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A novel anti-ageing mechanism for retinol: induction of dermal elastin synthesis and elastin fibre formation

D. Rossetti*, M. G. Kielmanowicz†, S. Vigodman†, Y. P. Hu*, N. Chen*, A. Nkengne‡, T. Oddos‡, D. Fischer†, M. Seiberg* and C. B. Lin*

*The Johnson & Johnson Skin Research Center, Consumer Product Worldwide, A Unit of Johnson & Johnson Consumer Companies, Inc., Skillman, NJ, USA, †ColBar LifeScience Ltd., Industrial Park, Rehovot, Israel and ‡Johnson & Johnson Consumer France, Pharmacology & Skin Care Research Center Campus de Maigremont, Val de Reuil, France

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Synopsis

Dermal elastic fibres are extracellular matrix protein complexes produced by fibroblasts and involved in skin elasticity. Elastin fibres decrease with age as a result of reduced synthesis and increased degradation, resulting in skin sagging and reduced skin elasticity. In this study, we show that retinol (ROL), known to enhance dermal collagen production, is also enhancing elastin fibre formation. ROL induced elastin gene expression and elastin fibre formation in cultured human dermal fibroblasts. Topical treatment of cultured human skin explants with a low dose (0.04%) of ROL increased mRNA and protein levels of tropoelastin and of fibrillin-1, an elastin accessory protein, as documented by QPCR and immunohistochemistry staining. Luna staining confirmed the increased elastin fibre network in the ROL-treated skin explants, as compared with untreated controls. These data demonstrate that ROL exerts its anti-ageing benefits not only via enhanced epidermal proliferation and increased collagen production, but also through an increase in elastin production and assembly.

Correspondence: Dr Connie B. Lin, The Johnson & Johnson Skin Research Center, Consumer Product Worldwide, A Unit of Johnson & Johnson Consumer Companies, Inc., 199 Grandview Rd., Skillman, NJ 08558, USA. Tel.: 908-874-1532; fax: 908-874-1254; e-mail: blin1@its.jnj.com

Résumé

Les fibres élastiques du derme sont des complexes de protéines matricielles extracellulaires produits par des fibroblastes et impliquées dans l'élasticité de la peau. Les fibres d'élastine diminuent avec l'âge en raison d'une synthèse réduite et d'une dégradation accrue, qui aboutissent à l'affaissement de peau et à la réduction de son élasticité. Nous montrons que le rétinol (ROL), connu pour favoriser la production de collagène dermique, améliore aussi la formation des fibres d'élastine. ROL a induit l'expression du gène de l'élastine et la formation des fibres dans des fibroblastes dermiques humains en culture. Le traitement topique d'explants de peau humaines en culture avec une faible dose de ROL (0.04%) a augmenté le taux d'ARNm et le marquage immunohistochimique et QPCR a montré l'augmentation du taux des protéines tropo élastine et fibrilline-1, une protéine associée à l'élastine. La coloration Luna a confirmé un réseau de fibres élastiques accru sur les explants cutanés ROL-TRAITÉES, en comparaison des explants non traités. Ces données démontrent que ROL exerce ses propriétés anti-âges non seulement via une amélioration de la prolifération épidermique et de l'augmentation de la production de collagène, mais aussi par une augmentation de la production d'élastine et de l'organisation du réseau élastique.

Introduction

Skin ageing is associated with wrinkles, sagging, uneven pigmentation, skin roughness and laxity, which are induced by chronological metabolic processes, and by external insults such as solar irradiation (photoageing). Epidermal ageing includes epidermal thinning and pigmentary effects such as unevenness or 'age spots'. Dermal ageing includes dermal thinning, as a result of a decrease in fibroblast number, reduced collagen synthesis and increased UV-induced collagen degradation (reviewed in [1–4]). Additionally, the dermal elastin network is destroyed by reduced synthesis of elastin and its accessory proteins, by increased elastin fibre degradation, and by UV-induced solar elastosis (reviewed in [1, 5]), resulting in reduced skin elasticity and resilience.

