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Original article

The effect of low-intensity pulsed ultrasound on wound healing using scratch assay in epithelial cells

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ABSTRACT

Purpose: Low-intensity pulsed ultrasound (LIPUS) is widely used in medical fields because it shortens the time required for biologic wound healing in fracture treatment. Also, in dental fields, LIPUS should be effectively employed for implant treatment.

However, most of the relevant reports have been published on its effects on bone formation around implants, and the effects of LIPUS on soft tissue healing remain unclear. In the present study, we examined the effects of LIPUS on soft tissue healing using gingival epithelial cells.

Methods: Gingival epithelial cells were cultured on a dish, followed by LIPUS exposure at a frequency of 3 MHz for 15 min. The cells were counted with a hemocytometer, and a scratch assay was conducted by measuring the closing area of the scratch wound using a microscope. Following LIPUS exposure, total RNA was collected for microarray analysis. In addition, real-time PCR was performed to examine the mRNA expression level of integrin α6β4. Furthermore, total protein was collected to examine the protein expression level of integrin α6β4 by western blotting.

Results: The cell count and scratch assay demonstrated that LIPUS exposure promoted cell proliferation and scratch-wound closure. Microarray analysis demonstrated the increased expression levels of adhesion-related genes, including integrin. Real-time PCR analysis demonstrated that LIPUS exposure significantly up-regulated the mRNA expression level of integrin α6β4. Western blotting showed intense staining of integrin α6β4.

Conclusion: LIPUS exposure promotes wound closure in the scratch assay and up-regulates the expression level of integrin α6β4 as compared with the control.

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1. Introduction

Free gingival graft is an effective surgical treatment in cases with an insufficient width of keratinized gingiva around natural teeth [1] or implants [2–4]. However, this technique causes an open secondary wound with post-surgical pain and bleeding at a donor site [5,6]. Hence, in cases for which multiple grafts or procedures are needed, shortening the time required for wound healing is critical for reducing the patient burden, such as postoperative discomfort and pain.

Physical therapy by applying physical energy to a wound site to promote healing has attracted attention [7]. Physical therapy is a therapeutic approach to utilize physical actions, such as heat, electricity, light, X-ray, and air. The energy used is roughly classified into electromagnetic and mechanical energies [8,9]. In addition, ultrasound therapy has attracted attention for decades as physical therapy.

Low-intensity pulsed ultrasound (LIPUS) is a medical device, developed as a therapeutic approach to promote fracture healing in the 1950s. This device applies acoustic waves as physical energy to living tissue [10,11].

An *in vivo* study demonstrated that LIPUS exposure promoted wound healing at all stages during fracture healing [12–15] and increased mechanical strength in a fibula fracture model [16]. Moreover, LIPUS up-regulated the expression levels of aggrecan mRNA in a rat femur fracture model [17]. An *in vitro* study showed that Cbfa-1/Runx2, Alk-3, alkaline phosphatase, osteopontin, TGF-β1 and BMP-7 in the rat bone marrow stromal cells [18], up-regulated osteoblasts and down-regulated osteoclasts in the rat alveolar mononuclear cells [19].

Thus, LIPUS is a noninvasive and safe device for fracture healing, and has been employed in daily clinical practice in orthopedic fields.

Reportedly, in dental fields, LIPUS exposure prevents insufficient salivary secretion due to autoimmune sialadenitis [20] and enhances bone formation around miniscrew implants [21], gradually demonstrating its usefulness.

Only a few studies have been conducted on soft tissue healing. For example, an *in vivo* study demonstrated that LIPUS enhances palatal mucosa wound healing in rats [22] and accelerates periodontal wound healing after flap surgery [23]. An *in vitro* study indicated that the expression of connective tissue growth factor (CTGF) in gingival cells (GE1) was enhanced by LIPUS exposure [24]. However, the detailed mechanisms remain unclear.

