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Biomedicine & Pharmacotherapy 58 (2004) 123–128
BIOMEDICINE & PHARMACOTHERAPY
www.elsevier.com/locate/biopha

Original article

Effect of L-fucose and fucose-rich polysaccharides on elastin biosynthesis, in vivo and in vitro

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Received 5 May 2003

Abstract

With increasing age elastic fibres in human skin are progressively lysed and skin elasticity is also decreasing. Still there is an age-dependent increase of elastic fibre surface density, mostly due to an alteration of the fibres. The present experiments were undertaken to explore if L-fucose and fucose-rich polysaccharides (FROP-s) could influence elastin biosynthesis. We show here, that topical application of a fucose-containing preparation to the skin of hairless rats increased after 4 weeks the elastic fibre surface density by about 40%, shown by quantitative morphology. Using human skin fibroblasts in explant cultures, the addition of L-fucose or of FROP-3 increased the biosynthesis of immunoprecipitable tropoelastin by about 40%. No increase was found however of desmosine–isodesmosine in skin explant cultures after 72 h of incubation. The effect of L-fucose and FROP-3 on the biosynthesis of collagen and non-collagen proteins excreted by the skin explant cultures was also investigated. L-Fucose, but not FROP-3, decreased collagen biosynthesis but both increased non-collagen protein biosynthesis. These results show that L-fucose and FROP-3 stimulate tropoelastin biosynthesis in vitro, and elastic fibre formation in vivo. This stimulation concerns also several non-collagen proteins secreted by skin explant cultures. Elastic fibre formation necessitates the simultaneous synthesis of several microfibrillar glycoproteins as well as of tropoelastin. The increased elastic fibre density in the in vivo experiments suggests that this is indeed achieved by L-fucose and FROP-3, further demonstrating their efficiency in the control of age-dependent modifications of connective tissues in general and of skin in particular.

Keywords: Human skin; Fibroblasts; Explant cultures; Tropoelastin biosynthesis; Hairless rat skin; Elastic fibre surface density

1. Introduction

Among the age-dependent modifications of the extracellular matrix (ECM) one of the most intriguing process concerns skin elastic fibres. They form an intricate network in human skin with vertical, candelabre-type (oxytalan) fibres in the superficial dermis, anchored in arciform (elaunin) fibres, continuous with the more disordered elastic network of the deeper dermis [5,6]. With age, the superficial vertical fibres are progressively lysed and replaced by a mostly horizontally running fibre system, the net change is an age-dependent increase of the elastic fibre surface density of human skin [15]. In contradiction to this increase of elastic fibres, the elasticity of human skin decreases with age, somewhat faster in females than in males [14]. The loss of skin elasticity varies from 2.2% to more than 5% per decade, according to socioprofessional conditions [14]. These modifications of elastic fibres differ from those observed in elastic arteries as the aorta, where histochemical and biochemical methods showed an age-dependent loss of elastic fibres as well as elasticity [16,17]. The fragmentation of the concentric arterial elastic system is attributed to an age-dependent up-regulation of elastase-type proteases produced by the vascular smooth muscle cells [9,17]. Although some neosynthesis was demonstrated in injured vessels [12], no systematic, age-dependent resynthesis of vascular elastin was however demonstrated.

In order to interfere with this complicated age-dependent modifications of skin elastic fibres, we tested the possibility to modulate pharmacologically elastin biosynthesis by skin fibroblasts, in explant cultures, cell cultures and also in vivo in hairless rat skin, using L-fucose and FROP-3 as active principles [13]. Their favourable action on several mecha-

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doi:10.1016/j.biopha.2003.12.001

nisms involved in the age-dependent modifications of skin was previously demonstrated [3,7,13]. Here we show that both L-fucose and FROP-3 increased elastic fibre surface density in rat skin and increased also tropoelastin biosynthesis in vitro.

2. Materials and methods

Hairless rats, male, initial weight about 250 g, came from Evic-Ceba, Bordeax, France, and were kept in individual cages on industrial laboratory chow (UAR, France) and drinking water ad libitum.

A pre-marked surface on both sides of the animals was treated. On the left side was applied a base-cream (Chronos line) containing 1% L-fucose. Ten milligrams cream was applied per cm^2 of skin surface, by rubbing in the preparation.

