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Localized Proliferative Effect of Preadipocytes on Cultured Human Epidermal Keratinocytes

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The effect of preadipocytes (ST 13) on cultured normal human epidermal keratinocytes (NHEK) was investigated. The growth of NHEK was accelerated with co-cultured ST 13 cells. This stimulative effect must have been localized around viable ST 13 cells, because neither the medium nor the surface conditioned by ST 13 or the ST 13 cell fragments showed any promotion of NHEK growth, and NHEK showed a compact, paving-like arrangement only when they were attached directly to ST 13 cells. It became clear that these compactly arranged keratinocytes have an active proliferative ability, since their nuclei showed a marked uptake of 5-bromodeoxyuridine (BrdU) and they were positively stained with anticytokeratin 37, a monoclonal antibody against the basal epidermis. Under electron microscopy, ST 13 preadipocytes were closely connected with NHEK. These results, together with those of previous reports, suggest that the localized proliferative effect of ST 13 cells on NHEK is due to cell-to-cell contact. ***Key word: Cell-to-cell contact.***

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Epidermal keratinocytes are often co-cultured with fibroblastic 3T3 cells (1–3). The effect of 3T3 cells on keratinocyte growth has been studied from three viewpoints: soluble factors secreted by these cells to form a conditioned medium (CM), extracellular matrices synthesized by them to form a conditioned surface (CS), and cell-to-cell contact between keratinocytes and 3T3 cells. However, different conclusions about the effects of 3T3 cells on the growth of keratinocytes have been reached by different authors (2, 4–6).

3T3 cells have been converted into adipose cells in vitro (7). We have previously studied the effect of mouse preadipocytes (ST 13) (8) on the growth of cultured mouse epidermal keratinocytes, and showed that ST 13 cells in the undifferentiated (fibroblast-like) state exhibited a promoting effect on the growth of mouse keratinocytes, but that those in the differentiated (adipocytes) state did not (9). The epidermis (epidermal keratinocytes) is connected to the dermis (dermal fibroblasts). Adipose cells exist under the dermis and are not usually in contact with the epidermis, because of the dermal layer. It is possible that preadipocytes in the undifferentiated state are in contact with the epidermis during wound healing in vivo, and it is unlikely that those in the differentiated state (adipose cells) are. Therefore, our previous findings, described above, appear to fit a control in vivo. Thus, preadipocytes (ST 13), like 3T3 cells, are very useful in studying the tripartite epidermis-dermis-fatty tissue interaction. In the present study, we investigated the effect of preadipocytes (ST 13) in the undifferentiated state on the growth of cultured normal human epidermal keratinocytes (NHEK) and examined the type of effect of preadipocytes (dermis) on keratinocytes (epidermis).

MATERIAL AND METHODS

Cell culture

NHEK were purchased from Sanko Junyaku Co., Tokyo, Japan, and were grown in K-GM (Sanko Junyaku). These NHEK were obtained from female breast skin and were used at the third passaged culture. K-GM are composed of modified MCDB 153 medium supplemented with bovine pituitary extracts 0.4% v/v, hydrocortisone 0.5 μg/ml, gentamicin 50 μg/ml, amphotericin B 0.25 μg/ml, EGF 10 ng/ml and insulin 5 μg/ml. Preadipocytes (ST 13) were a gift from Dr. A. Hiragun (Department of Oncology, The Tokyo Metropolitan Institute of Medical Science). A stock culture of ST 13 was grown in Eagle's minimal essential medium (MEM) (Nissui, Tokyo), containing 5% fetal calf serum (FCS) (Gibco Lab, Life Technologies Inc, Grand Island, N.Y.). These cells were grown at 37°C in a humidified 5% CO_2 atmosphere.

Preparation, and determination of suitable titer, of ST 13

ST 13 cells were treated with 4 μg of mitomycin C (MMC) per milliliter of medium and incubated at 37°C for 2 h. The cells were then washed, harvested and seeded onto 3.5-cm dishes at the titer of 5×10^3, 8×10^3 and 10×10^3 cells/dish.

Confirmation of ST 13 growth arrest induced by MMC treatment

To confirm the growth arrest of MMC-treated ST 13 cells, only ST 13 cells treated with MMC were seeded at 5×10^3 cells/dish. Non-treated ST 13 cells for control were seeded at the same titer. The medium was replaced by a new one every other day. The cell growth was determined 8 days later by counting trypsin-dispersed cells on a hemocytometer.