Integrin is a transmembrane glycoprotein that forms 24 different heterodimers by combining any of 18α and 8β subunits. It is a bidirectional signaling receptor located at the interface between the extracellular matrix and the intracellular milieu, a major receptor in keratinocyte adhesion in the basement membrane that underlies the skin epidermis [25]. Recently, the role of integrin in wound healing has attracted attention. Wound healing occurs in the soft tissue not through cell division and blood clot formation, but through resealing by the active movement of surrounding cells. As previously reported, integrin α6β4 controls cell migration by regulating the transcription and translation of different integrin subunits in a wound site [26]. In addition, integrin plays a central role in wound healing by inducing tissue remodeling and controlling various cell functions (cell proliferation and survival and extracellular matrix remodeling).

Thus, in the present study, the effects of LIPUS exposure on wound healing and integrin α6β4 expression were examined using gingival epithelial cells (GE1).

2. Materials and methods

2.1. Cell culture

A GE1 cell line collected from the gingival epithelial tissue of an SV40 large T-antigen transgenic C57BL/6 mouse [27], purchased from RIKEN BRC CELL BANK (Tsukuba, Japan), was cultured in a chemically-defined medium SFM101 (Nissui, Tokyo, Japan) supplemented with 1% fetal bovine serum (FBS), 100 U/mL penicillin G, 100 mg/mL streptomycin, and 10 ng/mL murine epidermal growth factor (mEGF) in a humidified atmosphere of 5% CO_2 at the optimal temperature of 33 °C.

The cells were cultured in 3.5-cm culture dishes, with the media exchanged every other day.

2.2. Ultrasound treatment

Ultrasonic irradiation was performed with a LIPUS exposure system (BR-Sonic PRO, ITO, Tokyo, Japan). LIPUS exposure was carried out from the lower surface of the 3.5-cm culture dish through an ultrasound gel placed between a LIPUS probe (L size) and the dish.

LIPUS exposure was conducted at a frequency of 3 MHz, a temporal average intensity of 160 or 240 mW/cm^2, and exposure time of 15 min [22,24,28]. Ultrasound was delivered with pulse ratio of 1:4 in order to deliver only non-thermal effect to the cells [29]. During the LIPUS exposure, GE1 cells were maintained in a humidified atmosphere of 5% CO_2 and at an optimal temperature of 33 °C. To prepare a control, the same conditions were employed, except for the absence of LIPUS exposure.

2.3. Cell counts

For cell counting, cells were detached from the dish with trypsin–EDTA (0.05% trypsin and 0.02% EDTA in Hanks Balanced Salt Solution) (SAFC Bioscience, KS, USA). GE1 cells were seeded at 1.0×10^5 cells/mL, and were collected every other day from the day after seeding (1, 3, 5 and 7 days after the seeding), followed by measurement using a standard microscope with a hemocytometer. LIPUS exposure at 3 MHz frequency, 160 mW/cm^2 intensity was performed every 24 h from the day after seeding.

2.4. Scratch assay

GE1 cells were seeded at 4.0×10^5 cells/mL. Two days later, confluent cells were linearly scratched using a 20-μL pipette chip. The scratched region was photographed immediately and every 12 h after scratching using a microscope equipped with a camera.

The photograph was traced to tracing paper, followed by the coloring of cells with image editing software (FireAlpaca).

Subsequently, the area with cells as a percentage of the total area was determined using the area measurement function of VHX-5000 (Keyence, Osaka, Japan). LIPUS exposure at 3 MHz frequency, 160 and 240 mW/cm^2 intensity was performed immediately and every 24 h after scratching.

2.5. *RNA isolation*

GE1 cells were seeded at 4.0×10^5 cells/mL. Two days later, confluent cells were exposed to LIPUS at 3 MHz frequency, 160 mW/cm^2 intensity. Immediately after the LIPUS exposure, total RNA was extracted with a RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA purity was determined from the ratio of 260- and 280-nm absorbance.

2.6. *Array comparative genomic hybridization analysis*

Array comparative genomic hybridization analysis was conducted using a SurePrint G3 Mouse Gene Expression 8x60K Microarray Kit (Agilent Technologies). For cDNA synthesis, RNA from two samples (100 ng, control or LIPUS) was reverse-transcribed using a poly dT primer with a T7 promoter and reverse transcriptase. By adding Cy dye (Cy3) to cDNA and T7 RNA polymerase, Cy3-labeled RNA was synthesized with a Low Input Quick Amp Labeling Kit. Subsequently, excessive Cy dyes were removed with an RNeasy mini kit for probe purification.