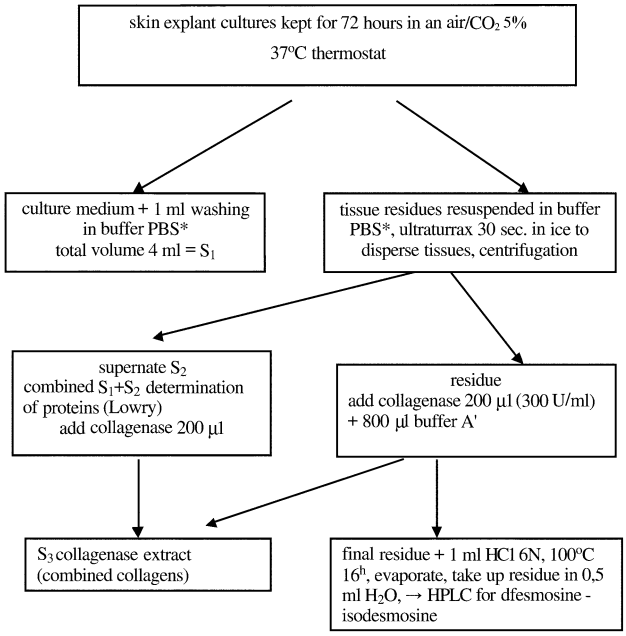
The other (left) side of the animals was used as control, treated only with the base-cream without L-fucose. One daily treatment was made on both sides of the animals during 4 weeks. The animals were than sacrificed by overanesthesia (Vetonarcol).

Before cutting out the biopsy-skin samples, a plastic ring was fastened on the skin surface by a glue, in order to avoid shrinking of the skin, and to keep the original size and area of the skin sample after excision. The excised skin sample was treated for histology, by fixation in Bouin's solution for 24 h and embedded in paraffin for sectioning.

The 5 μm sections (Reichert microtome) were stained for elastic fibres by Verhoeff's procedure with basic fuchsine and destained in successive alcohol baths until there was no more background staining. Some sections were stained by hematoxylin–eosin for control, and for the confirmation of epidermal thickness. The obtained preparations were used for the evaluation by image-analysis of the surface density (relative surface in a reference area) of the elastic fibres, using a Zeiss laboratory microscope fitted with a black-and-white Sony CCD video camera module. The final enlargement for the quantification of dermal elastic fibres was of 320×. The elastin surface density was determined with a Visiolab 1000 (Biocom, France) software. Each microscopic field is decomposed in 134 816 pixels (176 × 766). Elastic fibre density was measured on 60 different microscopic fields randomly selected in the dermis, and the result of 60 treated fields was compared to the results obtained on 60 control fields.

2.1. Explant and cell cultures

Human skin explant cultures were prepared by cutting in sterile conditions fresh human skin samples (mammary plastic operation of a 42-year-old Caucasian woman) in 1–2 mm^3 fragments, after scraping off the epidermal and subcutaneous fat layers. The fragments were incubated in Dulbecco-modified Eagle's MEM medium, with 10% fetal calf serum (FCS) and protease inhibitors (see Fig. 1) and 3 μCi/ml



* As PBS in Fig.2., but diluted 1:2 in water

Fig. 1. Flow-sheet for the method used to determine on human skin explant cultures the incorporation of ^{3}H-proline in total excreted proteins and in collagen, as well as the elastin crosslinks, desmosine and isodesmosine by HPLC. For other details see Section 2.

L-L_2-3′^{3}H-proline (Amersham, 370 kBq/ml). Incubation was carried out in a air/CO_2 (95%/5%) thermostat, with or without 1% L-fucose or FROP-3 added to the culture medium at a concentration of 10 μg/ml. After 72 h incubation the medium was separated from the tissue fragments, which were further treated as shown on the flow-sheet of Fig. 1. Proteins and incorporated radioactivity were determined in the recovered fractions, according to Lowry et al. [10] and on a Rack-β-counter. These explant cultures were used for the determination of total non-collagen proteins and collagen biosynthesis after separation of collagens from non-collagen proteins by digestion with a highly purified bacterial collagenase (Sigma C 0773 Type VII 1–3000 CDU/mg). The determination of elastin crosslinks, desmosine and isodesmosine, was done according to Cumiskey et al. [1], using a HPLC procedure. After 24 h hydrolysis at 100 °C in 6 N HCl, desmosine and isodesmosine were separated on a AccQ.Tag 3.9 × 150 mm silicium column, crosslinked with C18, on a Waters 600 E instrument, according to the method described [1]. Linear calibration curves were obtained, both for desmosine and isodesmosine between 50 and 600 pmol.