Influence of ST 13 cells on growth of NHEK

ST 13 cells treated with MMC were seeded at 5×10^3 cells/dish. On the following day, NHEK were seeded at 5×10^4 cells/dish. The medium used for the co-culture of ST 13 and NHEK was Eagle's MEM containing 10% FCS. The medium was renewed every other day. Cell growth was determined after 4 and 8 days.

Influence of ST 13 conditioned medium (CM) and conditioned surface (CS) on growth of NHEK

CM: All of the culture medium in each dish incubated with MMC-treated ST 13 cells for 2 days was centrifuged at 350 g for 5 min to remove cell debris and immediately used for culturing NHEK by replacing all of the culture medium in which the NHEK were incubated on the 1st, 2nd, 3rd, 5th and 7th day of culture.

CS: MMC-treated ST 13 cells were seeded, then removed 3 days later with 0.02% ethylenediaminetetraacetic acid (EDTA), and were immediately used for the culture of NHEK.

CM + CS: NHEK were cultured with the above conditioned medium on the above conditioned surface.

The medium used for the conditioning experiments (CM, CS) was Eagle's MEM containing 10% FCS. All cell growth was determined 4 and 8 days later.

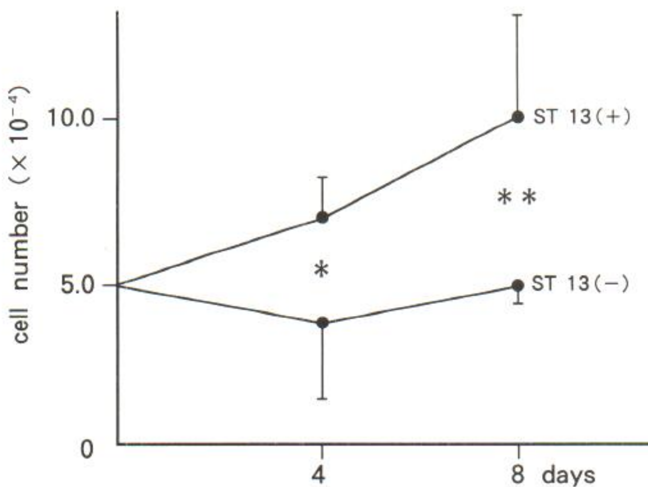


Fig. 1. Effect of preadipocytes (ST 13) on growth of normal human epidermal keratinocytes (NHEK). ($n=4$, values are mean ± SD) $^{*}p<0.1$; $^{**}p<0.05$.

Influence of ST 13 cell fragments

ST 13 cells were washed, harvested and fragmented with ultrasound. The solution containing ST 13 cell fragments (38 μl at once; ST 13 cell titer in each dish, 1×10^4 cells/dish) were added to NHEK cultures on the 2nd, 4th and 6th day. The medium used for the culture of NHEK with ST 13 cell fragments was Eagle's MEM containing 10% FCS. Cell growth was determined on the 8th day.

Immunohistochemical staining

5-bromodeoxyuridine (BrdU) staining: NHEK cultured with ST 13 cells on Lab-Tek chamber slides (Nunc, Inc., Naperville, Illinois, USA) were reacted 8 days later with BrdU (Seikagaku Kogyo Co., Ltd., Tokyo, Japan) (10 μM) for 1 h, then fixed with 70% ethanol for 30 min. Next, they were washed with Ca^{++}-free, Mg^{++}-free Dulbecco's saline, PBS^-, and treated with 4 N HCl at room temperature for 30 min. Finally, immunohistochemical staining of the cells was performed according to the ABC method (10), using anti-BrdU antibody (at a 1:60 dilution; Becton Dickinson, CA, USA). The specimens were observed by light microscopy, and the labeling index was calculated.

Monoclonal anti-cytokeratin 37 antibody staining: NHEK cultured with ST 13 cells on Lab-Tek chamber slides (Nunc) were fixed 8 days later with pre-cooled acetone (-20°C) for 20 min. They were then washed with PBS^-, and immunohistochemically stained by the ABC method, using monoclonal anticytokeratin 37 antibody (at a 1:400 dilution; BioMakor, Rehovot, Israel). The specimens were observed by light microscopy.