The purified probe (480 ng) from two samples was hybridized onto a DNA microarray chip (Gene Expression Hybridization Kit, Agilent Technologies) using a hybridization oven (Agilent Technologies) at 65 °C and 10 rpm for 17 h. Subsequently, the image data read by a scanner (Agilent Technologies) with a scan control (Agilent Technologies) were digitized using dedicated software, Future Extraction (Agilent Technologies). The digitized data were analyzed using GeneSpring GX (Agilent Technologies).

2.7. *Real-time PCR*

Complementary DNA (cDNA) was extracted from the total RNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems, USA). A reaction mixture was incubated for 60 min at 37 °C, heated for 5 min at 95 °C, and cooled at 4 °C. DNA amplification was carried out by real-time polymerase chain reaction against target genes, integrin α6 (Mm00434375_m1), integrin β4 (Mm01266840_m1), and 18s rRNA (Hs99999901_s1) as an endogenous control, using Taqman Universal PCR Master Mix (Applied Biosystems) with thermal cycling parameters (50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, followed by 60 °C for 1 min). At that time, ABI Prism 7300 (Applied Biosystems, USA) was employed as a real-time PCR system in a 96-well plate (Applied Biosystems, USA).

2.8. *Western blotting and analysis*

GE1 cells were seeded at 4.0×10^5 cells/mL. Two days later, the confluent cells were exposed to LIPUS for 15 min. Immediately after the LIPUS exposure, proteins were extracted using an EzRIPA Lysis kit with protease and phosphatase inhibitors (ATTO Corp, Tokyo, Japan).

The samples, supplemented with Ez Apply (ATTO Corp, Tokyo, Japan), were electrophoresed on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Nonspecific binding was blocked by immersing the membranes in protein-free blocking buffer (EzBlock Chemi, ATTO Corp, Tokyo, Japan) for 1 h at room temperature. Then, the membranes were incubated with primary antibody (rabbit monoclonal (EPR18124) to integrin α6, diluted to 1:2000 for 1 h at below 4 °C, rabbit monoclonal (EPR17517) to integrin β4, diluted to 1:1000 for 1 h at below 4 °C, and rabbit polyclonal to β actin, diluted to 1:1000 for 1 h at below 4 °C, Abcam, Cambridge, UK) followed by peroxidase-conjugated IgG fraction monoclonal mouse anti-rabbit IgG secondary antibody (Jackson ImmunoResearch, Europe, Ltd) for 1 h. After washing, immunoreactive proteins were visualized on the membranes using enhanced chemiluminescence (P membrane, ATTO Corp, Tokyo, Japan) and analyzed with a luminescent image analyzer (ImageQuant LAS 500, GE Healthcare, Cambridge, UK).

2.9. *Statistical analysis*

Computation and Statistical analyses were conducted with GraphPad Prism 5.0 for Mac OS X (GraphPad Software Inc., San Diego, CA, USA). The data are expressed as the mean ± SD. The unpaired *t*-test with Welch's correction was employed for comparison between the groups (control *vs.* LIPUS). A *P*-value of <0.05 was used to determine a significant difference.

3. Results

3.1. *Cell counts*

The number of cells at 5 days after LIPUS exposure was significantly higher than that of the control ($P < 0.05$, 1.4-fold), while that at 7 days was about 35% higher than that of the control, although there was no significant difference ($P < 0.05$, 1.3-fold) (Fig. 1).

3.2. *Scratch assay*

The scratch assay demonstrated that the cell area of 0% in the control immediately after scratching increased to 21% at 12 h, 32% at 24 h, 51% at 36 h, and 78% at 48 h after scratching, while that in the LIPUS exposure at 160, 240 mW/cm^2 intensity increased to 36%, 29% at 12 h, 50%, 42% at 24 h, 86%, 82% at 36 h and 97%, 91% at 48 h after scratching, respectively. Thus, the cell areas in the LIPUS exposure at 160 and 240 mW/cm^2 were significantly increased at all time points compared to that in the control ($P < 0.05$) (Fig. 2).