For tropoelastin determinations fibroblast cultures were used, derived from the ear-skin (otoplasty) of an 8-year-old boy. Cells were cultured as described [4] and passaged at a 1:2 split ratio. At the eighth passage 3×10^5 cells were seeded in 60 mm diameter culture-plates (Nunc) and incubated with 3 μCi/ml [^{3}H]-proline as described on the flow-sheet of Fig. 2.

A polyclonal anti-human elastin antibody was obtained from Elastin Products (PR 398, lot 981), diluted 1:200 for use

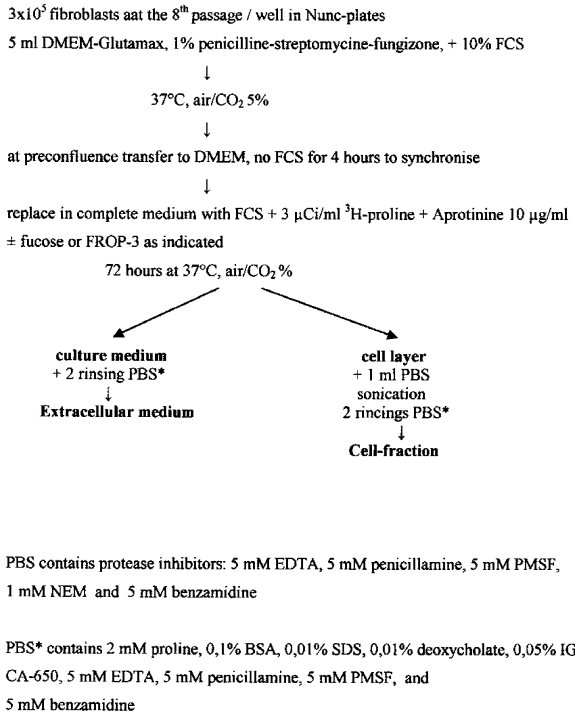


Fig. 2. Flow-sheet for the study of tropoelastin biosynthesis by human skin fibroblasts. For other details see Section 2.

in sterile saline. A monoclonal anti-κ-elastin antibody (A2-1), obtained from Dr. Fülöp (University of Sherbrook, Canada) was also used. After one night incubation at +4 °C a protein-A preparation (Sigma) was added, followed by centrifugation, three washings in PBS, resolubilised in a denaturing medium by heating to 100 °C for 10 min. After spinning for 10 min at 10 000 × *g* in the cold, the radioactivity of an aliquot was determined and total immunoprecipitated tropoelastin calculated. The specificity of tropoelastin immunoprecipitation was controlled by immunoblots after separation by PAGE of the precipitated proteins as well as staining the gels for proteins. Standard curves were prepared with recombinant human tropoelastin, kindly provided by Dr. Robert Mecham (Cell Biology Department, Washington University, St. Louis, MO, USA).

3. Results

3.1. In vivo experiments

As shown in Table 1, the surface density of elastic fibres in the hairless rat skin was increased by about 40% after 4 weeks of treatment with L-fucose.

When calculated as the total number of fibres per microscopic field, the average surface per fibre did not change significantly, suggesting no change in the quality of fibrillogenesis. Still, total fibre surface per field increased by 21%. Fig. 3 further illustrates this increase in fibre number per field. These experiments show that 4 weeks of treatment with the L-fucose-containing preparation resulted in a significant

Table 1
Effect of 4 weeks of topical application of a 1% L-fucose-containing cream on skin elastic fibre density of hairless rats. For details see Section 2

	Total surface density of fibres per field	Average surface density per fibre	Total number of fibres per field
Control rats	9536	49.64	192
Treated rats	11 568	43.2	268
Difference	+21.3%	N.S.	+40%