Electron microscopy

NHEK cultured with ST 13 cells on plastic cover slips (Thermonox®) (Nunc) were fixed 8 days later with 3% glutaraldehyde in 0.025 M phosphate buffer (pH 7.4) at 4°C for 30 min, post-fixed with 2% osmium tetroxide in the same buffer at 4°C for 30 min, then dehydrated in graded concentrations of alcohol and propylene oxide, and finally embedded in epon. Ultrathin sections were prepared using an ultramicrotome (LKB 8800 Ultrotome® III), mounted on grids, double-stained with 1% uranyl acetate and lead citrate, and viewed under a JEM-100C electron microscope.

RESULTS

MMC-treated ST 13 cells resulted in confluent cultures when seeded at 10,000 cells/dish, and were subconfluent at 8,000

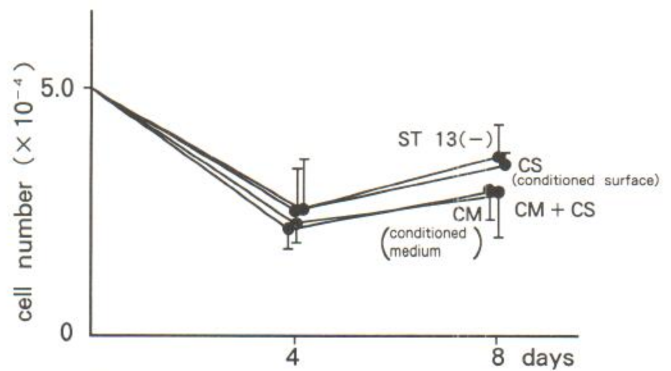


Fig. 2. Effect of medium and of surface conditioned by ST 13 cells (CM, CS) on growth of NHEK. ($n=4$, values are mean ± SD).

cells/dish. At 5×10^3 cells/dish, ST 13 cells were moderately dispersed, and so we studied the influence of ST 13 cells at that titer on the growth of NHEK.

We confirmed that the growth of ST 13 cells was arrested by MMC, because the number of cells counted 8 days after seeding was almost the same as that counted at seeding (5×10^3 cells/dish) (data not shown), and there was no difference in morphology between the ST 13 cells 8 days and 2 days after seeding. Non-treated ST 13 cells became confluent 8 days later.

The growth of NHEK both with ST 13 cells and without them is quite poor on the 4th day of culturing. This may be in part accounted for by the fact that the cells were derived from an adult donor. But the growth of NHEK 8 days after seeding was significantly better with ST 13 cells than without them ($p<0.05$) (Fig. 1), indicating that the co-cultured ST 13 cells accelerated NHEK proliferation. In the determination of cell growth, the markedly smaller size of NHEK clearly distinguished them in the hemocytometer from ST 13 cells.

Next, we tested whether the medium and the surface conditioned by ST 13 cells (CM, CS) promoted the growth of NHEK, and found that neither CM, nor CS or CM with CS stimulated NHEK growth. In all cases, the growth of NHEK was extremely poor, showing a decrease in number 4 and 8 days later, just as when no ST 13 cells were present (Fig. 2).