3.3. *Array comparative genomic hybridization analysis*

Comprehensive analysis of all 55,821 genes by array comparative genomic hybridization demonstrated more than two fold up- and down-regulations for 1829 and 2206 genes in the LIPUS exposure group, compared with the control, respectively (Fig. 3a). We showed some kind of summary with regard to the up- or down-regulation of adhesion-related molecules (Fig. 3b).

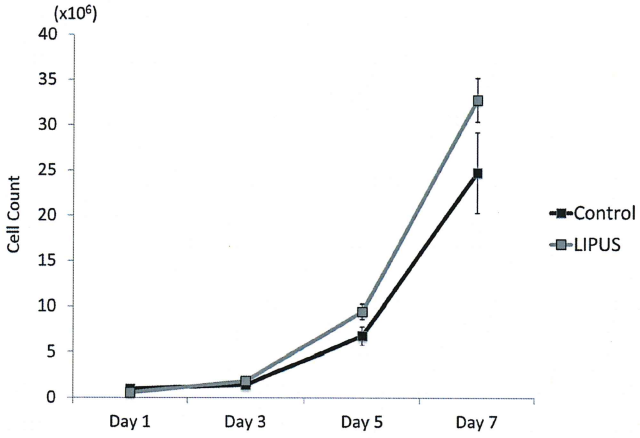


Fig. 1 – LIPUS exposure promoted the proliferation of GE1 cells. From the day after seeding, GE1 cells were continuously exposed to LIPUS every 24 h to be measured with a microscope using a hemocytometer. On day 5 after LIPUS exposure, the number of GE1 cells significantly increased as compared with the control. On day 7, cell proliferation was promoted, although there was no significant difference in the number of cells ($n = 3$, $^*P < 0.05$).

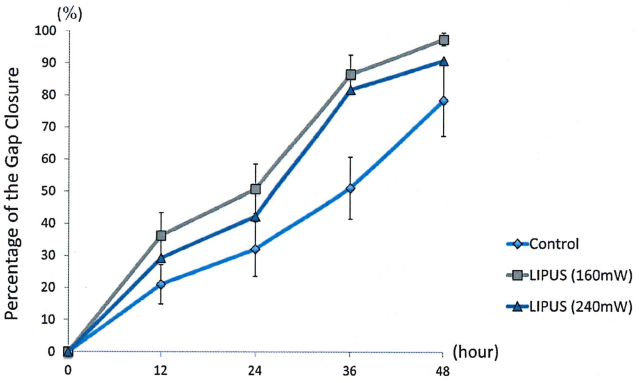


Fig. 2 – LIPUS exposure promoted gap closure in GE1 cells. Two days after seeding, confluent GE1 cells were exposed to LIPUS to conduct the scratch assay. The percentages of gap closure were determined every 12 h after scratching. As a result, LIPUS exposure significantly promoted gap closure as compared with the control ($n = 5$, $^*P < 0.05$).

3.4. *Real-time PCR, western blotting and analysis*

The real-time polymerase chain reaction demonstrated 2.5- and 3.2-fold significantly increased mRNA expression levels for integrins α6 and β4 in the LIPUS exposure group, as compared with the control, respectively (Fig. 4a). Western blotting demonstrated increased expression levels of integrins α6 and β4 in the LIPUS exposure group as compared with the control (Fig. 4b).

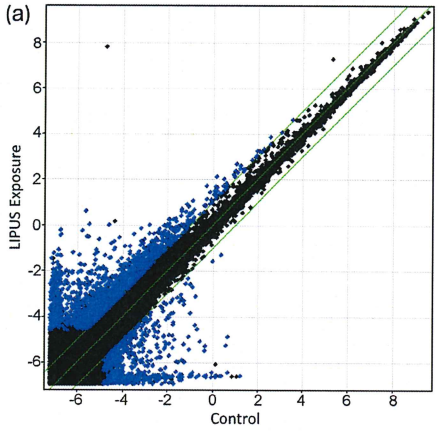
4. Discussion

The scratch assay in the *in vitro* study demonstrated that LIPUS exposure promoted the proliferation and migration of gingival epithelial cells and accelerated the wound healing of soft tissue. This supports previous findings that LIPUS enhances palatal mucosa wound healing in rats [22] and that LIPUS accelerates periodontal wound healing after flap surgery [23].