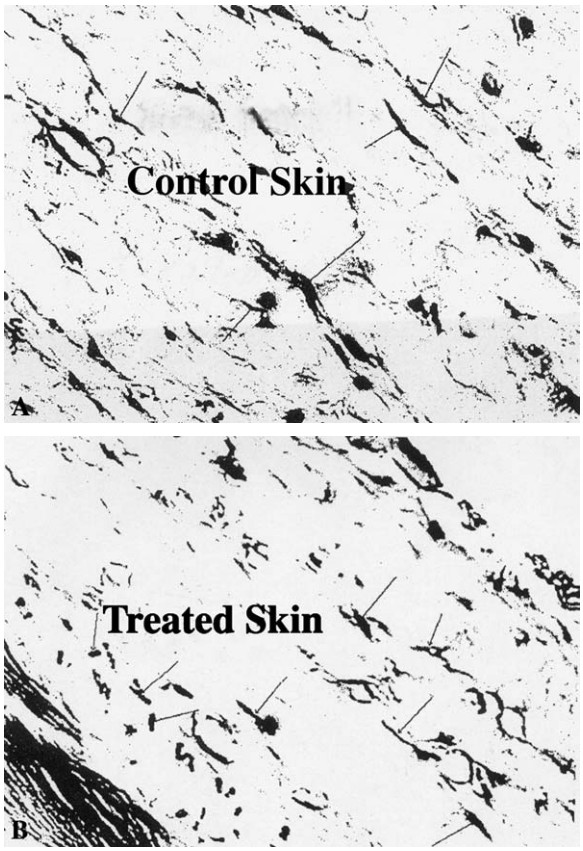


Fig. 3. Histological sections showing elastic fibres in the hairless rat skin, untreated (top) and treated (bottom) with a 1% fucose-containing cream. For other details see Section 2.

increase in elastic fibre surface density of the hairless rat skin as compared to the skin samples treated with the cream without L-fucose.

3.2. In vitro experiments

3.2.1. Tropoelastin biosynthesis in fibroblast cell cultures

As shown in Fig. 4, both L-fucose and FROP-3 increased tropoelastin biosynthesis. At 1 µg/ml L-fucose had no effect yet, but at 10 µg/ml it produced an increase of about 40%. FROP-3 did produce a significant increase already at 1 µg/ml. Further increase of its concentration resulted however in a decrease of stimulation.

Immunoblots of the dissociated immunoprecipitates showed a strong double-band at about 72 kDa, and also a weaker band at about 118 kDa (Fig. 5A). Control immunoblots, using the recombinant tropoelastin preparation (Fig. 5B)

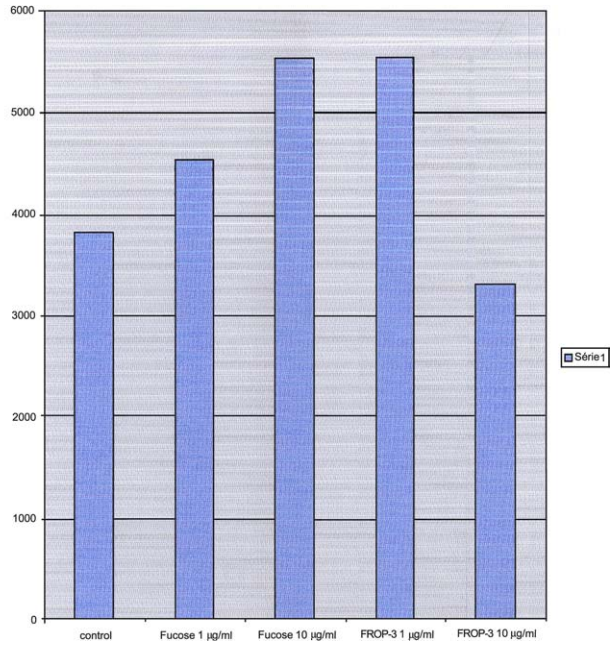


Fig. 4. Biosynthesis of tropoelastin in human skin fibroblast cultures. For details see Section 2 and Fig. 2. Results expressed as radioactivity (cpm) in immunoprecipitable tropoelastin per 10^6 cells.

gave a strong band at about 72 kDa and also a weaker band at about 56 kDa. Only the 72 kDa band showed a dose-dependent increase of intensity between 0.1 and 2 µg s of TE applied to the gel. The 56 kDa band might well correspond to a breakdown product of TE.