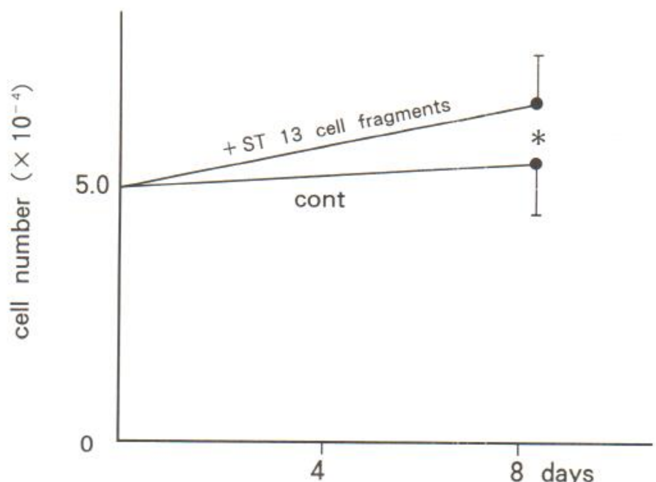
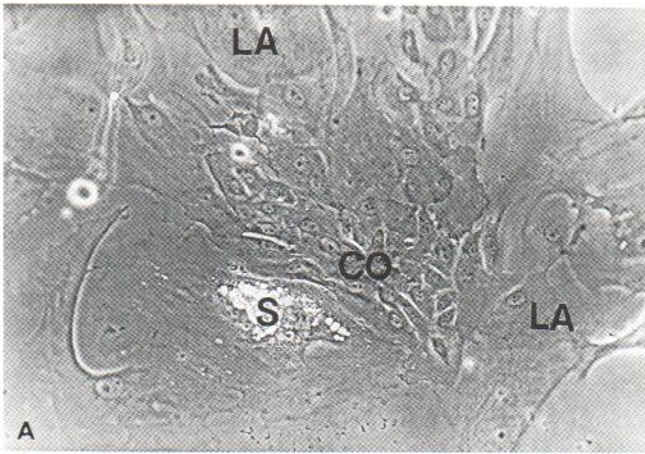


Fig. 3. Effect of ST 13 cell fragments on growth of NHEK. ($n=4$, values are mean ± SD) * not significant.



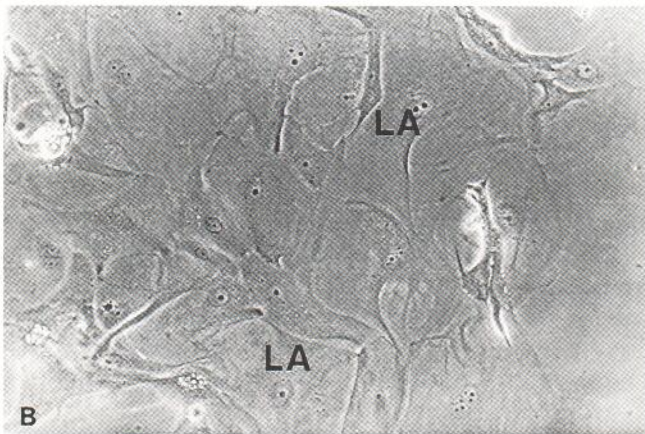


Fig. 4A, B. Morphology of cultured NHEK. A: With ST 13 cells. S, ST 13 cells; CO, NHEK showing compact, paving-like arrangement; LA, large, flat NHEK. B: Without ST 13 cells.

Furthermore, ST 13 cell fragments caused no significant stimulation of NHEK growth (Fig. 3).

When MMC-treated ST 13 cells were seeded at the titer of 5×10^3 cells/dish, the subsequently seeded NHEK formed the colonies in direct contact with one or two ST 13 cells. Interestingly, those NHEK attached directly to ST 13 cells exhibited a compact, paving-like arrangement (Fig. 4A). The other NHEK had a large, flat shape. All NHEK without ST 13 cells were large, flat cells without any tendency toward compactness (Fig. 4B). In order to ascertain whether the compactly arranged cells had any mitotic ability or not, we performed BrdU staining to determine the distribution of cells in the S-phase (11). In our specimens, the nuclei of the compactly arranged cells were conspicuously labeled (Fig. 5), but no labeled nuclei were seen in the large, flat cells. The labeling index of these compactly arranged cells showed almost the same values as when NHEK were cultured in serum-free growth medium, K-GM (Fig. 6). Furthermore, the compactly arranged cells showed positive staining with anti-cytokeratin 37 antibody (Fig. 7A). The large, flat cells, in contrast, showed negative or weak positive staining (Fig. 7B).

Under electron microscopy, ST 13 cells were closely connected with NHEK (Fig. 8). Numerous smooth- and rough-surfaced endoplasmic reticula can be observed in the ST 13 cell. Many tonofilaments were seen in NHEK.

