C. acnes* suspensions were injected in 20-μL aliquots intra dermally into four sides of the backs of the six-week-old female Hos:HR-1 mice (HR-1; SLC Inc., Hamamatsu, Japan). These mice made an oral contribution with RG in water for 2 weeks from next day after *C. acnes* injection. Thereafter, size of inflammatory nodules was measured. In addition, mice were killed (after 2 weeks and 4 weeks), and the injected regions of dorsal skin were stained with hematoxylin and eosin.

**Immunofluorescence staining for mouse skins**

Tissue samples were obtained from the inflammatory nodules of each mouse, and the samples were placed in cryomolds using an embedding medium (OCT compound) in a freezer at −80°C. The block was cut into sections (7 μm slices) using a cryostat (Leica CM3050 S), and the sections were applied on glass slides. Immunostaining was performed as previously described (20). Antibodies against neutrophils (1:80; Abcam), myeloperoxidase (MPO, 1:200; Abcam), interleukin (IL)-1β (1:150; Abcam), matrix metalloprotease (MMP-2, 1:300; Abcam), MMP-3 (1:100; Abcam), MMP-9 (1:250; Abcam), and LL-37
(1:300; Abcam) were used. Histological changes were compared among the mice, specifically changes in inflammation, and the expression level of protein was quantified using Image-J program.

**Statistical analysis**

Data are expressed as means ± standard deviation (SD). ANOVA was used for statistical analysis of the data. P < 0.05 was considered statistically significant.

**Results**

**Red ginseng water extract augments the increased expression of inflammatory cytokines in the LPS-treated sebocytes and ORS cells.**

First, we were characterized human cultured sebocytes and ORS cells of scalp. Sebocytes were positive for CK-1-3, CK-15, CK17 and CK19, but ORS cell were positive only for CK-1-3. Sebocytes were different from ORS cells in the expression of cytokeratin and were expressed sebocyte maker, CK19, in immunofluorescence staining (Supplement Figure 1).

Next, to investigate the effect of red ginseng (RG) on the cell viability of human cultured sebocytes and ORS cells, we performed MTT assay by treated the various concentration (Supplement Figure 2). In this study, RG treated the concentration that were not cytotoxic in sebocytes and ORS cells.

We showed the change of inflammatory cytokines whether RG causes an increase of inflammation in the LPS-treated sebocytes and ORS cells. The treatment of LPS resulted in the elevation of mRNA of inflammatory cytokines (IL-β, IL-6, IL-8 and TNFα) in sebocytes and ORS cells for 6 hours and 24 hours in real time PCR. Interestingly, addition of both LPS and RG induced the mRNA expression of inflammatory cytokines (IL-β, IL-6, IL-8 and TNFα) rather than LPS only treatment in sebocytes and ORS cells (Figure 1 A and B). In line with the above results, treatment with LPS resulted in increment of inflammatory cytokines (IL-β, IL-6, IL-8 and TNFα) protein level in conditioned medium, and LPS-induced induction of inflammatory cytokines were augmented when RG was added together with LPS (Figure 1 C-F). We evaluated the effect of RG on the production of inflammatory cytokines in LPS-treated sebocytes and ORS cells by real time PCR and ELISA. Compared to LPS untreated cells, cell treated with only RG decreased the expression of inflammatory cytokines in sebocytes and ORS cell without stimulation of LPS by ELSIA (supplement Figure 3).

**Red ginseng water extract causes an increased expression of inflammatory cytokines through the pathway of NFkB and JNK in the LPS-treated sebocytes and ORS cells.**

When cells are activated with LPS, cells are caused the process of activating NF-κB, which leads to the translocation of NF-κB into the nucleus or the activation of c-Jun N-terminal kinase (JNK), thereby initiating the transcription of various inflammatory genes. Therefore, we assessed whether RG induces the activation of the NFkB and JNK pathway. First, we checked the expression level of p-NFκB in nucleus. The level of p-NFκB in the nucleus significantly increased by exposing LPS for 6 h. LPS-induced
accumulation of p-NFκB was augmented when RG was added together with LPS in sebocytes and ORS cells in western blot analysis (Figure 2A). Consistent with this results, translocation of NFκB into nucleus increased in LPS-treated sebocytes and ORS cells after treatment with RG in immunofluorescence staining (Figure 2B).

Next, we showed the expression level of JNK pathway. In western blot analysis, expression of p-c-jun and p–JNK was increased in the LPS-treated sebocytes and ORS cells 6 hours after treatment with RG (p<0.05) (Figure 2C and D). In addition, the expression of p-c-jun was increased in the LPS-treated sebocytes and ORS cells 6 hours after treatment with RG using immunofluorescence staining (Figure 2E).