Elastin fibres are an essential element of the dermal connective tissue. Elastic fibres are produced from extracellular matrix proteins that are synthesized and secreted by dermal fibroblasts. This is followed by the assembly of the elastin protein and numerous microfibrillar components, to create a fibre network that can stretch and relax ([6–8] and reviewed in [9–11]). The mature elastin fibre is made primarily of the elastin protein, which is encoded by a single gene. Tropoelastin, a 72 000-dalton polypeptide, is secreted as a soluble protein and is then cross-linked by the enzyme lysyl oxidase (LOX) [12–14]. Microfibrils consist mainly of fibrillins, but they also include, or are associated with microfibril-associated glycoproteins, fibulins and EMILIN-1 [9–11, 15]. The microfibrils direct the deposition of tropoelastin during elastic fibre production and facilitate the alignment of the elastin monomers prior to their cross-linking by LOX. The controlled and balanced synthesis of elastin and the microfibril components, as well as their interactions, are essential for the formation of normal elastin fibres. Aberrations in the structure, metabolism or assembly of elastin result in heritable and acquired diseases, affecting skin and other connective tissues, such as cutis laxa, pseudoxanthoma elasticum and elastosis perforans serpiginosa [16]. Mutations in fibrillin-1, the main structural component of microfibrils, lead to Marfan syndrome, a heritable disease with severe skeleton, skin and joints defects [17].

The elastin fibre network in the skin performs best in adolescence and early adulthood, and declines thereafter. Tissue regeneration slows upon ageing, and fibroblasts increase the release of human neutrophil and macrophage elastases, which are known to degrade mature elastic fibres [18–20]. Additionally, environmental factors, and in particular ultraviolet (UV) exposure, induce a massive accumulation of non-functional elastotic material in the upper and middle dermis, termed solar elastosis [1, 20]. Known strategies aimed to prevent or reverse skin ageing include sun avoidance and sunscreens, the use of anti-oxidant combinations, and the use of retinoids to inhibit collagenases and to promote collagen production, yet strategies aiming at enhancing the elastin fibre network are limited (reviewed in [3]). Recently, the use of dill [21] and non-denatured soybean [22] extracts were suggested for enhancing skin elasticity.

The anti-ageing effects of retinol (ROL) on the skin are extensively documented. Clinically, ROL was shown to enhance keratinocyte proliferation [23–25], to increase the production of collagen [23, 26], and to reduce the formation and the pigmentary levels of dyspigmentation [27]. Recent studies of the clinical effects of ROL on wrinkles and photo ageing document an increase in skin elasticity, as assessed by dermatologists [28, 29]. Retinoic acid, but not ROL, was shown to increase elastin synthesis in chick embryonic skin fibroblasts [30], and topical tretinoin treatment was shown to increase the tropoelastin content of photoaged hairless mouse skin [31]. These data might suggest that the topical application of ROL may also stimulate elastin synthesis in human skin; however, definitive research to that effect is currently lacking. Here, we report that ROL is able to stimulate elastin and fibrillin-1 gene expression and elastin fibres formation in normal human dermal fibroblasts, in 3D dermal cultures and in human skin explants. These data point to a novel mechanism of action, adding to the anti-ageing effects of ROL.

Materials and methods

Chemicals

Unless otherwise specified, all chemicals were from Sigma-Aldrich (St Louis, MO, U.S.A.). Human skin Etna-Elastin was purchased from Elastin Products Company, Inc. (Owensville, MO, U.S.A.). TGF-β was purchased from PeproTech Inc. (Rocky Hill, NJ, U.S.A.). ROL was dissolved in DMSO as stock solution of 10^{-1} M and was further diluted in

culture media. An oil-in-water emulsion containing 0.04% ROL was used for topical treatments.

Monolayer dermal fibroblast culture

Primary normal human adult dermal fibroblasts were purchased from PromoCell (Sickingenstr 63/65, Heidelberg, Germany), and were maintained according to manufacturer's instructions. Cells were plated at 1×10^4 cells/96 well in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp., Carlsbad, CA, U.S.A.) with 5% fetal bovine serum (FBS; Biological Industries, Beit Haemek, Israel) and were incubated for 48–72 h. Cells were then treated, in triplicates, with ROL at the concentrations indicated, in DMEM supplemented with 1% FBS, and incubated for additional 72 h. Cells were washed thrice with 300 μL of 1× phosphate-buffered saline (PBS; Neopharm, Emek Hefer Industrial Park, Israel) and were lysed with 100 μL of lysis buffer (0.1 M Tris, 0.15 M NaCl, 0.5% Triton X-100) for analysis of elastin protein levels by ELISA.