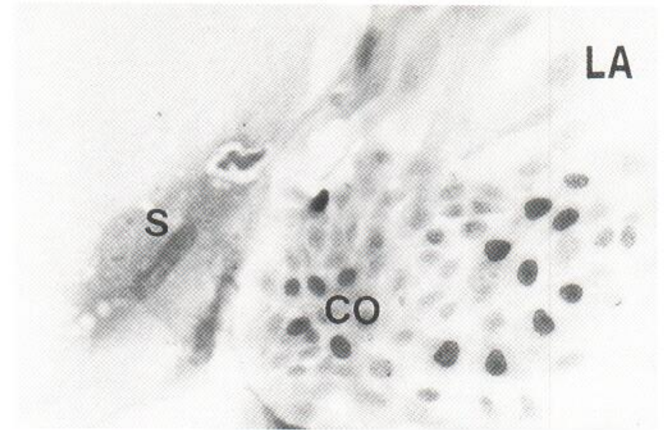


Fig. 5. BrdU staining of NHEK with ST 13 cells. S, ST 13 cells; CO, NHEK showing compact, paving-like arrangement. LA, large, flat NHEK.

DISCUSSION

We have investigated the effect of preadipocytes (ST 13) on the growth of cultured normal human epidermal keratinocytes (NHEK). ST 13 cells had a proliferative effect on NHEK growth (Fig. 1). Neither the medium (CM) nor the surface (CS) conditioned by ST 13 cells or the ST 13 cell fragments caused any promotion of NHEK growth, and NHEK showed a compact arrangement only when attached to ST 13 cells. The

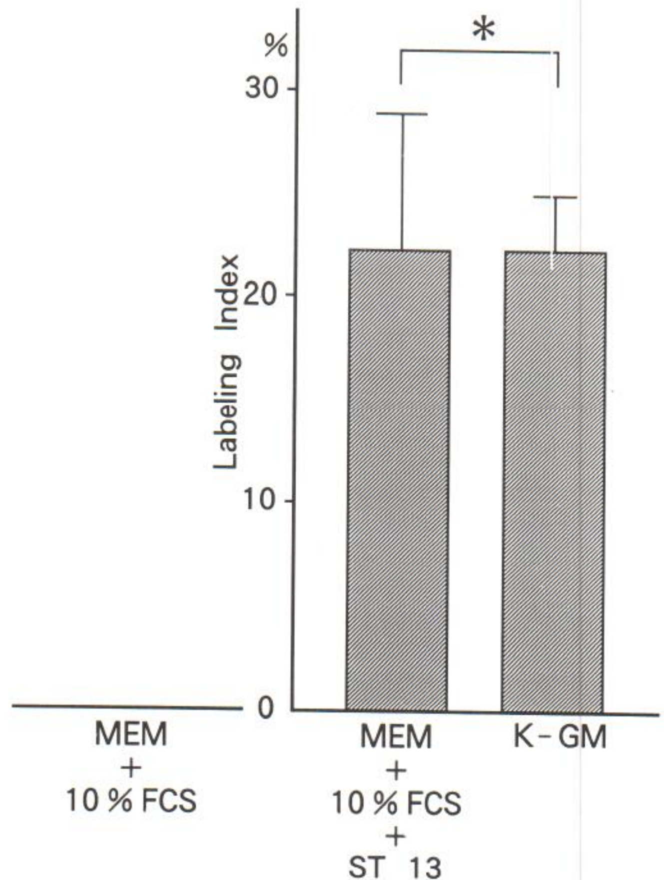
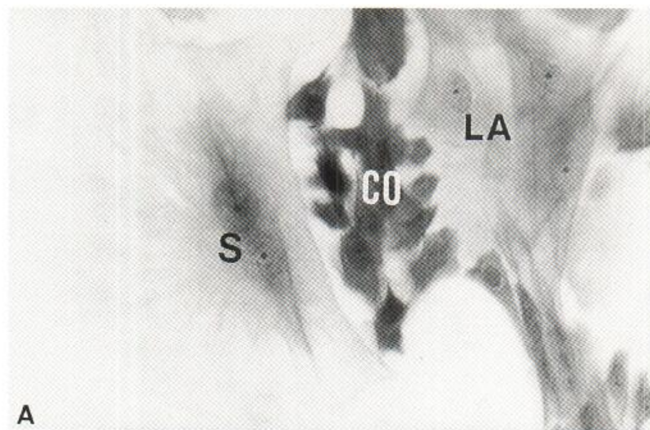


Fig. 6. Labeling index of the compactly arranged cells. ($n=3$, values are mean ± SD) * not significant.