**Red ginseng water extract increases the production of sebum in LPS-treated sebocytes.**

LPS is a component of gram negative bacteria and is also immunostimulatory factor (8). Toll-like receptor 4 (TLR4) primarily mediates cellular signaling induced by gram negative bacteria (9). Therefore, we assessed whether LPS treatment increases the expression level of TLR4 in human sebocytes. We observed that the expression of TLR4 increased by LPS treatment. Especially, RG more increased the expression of TLR4 in LPS-treated sebocytes for 24h in RT-PCR analysis (Figure 3 A and B). Next, we investigated whether RG is involved in sebum production in LPS treated sebocytes. The production of sebum increased in LPS-treated sebocytes after treatment with RG for 24h (Figure 3C). Nile red staining and oil red o staining revealed that the accumulation of lipid droplets by treatment with RG was increased in the perinuclear cytoplasm in LPS-treated sebocytes (Figure 3D). To verify the production of sebum in sebocytes, we examined the expression of sebum secretion related genes after RG treatment in LPS-treated sebocytes. In RT-PCR analysis, RG augmented the expression of sebum secretion related genes in LPS-treated sebocytes (Figure 3E). Consistent with the result of RT-PCR, RG induced the level of several lipogenic-regulated genes in real time PCR (Figure 3F).

**Red ginseng water extract delayed the improvement of inflammatory nodules in mice.**

To evaluate the effect of RG on *C-acne* induced skin, we injected *C. acnes* suspensions into intra dermally of four sites of mice back skin. After 24h, mice made an oral contribution with RG for 2 weeks. The group of oral contribution with RG aggravated inflamed nodule than in the control group (Figure 4 A). H&E staining showed more severe inflammatory nodules in RG-treated mice than in control at week 2 and week 4 (Figure 4B). *C. acnes* triggers the activation of TLR2 activation and causes the release of the neutrophil chemoattractant cytokine. In addition, inflammatory nodules by induced *C. acnes* were composed of inflammatory cells, such as neutrophils and T cells (10). Therefore, we checked the expression of inflammatory makers and tissue remodeling markers. First, we showed the group of RG-treated *C. acnes* increased the expression of TLR2 than the group of only *C. acnes*. (Supplement Figure 4A). Immunofluorescence staining showed an increase in the expression both inflammatory biomarkers, such as neutrophil, MPO and IL1β and tissue remodeling biomarker, such as MMP3 and MMP (Figure 4 C and D). Moreover, antimicrobial peptides can play a protective role against *C. acnes* or induce inflammatory signal in acne vulgaris, LL37 that is one of the antimicrobial peptide increased in LPS-treated sebocytes and ORS cells with RG and RG-treated *C. acnes* mice (Supplement Figure 4 B and C).
Discussion

Inflammation is an integrated response to pathogenic invaders and self-defense mechanisms. However, inflammation can lead to overexpression of inflammatory cytokines and chemokines. Panax ginseng, which has anti-inflammatory effects, has been used to control inflammation of human body. In addition, Panax ginseng shows antioxidative effects to remove reactive oxygen species. Furthermore, Panax ginseng has immune-stimulating activities. It was reported that red ginseng possesses good antioxidative and immune-stimulating effects comparing with black ginseng and fermented red ginseng. In particular, Rg3-enriched red ginseng extract has been investigated for its anti-inflammatory and antioxidant properties (11). The mechanisms of anti-inflammatory effect of Panax ginseng include the inhibition of enzyme expression and proinflammatory cytokines. Panax ginseng also has inhibitory effect on the synthesis of interleukins and inflammation-related pathway, such as NF-κB and TLR (2-7). It was reported that Rg3-enriched red ginseng can be considered a potent anti-inflammatory agent by mediating the nuclear receptor RXRα-PPARγ heterodimer. Rg3-enriched red ginseng extract can also suppress TLR4-activated inflammatory signaling pathway by LPS (12). The cascade of proinflammatory cytokines, such as IL-1β, IL-6 and TNF-α, by induced the pathway can also be suppressed by Rg3-enriched ginseng (13, 14). It was demonstrated that Rg3 of red ginseng extracts inhibited the levels of nitric oxide in vitro and in vivo studies using LPS-treated cells and mice. LPS binds with Toll-like receptor 4 and activates the inflammatory signaling pathway via inducible nitric oxide synthase and cyclooxygenase-2. These mediators stimulate proinflammatory cytokines, such as IL-1β and TNF-α. When the bind of TLR4 with LPS takes place, TGF-β activated kinase1 (TAK1) is activated via phosphorylation. Subsequently, phosphorylated TAK1 moves downstream to activate IκB kinase (IKK α/β) by phosphorylation, which then phosphorylates and activates IκBα. Phosphorylated IκBα then sets NF-κB free, whereby it translocates into the nucleus and initiates the transcription of inflammation-associated genes (15).