3D dermal fibroblast cultures

A 3D collagen scaffold was prepared by ColBar LifeScience Ltd (Rehovot, Israel) using collagen Type I purified from porcine tendons and cross-linked by glycation [32]. The collagen scaffolds ($5 \times 1 \times 5$ mm) were placed in 6-well plates and were seeded drop-wise with 2×10^6 human adult dermal fibroblasts, suspended in 50 μL growth medium (DMEM; Invitrogen Corp.), supplemented with 10% FBS (Biological Industries). Cultures were kept at room temperature in a Laminar Flow Hood to allow cell adherence. After 2 h, the volume of culture medium was increased to 5 mL, and the 3D dermal fibroblast cultures were placed in a 37°C, 5% CO_2 incubator on an orbital shaker, for 2 days. After the 2 days pre-incubation, the medium was replaced, and ROL or TGF-β, at the indicated concentrations, were added, in DMEM supplemented with 1% FBS, for additional incubation of 2 weeks. Media and treatments were refreshed thrice a week. Following 2 weeks of incubation, the 3D cultures were placed into 4% buffered formalin solution and were processed for histological analysis.

Human skin explants

Human skins were obtained with informed consent from abdominal skins of healthy individuals undergoing plastic surgery (The Johns Hopkins Outpatient Center, Plastic Surgery, Baltimore, MD, U.S.A.). Patient identities were not disclosed to preserve confidentiality, in compliance with US HIPAA regulations. Punch biopsies (12 mm) were disinfected at room temperature for 30 min with DMEM with high sucrose content (Invitrogen Corp.), supplemented with Pen/Strep, fungizone and gentamycine (all from Invitrogen). Explants were then rinsed with DMEM and placed in a 1 : 1 mixture of DMEM and F12 nutrient mixture (F-12) (Invitrogen Corp.) supplemented with 2% FBS (Invitrogen Corp.) and a cocktail of growth factors [33]. After an overnight incubation in a humidified chamber, in a 5% CO_2 atmosphere at 32°C, explants were transferred to 37°C for the rest of the culture duration. Explants were treated topically with a ROL formulation (0.04%, 5 μL) at days 1 and 3, and were harvested at different time points for RNA extraction or histological staining. Culture media were refreshed daily, except weekends. Experiments were repeated thrice.

ELISA

Cells were lysed using TritonX-100 based lysis buffer and were tested by a direct ELISA using rabbit anti-Elastin polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.), and a biotinylated goat anti-rabbit IgG (Abcam Inc., Cambridge, MA, U.S.A.). Human skin Etna-Elastin was used as a standard. Elastin standard (1 mg/mL) was diluted in PBS with 0.5% Bovine Serum Albumin to obtain working solutions. The wells of the ELISA plates were coated with the appropriately diluted cell lysates (1 : 31 or 1 : 100 in PBS with 0.5% BSA for TGF-β-treated cells; 1 : 1 or 1 : 31 for ROL-treated cells) to obtain elastin concentrations within the linear portion of the standard curve.

Histological and immuno-fluorescence staining

3D dermal cultures were placed in 4% buffered formalin solution (Richard-Allan Scientific, Kalamazoo, MI, U.S.A.), dehydrated and then embedded in paraffin. Two, 5 μm thick consecutive sections were collected from each block at 150 μm intervals. Skin tissues were fixed overnight in 10% buffered formalin, and followed by embedding into paraffin blocks and sectioning (5 μm) using standard procedures. Sections were stained with

Herovici staining, for documenting newly synthesized collagen [34] or with Luna staining, for documenting Elastin fibres [35]. Images of 3D cultures were captured using Nikon microscope ECLIPSE 50i (Nikon Instruments, Melville, NY, U.S.A.) and Nikon Digita Sight DS-5M; Software: NIS Elements AR 3.0 SP4 Build 384 (Nikon Instruments). Images of skin explants were obtained using Leica microscope (Leitz DM1L, Leica, Allendale, NJ, U.S.A.) and a QiCAM camera (QIMAGING, Surrey, BC, Canada).