LIPUS has been widely used in clinical practice for fracture healing. The effects of LIPUS on hard tissue healing are well known. LIPUS exposure for fracture healing has been performed at a frequency of 1 or 1.5 MHz [12–17]. Previous studies demonstrated that the bone mineral content in a rabbit fibula fracture site after LIPUS exposure at 1.5 MHz was higher than that at 3 MHz [30] and that, when calvarial osteoblasts were exposed to LIPUS at 1 MHz, the expression levels of Runx2-associated bone formation-related genes increased [31]. Moreover, LIPUS exposure at 1 MHz accelerates healing on low-turnover osteoporosis by promoting bone formation [32]. Thus, the frequency of 1 or 1.5 MHz, which are effective for wound healing of deep tissue, may be desirable for fracture healing in orthopedic fields.

For soft tissue, LIPUS exposure is performed in clinical practice at a frequency of 3 MHz, which is effective for relatively shallow wounds [33]. Thus, in the present study, LIPUS exposure was performed at a frequency of 3 MHz through a gel from the bottom of a culture dish.

(a)



(b)

Representative up-regulated genes	Representative down-regulated genes
Integrin α6, β4	Integrin β6
Claudin 1, 4, 5	Claudin 7, 18, 24
Keratin 5, 13,14	Keratin 19, 28, 32
Involcrin	Cadherin 6, 20, 23
Filaggrin	Occludin

Fig. 3 – The results of array comparative genomic hybridization analysis are shown as a scatter plot diagram (a). The array comparative genomic hybridization was conducted with mRNA collected from GE1 cells immediately after LIPUS exposure using a SurePrint G3 Mouse Gene Expression 8x60K Microarray Kit (Agilent Technologies). As a result, 1829 and 2206 genes were up- and down-regulated more than twofold after LIPUS exposure, respectively, as compared with the control. We showed some kind of summary with regard to the up- or down-regulation of adhesion-related molecules (b).

For the other operating parameter for LIPUS, intensity, Min et al. confirmed that LIPUS exposure is most effective at an intensity of 200 mW/cm^2 (considered 40–700 mW/cm^2) for soft tissue [28]. The adopted device (Br-Sonic PRO) in the present study had selective ultrasound power of 160, 240 and 320 mW/cm^2, and in considering the effective intensity for GE1 cells, scratch assay was applied using 160 and 240 mW/cm^2. As a result, gap closure occurred earlier at 160 mW/cm^2 as compared with 240 mW/cm^2 (Fig. 2). Thus, further experiments were conducted at an output of 160 mW/cm^2.

Cell proliferation and migration are closely involved in epithelial wound healing in the scratch assay. The wound site contracts as cells surrounding the wound are retracted, and, finally, lamellae are produced to complete wound closure. At that time, integrin α6, especially α6β4, promotes contraction of the wound site; Integrin α6 and α3β1 contribute to lamella formation [34].

Furthermore, in the present study, comprehensive gene analysis by microarray assay demonstrated that LIPUS exposure increased the expression levels of adhesion-related genes, such as integrin, claudin, involucrin and filaggrin.

Among those, claudin1, 4, 5, involucrin, filaggrin showed increased expression levels of mRNA with microarray assay but did not show significant up-regulation with real-time PCR (data not shown). On the other hand, integrin α6, β4 showed markedly increased expression levels of mRNA with microarray assay and also showed significant up-regulated expression levels of mRNA with real-time PCR. However, other integrin subunits did not show pronounced expression levels of mRNA with microarray assay.

Hence, we examined the proliferation, survival, extracellular matrix remodeling, and the effects on integrin α6β4, a gene involved in wound healing, in a mouse-derived gingival epithelial cell line.

As a result, the LIPUS exposure group showed up-regulated mRNA and protein expression levels of integrin α6β4, as well as a significant increase in the cell growth and early closure of a wound site by the scratch assay. These results cannot reveal mechanisms for promoting wound healing by LIPUS exposure, but show that up-regulated integrin α6β4 expression by LIPUS exposure may promote soft tissue healing *in vitro*.