Similar to NF-κB pathway, MAPK also consists of a serious of upstream (MKK and MEK) and downstream (ERK, JNK and P38) factors that are responsible for initiation of inflammation (16, 17). Rg3-RGE inhibits the phosphorylation of all factors of NF-κB and MAPK pathway. In our study, RG increased the expression of inflammatory cytokines, such as IL-1β, IL-6, IL-8 and TNF-α, in both sebocytes and ORS cells after treatment with LPS. However, RG did not show an increase in the protein expression levels of IL-1β, IL-6, IL-8 and TNF-α in the LPS-not-treated sebocytes and ORS cells. In addition, RG activated the pathway of NF-κB/AP-1: expression of p-c-jun, p-JNK was increased after treatment of the LPS-pretreated sebocytes and ORS cells with RG.

RG also enhanced expression of LL-37 in the LPS-treated sebocytes and ORS cells. The antibacterial activity of Panax ginseng against C. acnes and Staphylococcus aureus has been reported. Norajit et al. (18) and Sung et al. (19) also found that red ginseng possessed stronger antibacterial activity than the white red ginseng. In our study, RG aggravated acneiform eruption in HR-1 mice induced by C. acnes. RG delayed improvement of inflammatory nodules in mice in H&E staining. In addition, immunohistochemistry showed an increase in the expression inflammatory biomarkers in RG-treated mice compared with control. LPS-pretreated sebocytes significantly increased production of sebum lipid after treatment with RG.
In conclusion, RG shows an increase in the expression of inflammatory biomarkers in cultured sebocytes and ORS cells after treatment with LPS. In addition, improvement of inflammatory nodules in mice was delayed after treatment with RG. Therefore, RG might be one of the aggravating factors of inflammatory acne and should recommended to be stopped in patients with inflammatory acne.

Declarations

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Conflicts of interest: None

Funding: None

References


**Figures**
Figure 1

RG induces expression of inflammatory cytokines in the LPS-treated sebocytes and ORS cells. (A) Sebocytes were treated with LPS and RG for 24 h and analyzed by real-time PCR. Relative levels of IL-1β, IL-6, IL-8 and TNFα are shown as mean±SD from three independent experiments (*P<0.05). (B) ORS cells were treated with LPS and RG for 24 h and analysed by real-time PCR. Relative levels of IL-1β, IL-6, IL-8 and TNFα are shown as mean±SD from three independent experiments (*P<0.05). Concentrations of (C)
IL-1β, (D) IL-6, (E) IL-8 and (F) TNFα in conditioned medium measured by ELISA. Sebocytes and ORS cells were treated with LPS or RG for 24 h. Data are the mean ± SD from three independent experiments (*P < 0.05).

Figure 2

RG increases expression of inflammatory cytokines through the activation of NF-κB and JNK in LPS-treated sebocytes and ORS cells. (A) Cells were treated with LPS and RG for 6 h and total nuclear factions
(10 μg/lane) were probed with anti-p-NF-κB antibody. Lamin B1 was used as an internal control. (B) Cells were treated with LPS and RG for 6 h and cells were immunostained with anti-p-NF-κB antibody. DAPI nuclear staining was also performed (lower panels). (C) Cells were treated with LPS and RG for 6 h and total cell factions (10 μg/lane) were probed with anti-p-c-jun and p-JNK antibody. Actin was used as an internal control. (D) Relative levels of p-c-jun and p-JNK protein are shown as mean±SD from three independent experiments (*P<0.05). (E) Cells were treated with LPS and RG for 6 h and cells were immunostained with anti-p-NF-κB antibody. DAPI nuclear staining was also performed (lower panels).

Figure 3

RG induces lipid production in LPS-treated sebocytes. (A) Sebocytes were treated with LPS and RG for 24 h and analyzed by RT-PCR and (B) relative levels of TLR4 are shown as mean±SD from three independent experiments (*P<0.05). (C) Sebocytes were treated with LPS and RG for 24h and measured concentration of neutral lipids and relative levels of lipid production are shown as mean±SD from three independent experiments (*P<0.05). (D) Sebocytes were treated with LPS and RG for 24 and intracellular lipid droplets were visualized using Oil red O staining and Nile red staining. Sebocytes were treated with LPS and RG for 24 h and analyzed expression of sebum production related genes by (E) RT-PCR and (F) real time PCR.
Relative levels of SREBP1a, SREBP1c and SCD are shown as mean±SD from three independent experiments (*P<0.05).

Figure 4

RG aggravates mice with Cutibacterium acnes-induced inflammatory nodules (A) Inflammatory nodules were larger in RG-treated mice than in control. (B) H&E staining showed more severe inflammatory nodules in RG-treated mice than in control. (C) Relative levels of proteins are shown as mean±SD from three independent experiments (*P<0.05). (D) In immunostaining, an increase in the expression both inflammatory biomarkers (neutrophil, MPO and IL1β) and tissue remodeling biomarker (MMP2, MMP3 and MMP9).

Supplementary Files

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- RGTableSupplementary1.docx
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