For immuno-fluorescence staining, human skin explants were embedded in Tissue-Tek OCT compound (EMS, Washington, PA, U.S.A.), flash frozen in liquid nitrogen and stored at −80°C. Frozen sections, 7 μm thick, were cut with a Leica CM 1950 cryostat (Leica, Microsystems GmbH, Wetzlar, Germany) and stored at −80°C until used. Slides were heated on a slide warmer to 65°C for 30 min, then fixed in acetone at −20°C for 10 min, and then allowed to air dry for 20 min. Sections were incubated with 5% donkey normal serum (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) in 1× PBS prior to incubating with primary antibodies. A tropoelastin rabbit polyclonal antibody (Abcam Inc.) diluted 1 : 200 and a fibrillin-1 mouse monoclonal (Lab Vision Corporation, Fremont, CA, U.S.A.) diluted 1 : 100 were applied to the tissue sections for 1 h at room temperature. The tissues were washed twice in 1× PBS at room temperature for 5 min and then incubated with a 1 : 200 dilution of donkey anti-rabbit Cyanine-2-conjugated and donkey anti-mouse Cyanine-3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) in 1× PBS and DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; Sigma, St Louis, MO, U.S.A.) at room temperature for 30 min prior to being mounted with cover slips. The slides were left to air dry for 24 h before being viewed with a fluorescence microscope (Leica DMIRE2, Leica, Microsystems GmbH) at wavelengths of 492 nm for Cy2 (green for tropoelastin) and 550 nm for Cy3 (orange for fibrillin-1).

RNA extraction and QPCR

Total RNA was extracted from skin explants using TRIzol (Invitrogen), followed by a purification step using Mini-Column Clean-Up (Qiagen, Valencia, CA). The purified RNA was converted to cDNA using Superscript® III reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.). QPCR analyses were set up using a custom-made template by SABiosciences Corp. (Frederick, MD, U.S.A.). The template was coated with primers for: Elastin, fibrillin-1, collagen I, and 18S RNA. Each 25 μL reaction contained 12.5 μL master mix (SABiosciences), 11 μL cDNA (~6 ng) and 1.5 μL of H_2O. QPCR was performed on 7500 Realtime PCR system (Applied Biosystems, Foster City, CA, U.S.A.).

Statistical analysis

Statistical analysis was performed with Student's *t*-test. All results are presented as the mean ± SE.

Results

ROL increases elastin protein levels and elastin fibres staining in cultured dermal fibroblasts

Normal human adult dermal fibroblasts were cultured in monolayers, and were remained untreated or were treated with ROL (10^{-5} and 10^{-6} M) for 72 h. Cells were then lysed and their elastin protein content was quantified using an ELISA assay. As shown in Fig. 1, the exposure to ROL (10^{-5} and 10^{-6} M) led to a dose-dependent increase in elastin protein levels in the treated cells, as compared with untreated control.

The ability of ROL to induce elastin synthesis was further demonstrated using 3D cultures of human adult dermal fibroblasts grown in collagen scaffolds. The 3D cultures were treated with ROL (1×10^{-5} M) or with TGF-β (10 ng/mL, positive

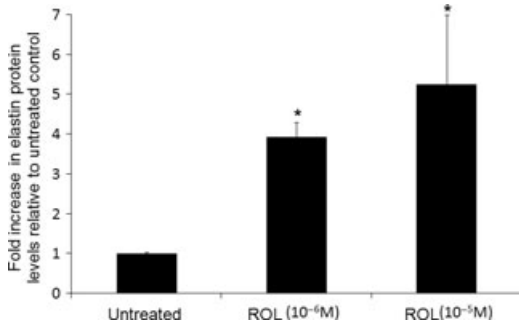

Figure 1 ROL increases elastin protein levels in dermal fibroblasts. Normal human adult dermal fibroblasts were treated with ROL at indicated concentrations for 72 h and cell lysates were examined for elastin protein levels by direct ELISA. ROL treatment led to a dose-dependent induction of elastin protein (*$P < 0.01$, relative to untreated).