Many reports have been published on the roles of integrin α6β4. For example, the loss of integrin α6β4 leads to separation of the epidermal from dermal layers of the skin at the site of the basement membrane zone, skin blistering, and post-natal lethality [35–39]. In addition, the increased expression level of integrin α6β4 facilitates stable adhesion when skin cell cause motile behavior associated with healing and tumorigenesis. Furthermore, integrins α2 and α3 may control whole cell movements. Particularly, integrin α3β1 promotes matrix proteolysis during wound healing or tumorigenesis [40], and skin cell migration is suppressed by inhibiting integrin α3β1[41]. Similarly, integrin α2β1 controls cell migration by promoting matrix proteolysis [42]. According to a recent report, integrin expression in skin cells plays an important role in promoting wound healing and tumorigenesis, although its

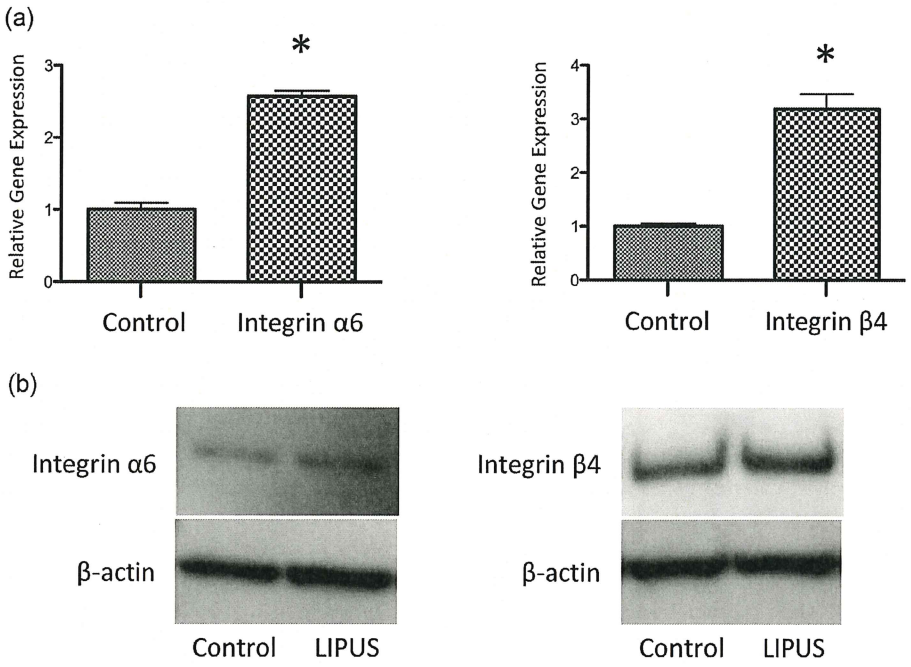


Fig. 4 – LIPUS exposure enhanced the expression levels of integrin α6 and β4 in GE1 cells by the real-time PCR (a) and western blotting (b). The expression levels of integrin α6 and β4 were analyzed using the real-time PCR with mRNA collected from GE1 cells immediately after LIPUS exposure. Western blotting analysis was conducted as described in Section 2. For both the real-time PCR and western blotting, culture media were collected from compressed GE1 cells immediately after LIPUS exposure.

mechanism remains unclear. According to another report, keratinocytes deficient in integrin α6 showed not only the same pattern of aberrant motility as integrin β4-deficient cells, but also loss of integrin α2β1 and α3β1 expressions, suggesting that integrin α6β4 regulates the expressions of integrins α2 and α3[26,43].

The comprehensive analysis of 55,821 gene expressions using a microarray demonstrated up-regulated expression levels for many adhesion-related genes, besides integrins α6 and β4. Thus, further investigation to analyze other adhesion-related genes, besides integrins α6 and β4 is warranted.

5. Conclusions

LIPUS exposure on GE1 cells was shown to promote their growth in number. Also, the scratch assay on GE1 cells revealed the acceleration of gap closure by LIPUS exposure. Furthermore, microarray, real-time PCR and western blotting assays have shown that LIPUS exposure on GE1 cells up-regulates mRNA and protein expressions of integrin α6 and β4. The present study should help reveal the effects of LIPUS exposure on the human body.

Conflict of interest

The authors have no conflict of interest with respect to the manuscript content or funding.

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