3.2.2. Skin explant cultures

Skin explant cultures were used for the determination of the elastin crosslink content—desmosines and isodesmosines—as well as for the determination of incorporation of the radioactive tracer in total secreted proteins and in secreted collagen.

Table 2 shows the results of [^{3}H]-proline incorporation in total non-collagen proteins and in collagen, determined as described in Section 2. Average incorporation in proteins was 2008 cpm/µg (±1636 S.D.) tissue fresh weight. This value increased both in presence of L-fucose and of FROP-3 by about 40% and 30%, respectively. Collagen biosynthesis decreased however in the presence of L-fucose by about 36%. No such inhibition of collagen biosynthesis was seen with FROP-3.

Table 3 shows the determination of desmosine and isodesmosine in the control and treated skin samples. No significant modification could be noticed. The values found are in agreement with literature data and calculated values [8].

4. Discussion

The described results indicate a stimulating effect of L-fucose and FROP-3 on elastin biosynthesis, both in vitro and in vivo. The synthesis and deposition of elastic fibres is a

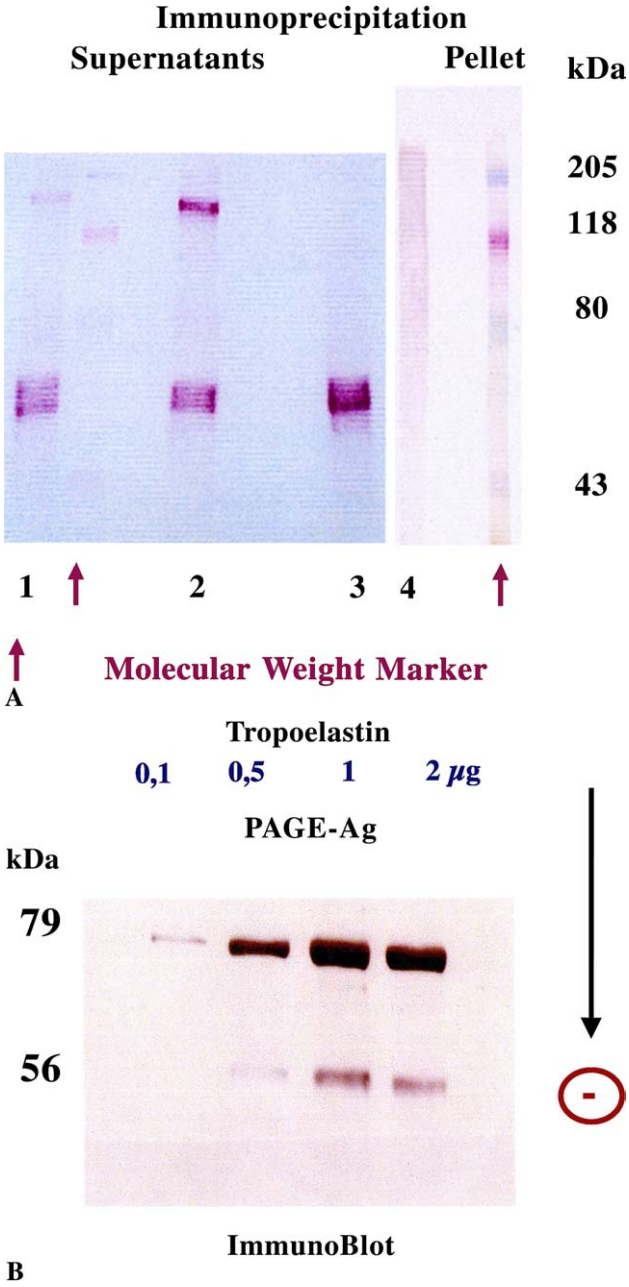


Fig. 5. Immunoblots of tropoelastin from fibroblast culture immunoprecipitates (A) and of recombinant tropoelastin (B). Approximate molecular weights are indicated. (A) 1 and 2: Anti-human tropoelastin (1/200 and 1/100 dil). 3: Anti-human κ-elastin A2-1. (B) Increasing concentrations of recombinant tropoelastin from 0.1 to 2 µg as indicated.