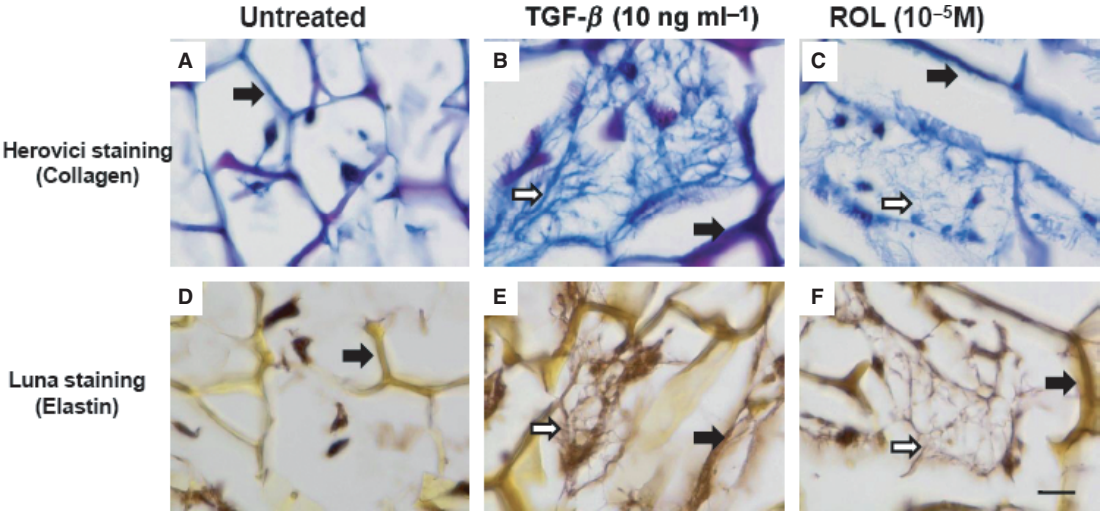

Figure 2 ROL increases elastin fibre staining in 3D dermal fibroblast cultures. 3D dermal fibroblast cultures, established in collagen scaffolds, were treated with TGF-β (10 ng/mL), or with ROL (1×10^{-5} M), or remained untreated for 2 weeks. Representative Herovici-stained (collagen), and Luna-stained (elastin) histological images of control, ROL and TGF-β-treated samples are shown. (A–C) Collagen staining of control (A), TGF-β (B) and ROL-treated sample (C). Newly synthesized pro-collagen is stained as fine blue fibres (open arrow) and collagen scaffolds are stained as thick red/blue fibres (solid arrow); (D–F) Elastin staining of control (D), TGF-β (E) and ROL-treated sample (F). Newly synthesized elastin fibres are stained as fine purple/brown fibres (open arrow) and collagen scaffolds are stained as thick yellow-brownish fibres (solid arrow). Bar = 10 μm.

control) [36], or remained untreated for 2 weeks. Histological staining of the 3D cultures (Fig. 2) was used to document the induction of collagen synthesis (a known ROL activity, shown by Herovici staining) and the enhancement of elastin fibre formation (shown by Luna staining). As expected, TGF-β induced the formation of new collagen fibres (Fig. 2B, compared with untreated control Fig. 2A), and also increased the formation of new elastin fibres (Fig. 2E, compared with untreated control Fig. 2D). ROL (10^{-5} M) induced collagen synthesis in this new 3D culture system (Fig. 2C, compared with untreated control Fig. 2A), confirming both the metabolic activity of the culture system and the activity of ROL under the tested conditions. Additionally, ROL was shown, for the first time, to enhance the formation of an elastic fibre network *in vitro* (Fig. 2F, compared with untreated control Fig. 2D). These results suggest that ROL can induce elastin protein synthesis and elastin fibre formation by adult skin fibroblasts.

ROL enhances the elastin fibre network of human skin explants

To further confirm the elastin-enhancing effect of ROL, studies were performed using full-thickness human skin explants, which better represent the physiological complexity of the skin. To study the effect of ROL on human skin, in a way that mimics the biological activity in human, topical treatment of ROL was used in skin explants. Human skin biopsies, obtained with informed consent from healthy donors undergoing abdominal surgeries, were topically treated with ROL (0.04%), at days 1 and 3, or remained untreated. Explants were harvested at different time points for gene expression analyses and for histological staining. As a control for the metabolic activity of the explants and for the known activity of ROL, a collagen staining (Herovici) was performed. As expected, at day 7 the ROL-treated explants (Fig. 3B) showed more newly synthesized pro-collagen (blue staining) and mature collagen (red staining) than their corresponding untreated controls (Fig. 3A), suggesting that the explants are metabolically active and responsive to ROL, suggesting that the explants are metabolically active and responsive to ROL. Note the documented increase in mature collagen, but not in procollagen staining, at day 7, suggesting the correct assembly of the newly synthesized collagen fibres in the cultured skin explants.