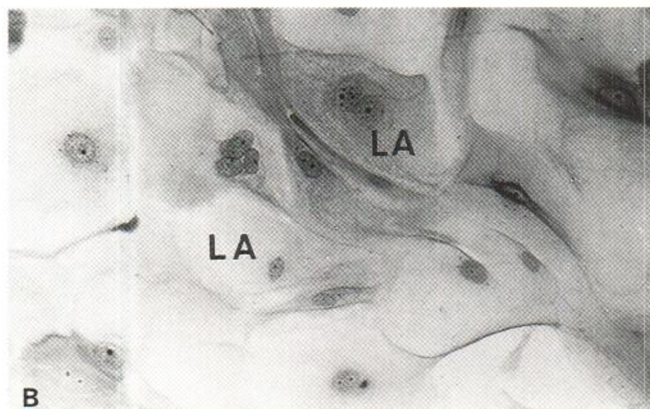


Fig. 7 A, B. Monoclonal anti-cytokeratin 37 antibody staining. A: With ST 13 cells. S, ST 13 cells; CO, NHEK with compact, paving-like arrangement. LA, large, flat NHEK. B: Without ST 13 cells.

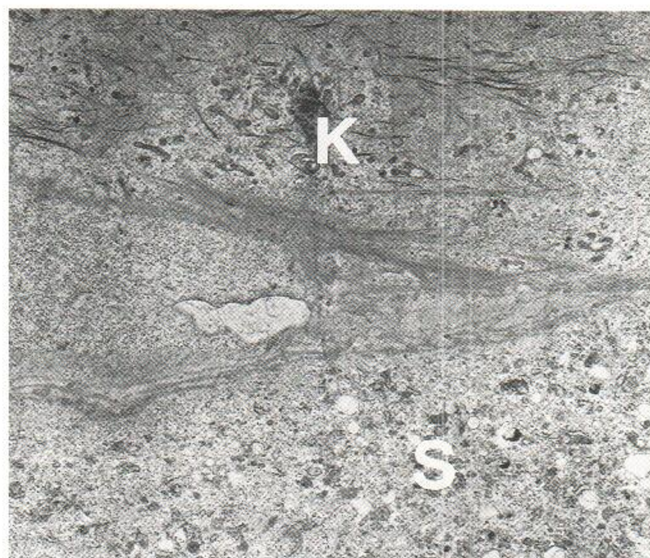


Fig. 8. Electron micrograph of preadipocyte (ST 13: S) and normal human epidermal keratinocyte (NHEK: K). × 3,200.

CM and CS that we used were similar to those in the environment described under influence of ST 13 cells on the growth of NHEK in Material and Methods. It may be that CM and CS obtained with a high cell density promote NHEK growth, as has been seen in a system with 3T3 cells (2). ST 13 cells were cultured at a low density because they are much larger than 3T3 cells. The stimulative effect of ST 13 on NHEK growth that we saw was probably derived from a localized micro-environmental effect around viable ST 13 cells.

The compactly arranged NHEK in direct contact with ST 13 cells proved to have an active proliferative ability, because they were distinctly labeled by BrdU staining (Fig. 5), and showed positive staining with anticytokeratin 37 antibody (Fig. 7A), indicating that they had keratins similar to those of the basal cells of epidermis (12). Electronmicroscopically, ST 13 cells were connected closely with NHEK. Murray & Fitzgerald (4) demonstrated the direct transfer of labeled uridine between the cells of a continuous epidermal line and Swiss 3T3 cells. Miller et al. (5) showed that epidermal cell contact with metabolizing BALB/c 3T3 clone A 31 cells was probably necessary for the feeder effect. Kamalati et al. (6) observed that SVK14 cells (SV40-transformed human keratinocyte line), when co-cultured with Swiss 3T3, form organized structures through specific cell-to-cell interactions. Cell-to-cell contact may be involved through either surface recognition or modification, or through direct transfer of important molecules (5). These previous reports, and our findings that NHEK directly attached to ST 13 cells proliferated actively, that ST 13 cells had to be viable in order to promote NHEK growth, and that close ultrastructural connections were formed between ST 13 cells and NHEK, suggest that viable ST 13 cells exert their stimulative effect on the growth of NHEK through cell-to-cell contact.

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