complex, lengthy procedure, and only its first and last phases could be studied, that is tropoelastin biosynthesis by human skin fibroblast culture, and elastic fibre formation in hairless rat skin. Both methods suggested a stimulation of the order of 40% of tropoelastin biosynthesis in cell cultures and elastic fibre formation in the rat skin. Both L-fucose and the fucose-rich polysaccharides, FROP-3, were comparably active in this respect. One advantage of FROP-3 over L-fucose is the lack of inhibition of collagen biosynthesis shown by L-fucose. These results confirm previous experiments carried out in different conditions, showing also a comparable

Table 2
Incorporation of ^{3}H-proline in total excreted proteins and in collagens by human skin explant cultures, incubated alone or with L-fucose 10 μg/ml. For details see Section 2 and Fig. 1. Results are expressed as radioactivity (cpm) per μg tissue fresh weight. Six parallel experiments are shown with average value and S.D.

	^{3}H-proline incorporated in					
	Total excreted proteins			Total collagen		
Treatments	Control	Fucose	FROP-3	Control	Fucose	FROP-3
Number 1	2085	2020	3655	666	759	1013
Number 2	418	4068	3255	723	1368	1272
Number 3	452	2296	3574	1070	66	1429
Number 4	4773	3125	2602	1963	843	681
Number 5	2766	1989	178	1620	965	670
Number 6	1554	3405	2434	435	122	1225
Average	2008	2817	2616	1080	687	1048
S.D.	1636	848	1295	598	505	318

Table 3
Desmosine–isodesmosine content of human skin explant cultures incubated alone or with L-fucose 10 μg/ml or FROP-3 10 μg/ml, for 72 h as described in Section 2 and Fig. 1. Results expressed in pmol/mg fresh weight. Six parallel experiments are shown with average values and S.D.

Number of	Control		L-Fucose		FROP-3	
experiment	Desmosine	Isodesmosine	Desmosine	Isodesmosine	Desmosine	Isodesmosine
1	33.26	65.42	26.91	54.13	33.76	69.72
2	32.36	55.61	42.35	79.48	24.88	55.01
3	48.99	90.73	31.52	56.49	41.68	80.37
4	44.66	78.90	43.96	77.45	31.21	60.88
5	34.83	73.78	42.42	74.05	29.62	60.32
6	34.86	63.76			32.74	72.30
Average	38.16	71.37	37.43	68.32	32.31	66.43
S.D.	6.92	12.48	7.70	12.06	5.55	9.36

inhibition of collagen biosynthesis by L-fucose. Newly synthesised tropoelastin can be rapidly degraded by a number of proteolytic enzymes [9,17]. We could show previously that both L-fucose and FROP-3 down-regulated the expression and activation of MMP-2 and MMP-9, both of these enzymes are endowed with elastolytic activity [7]. As suggested by the immunoblots (Fig. 5), newly synthesised tropoelastin is partially degraded in the peri-extracellular space. This above mentioned effect of L-fucose and FROP-3 on the down-regulation of MMP activity might contribute to the increased net yield of tropoelastin biosynthesis.

As far as skin elastic fibre formation is concerned, this is a complex, lengthy process, only partially understood [2,11]. A number of microfibrillar glycoproteins as well as lysyloxidase are necessary for fibre formation. Crosslink formation is a slow process. This explains the absence of increase of desmosines after 72 h of incubation. The results of morphometric evaluation of skin elastic fibre density of L-fucose treated hairless rats suggests that most if not all of these factors were present and contributed to the increased elastic fibre deposition in the L-fucose treated skins. Four weeks of treatment proved sufficient to produce the crosslinked elastic fibres.

Altogether these results indicate, that both L-fucose and FROP-3 efficiently stimulate elastin biosynthesis and deposition.

Acknowledgements

Supported by Institut DERM, Paris and NATURA Sao Paolo, Brasil. The hospitality and helpful discussions with Professor Gilles Renard are thankfully acknowledged. The Ophtalmology Research Laboratory is supported by a CRI-INSERM 9808 and by funds from University Paris 6 (Equipe d'Accueil 2395). Helpful discussions with M.M. Eduardo Luppi, Philippe Pommez, Jean-Luc Gesztesi and Pierre Fodor from NATURA Sao Paolo, Brasil are gratefully acknowledged.

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