The elastic fibre network of the skin explants was visualized at day 7 using Luna staining.

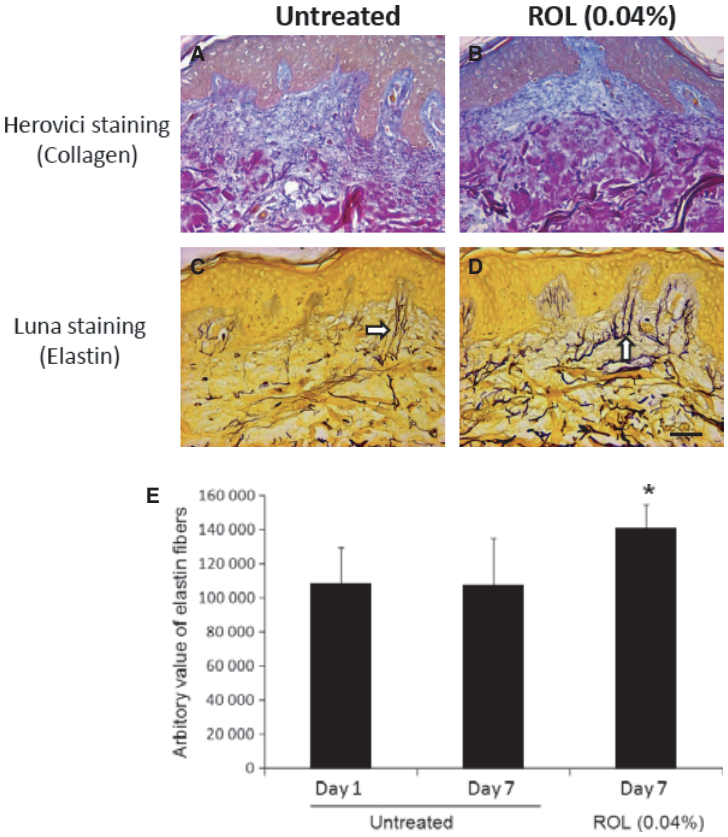

Figure 3 ROL enhances elastin fibre formation in human skin explants. Human abdominal skin biopsies, obtained with informed consent, were topically treated with ROL (0.04%) at days 1 and 3, or were remained untreated, and histochemical stainings were performed at day 7. Representative Herovici-stained (collagen), and Luna-stained (elastin) histological images of control and ROL-treated skin explants are shown at day 7. (A, B) Collagen staining of control (A) and ROL-treated sample (B). Newly synthesized pro-collagen is stained in blue and mature collagen is stained in red; (C, D) Elastin staining (open arrow) of control (C) and ROL-treated sample (D); Bar = 50 μm. (E) Quantitative analysis of Luna stained fibres (arbitrary units, dark pixles/area, $^*P < 0.05$, relative to untreated controls at days 1 and 7).

In the ROL-treated skin explants, an increase in stained elastic fibres was demonstrated immediately below the dermal–epidermal junction, and also in the deeper dermis (Fig. 3D, compared with untreated control Fig. 3C). Using computerized image analysis (Fig. 3E) the level of intact, Luna-stained elastin fibres was quantified for the duration of the study. Upon topical treatment with ROL, the quantity of the stained elastic fibres was increased, further suggesting the active induction of elastin synthesis and assembly by ROL (Fig. 3E).

ROL enhances elastin and elastin accessory proteins levels in human skin explants

The expression levels of collagen, elastin and fibrillin-1 were quantified next in the explants system. The expression levels of elastin and of fibrillin-1 were quantified to assess the effect of ROL on the two major components involved in elastin fibre formation. The expression of collagen was used as a positive control. As shown in Fig. 4A, ROL induced a two-fold increase in collagen-1 expression, as previously reported [23, 26], further confirming that the cultured skin explants are metabolically active and responsive to ROL. ROL induced the expression of both elastin and fibllin-1 at day 4 (Fig. 4A), suggesting that it could affect both elastin protein synthesis and elastin fibre assembly. IHC of tropoelastin and fibrillin-1 was performed to document the effect of ROL on the synthesis of these two proteins. As shown in Fig. 4B, the topical treatment of ROL resulted in a marked increase in tropoelastin (green) and fibrillin-1 (orange) proteins, immediately below the epidermal–dermal junction and in the deep dermis. The effect of ROL was seen as early as day 4, and was consistent throughout the duration of the study (day 7). The co-localization of tropoelastin and fibrillin-1 (yellow) is more profound in the ROL-treated samples (bottom panel of Fig. 4B) suggesting higher synthesis levels and better assembly of elastin fibres proteins upon ROL exposure.

Discussion

ROL is known to reduce the signs of human skin ageing by increasing epidermal proliferation to

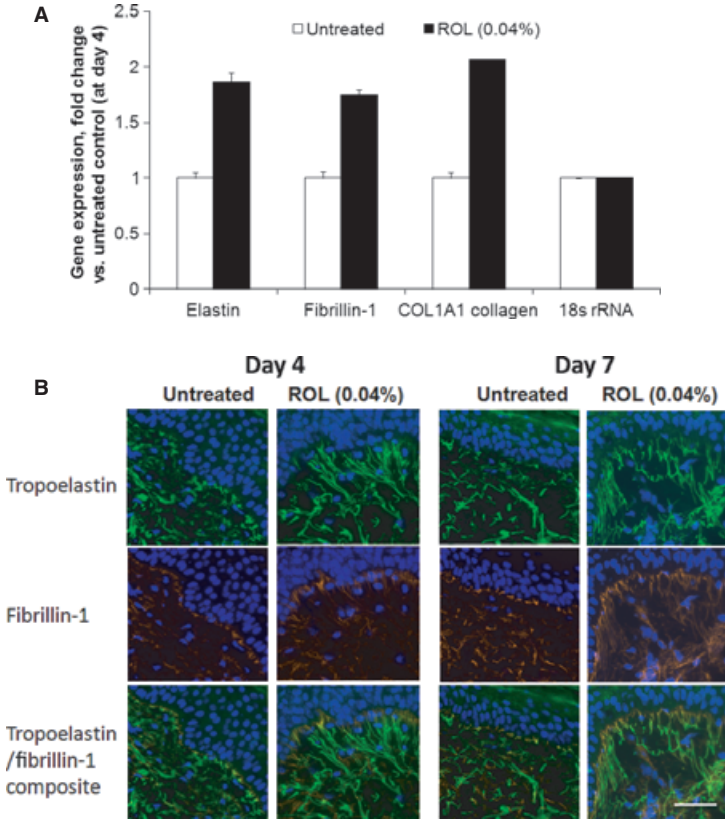

Figure 4 ROL increases mRNA and protein levels of elastin and fibrillin-1 in human skin explants. Human abdominal skin biopsies, obtained with informed consent, were topically treated with ROL (0.04%) at days 1 and 3 or were remained untreated. (A) Gene expression analyses (QPCR) of elastin, fibrillin-1, collagen-1 and 18S RNA. The expression of elastin, fibrillin-1 and collagen-1, was increased at day 4 following the ROL treatment. 18S RNA was used as housekeeping, control gene; (B) IHC staining of tropoelastin and fibrillin-1 in skin explants at days 4 and 7. Both tropoelastin (green) and fibrillin-1 (orange) protein levels were increased in the ROL-treated skin explants, compared with their corresponding untreated controls. The composite images of tropoelastin/fibrillin-1 (co-localization is yellow) are shown at the lower panel. Bar = 50 μm.

restore epidermal thickness, inhibiting tyrosinase activity to reduce the pigmentary changes associated with 'age spots', and enhancing collagen synthesis to restore age-induced dermal thinning [23, 25–29]. Here, we show that ROL also enhances elastin synthesis and elastin fibre assembly, which could result in enhanced skin elasticity.

The effect of retinoic acid on skin elasticity had been documented experimentally and in the clinic [30, 37]. However, no such effect has been reported for ROL. On the contrary, Tajima *et al.* documented retinoic-acid induced upregulation of elastin secretion into conditioned media of chick embryo fibroblasts, but could not detect any induction when the cells were treated with ROL [30]. Similarly, Hayashi *et al.* documented that retinoic acid, but not ROL, stimulated elastin gene expression in chick embryo vascular smooth muscle cells [38]. Using human dermal fibroblasts, we were able to document an increase in elastin protein levels in lysates of ROL-treated cells. This detection was aided by the development of a novel ELISA assay for elastin, which enabled the detection of as low as 20 ng/mL of elastin. The detection of ROL-induced elastin protein production was confirmed with histological and IHC staining of tropoelastin and of mature elastin fibres.

ROL-induced elastin synthesis is shown in well-defined 2D and 3D dermal fibroblast culture systems, as well as in the more physiologically complex *in vitro* system of human skin explants. We verified the dermal metabolic activity of our *in vitro* systems by reproducing the known effect of ROL on collagen synthesis [23, 26]. As the induction of collagen by ROL was confirmed in the clinic we believe that our findings of elastin enhancement by ROL are relevant to the enhancement of human skin elasticity.

The reduced functionality of the dermal elastic network provides a major contribution to the sagging, loose and lax appearance of aged skin. However, most searches for the fountain of youth have centered on dermal collagen enhancement and neglected to specifically address the elastin network. TGF-β induces elastin expression in cultured fibroblasts [39] and in transgenic mice [36, 40], but there are no published reports searching for TGF-β-inducing agents for reducing the signs of

skin ageing. Zhao *et al.* [22] demonstrated that non-denatured soybean extracts induce elastin expression and inhibit elastase activity *in vitro*, and documented enhanced elastic fibre network *in vivo* upon topical treatment with this extract. A recent clinical study documents enhanced skin elasticity by dermatologist's evaluation, as a part of an overall assessment of the effect of ROL in photoageing [28].

The induction of elastin synthesis is essential, but is not sufficient, for the formation of elastic fibres (reviewed in [15]). Cenizo *et al.* had identified a dill extract that stimulates LOXL gene expression in culture, and propose its use in enhancing human skin elasticity [21]. Watson *et al.* reported the enhanced IHC staining of fibrillin-1, upon 12-days occlusive treatment of human forearm skin with either retinoic acid or a commercially available cosmetic product with an undisclosed composition [41]. These results were correlated with an improvement in facial wrinkles following a 6-month topical treatment period. Here, we show enhanced tropoelastin and fibrillin-1 staining in cultured human skin explants topically treated with ROL, suggesting that ROL-enhanced elastin synthesis and elastin fibre assembly could contribute to facial wrinkle reduction.

Retinoic acid induces tropoelastin expression in cultured human fibroblasts and in mouse model systems [30, 31], but reduces the UV-induced enhancement in elastin gene expression in similar experimental systems [42]. It would be interesting to investigate the effect of ROL on UV-induced elastin production and to identify if, similar to retinoic acid, ROL could be effective in the prevention of solar elastosis.

It has been well established that topical retinoic acid is metabolized by human skin and dermal fibroblasts [43, 44]. On the other hand, the metabolism of topical ROL by dermal fibroblasts is not completely understood. Randolph reported that dermal fibroblasts actively metabolize retinoic acid but not ROL [43] and that ROL can be metabolized by human keartinocytes [45]. However, Bailly *et al.* reported that low but significant amounts of retinoic acid were detected in the epidermis and dermis 24 h after topical ROL treatment of skin explants. Additionally, they reported that retinoic acid and other metabolites were detected in cultured human dermal fibroblasts treated with ROL at 24 h [44]. Therefore, it is plausible that the elastin enhancement, anti-ageing effect induced by the topical treatment of ROL in our model systems is mediated by its active metabolite, retinoic acid.

Anti-ageing skin care treatments range from cosmetic preparations, fillers and neurotoxins, to surgical intervention and laser skin resurfacing [46, 47]. However, adverse effects have been associated with many of these methods, and the desire for safe and effective products that reduce wrinkles and improve skin elasticity remains high. Our studies show that significant stimulation of collagen and elastin could be achieved by using low levels of ROL (0.04%) compared with previously published studies where ROL was used at 1% [23]. Low levels of ROL are considered safe and non-irritating [25, 48]. Our findings that low levels of ROL enhance not only collagen synthesis, but also elastin synthesis and elastin fibre formation, provide an additional mechanism for the anti-ageing effects of ROL.

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