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An Association of Vitamins A and E with Hyaluronic and Lactobionic Acids may Prevent Molecular Changes Associated with Keratocyte to Myofibroblast Transition

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1. Introduction
The cornea is the most external tissue of the eye, and the main lens of the ocular dioptr, accounting for approximately 48 diopters [1]. In order to perform efficiently its role, the cornea must be highly transparent, so that the light arrives undisturbed to the photoreceptors.
in the retina, with no blurring or distortion of the images. Such transparency of the cornea is due to three different characteristics of this tissue. (I) The cornea is avascular. The pathologic presence of blood vessels in the cornea would alter the delicate hydration balance of this tissue, and in case blood vessels should invade the area covering the pupil, they could shield and deflect the light entering the eye. The avascularity of the cornea is maintained through the presence of a VEGF-trap molecule, a soluble, truncated form of the VEGF receptor-1 (sVEGFR-1) the product of the soluble Flt gene (sFlt-1). (II) The hydration state of the corneal stroma is optimized for transparency, and tightly controlled by the ionic pumps of the corneal endothelium. Failure of these pumps, for iatrogenic, traumatic or genetic reasons, leads to corneal edema and corneal opacification potentially invalidating for the patient. (III) Corneal stroma has a special architecture that results in a transparent matrix. Collagen fibrils are arranged in approximately 200 layers of lamellae, each one arranged regularly almost right angle to each other. This arrangement, critical for transparency, is maintained by proteoglycans such as lumican and keratan. In the case of local inflammatory events, such as after PRK, the release by injured epithelial cells of the inflammatory cytokine TGFβ induces the transformation of keratocytes into myofibroblasts, characterized by a decreased production of lumican and an increased expression of the cytoskeletal protein αSMA.

PRK triggers inflammation and a wound healing response in the corneal stroma. Keratocytes replicate under the influence of TGFβ, and undergo the transition towards myofibroblasts, thus causing corneal haze. Moreover, regenerating nerve endings after the lesion caused by PRK tend to avoid corneal regions populated by myofibroblasts, so that corneal haze may be accompanied by delayed nerve regeneration. Mitomycin C is a cytotoxic antibiotic that alkylates DNA and proteins, thus inducing apoptosis of proliferating cells. The intraoperative use of mitomycin C, by killing the replicating keratocytes, has been shown to be effective in reducing the risk of haze development and delayed nerve regeneration after PRK. Such treatment, however, despite being in use since many years with no reports of serious complications, is not devoid of potential side effects. In a preclinical study on rabbits, mitomycin C significantly reduced keratocyte density in the anterior stroma, with an effect lasting at least 6 months. Such reduction may cause biomechanical instability, iatrogenic ectasia and increased risk of corneal melting in case of a further delay of keratocyte repopulation. Endothelial toxicity and endothelial cell loss are other possible side effects, putting the patient at risk of corneal edema development because of a reduced function of the endothelial pump system.

Based on this knowledge, we set out to investigate whether we could identify some different natural, non-toxic compounds, which upon topical administration could prevent the myofibroblast transition of keratocytes, thus reducing the risk of corneal haze development after a traumatic corneal injury. In order to obtain an initial proof of concept, we have used a primary cell culture of human keratocytes, treated with TGFβ1 to induce the keratocyte to myofibroblast transition, monitored by the expression of the main determinants characterizing the two states: lumican, αSMA and Fn.

2. Materials and Methods

2.1 Cell Culture

Human primary corneal keratocytes (Innoprot, cat. No. P10872) have been used throughout the study. Human keratocytes (HK) were routinely grown in fibroblast medium (Innoprot, cat. No. P60108) with 2% fetal bovine serum (FBS) and 5% horse serum (HS), in a humidified incubator at 37°C, and 5% CO₂. HK from the original vial were expanded to generate freezing lots considered at passage 2. Freshly thawed cultures were normally splitted by trypsinization at 1/3 dilution for no more than 10 passages (roughly 15 population doublings).

2.2 Cell Treatment

Trypsinized cells from semi-confluent cultures were seeded in 6 multiwell plates at a cell density of 5000/cm² in growth medium. The next day, wells were rinsed with PBS, and cells shifted 4 hours to serum free fibroblast medium. A further 3-hour pre-incubation followed, with 50 μM vitamin E (Sigma-Aldrich Cat. No.258024), 10 μM retinyl palmitate (Sigma-Aldrich Cat. No. PHR1235), 2% w/v sodium lactobionate (SL) (Glenthamp Life Science, Wiltshire, United Kingdom Cat. No. GK2515), 0.15% w/v hyaluronic acid (HA) (MW 1.5 Mda) (kindly supplied by SOOF Italia SpA, Montegiorgio, Italy) prepared at the indicated final concentrations, either alone or in association. Then, TGFβ1 (R&D Systems, United Kingdom Cat. No. 240-B-002) was added at 10 ng/ml, and plates incubated for further 48 hours. At the end of the incubation period, cell monolayers were rinsed three times with PBS and extracted with RIPA (Calbiochem-Merck, Darmstadt, Germany, Cat. No. 20188) in presence of protease inhibitors (Protease Inhibitor Cocktail Set III EDTA-Free, Calbiochem-Merck, Darmstadt, Germany).
Cat. No. 539134) for 30 minutes on ice. Extracts were then clarified by centrifugation at 13000 rpm for 20 minutes at 4°C, and proteins in supernatants quantified by the BCA assay (Sigma-Aldrich, Cat. No. 2322).

2.3 Western Blotting

Thirty µg of proteins were then loaded on a pre-casted 4-12% SDS-PAGE, and blotted onto a nitrocellulose membrane, which was then saturated with skimmed milk and incubated with antibodies against αSMA (Abcam, cat. No. ab32575), lumican (Abcam, cat. No. ab168348), Fn (Abcam, cat. No. 6328) or GAPDH (Cell Signaling, cat. No. 2118) overnight at 4°C. The next day membranes were incubated with the respective peroxidase-labeled secondary antibodies (Amersham, GE Healthcare, Illinois, USA, Cat. No. NA934V) for 1h at room temperature. The peroxidase signal was then developed by chemiluminescence (ECL SuperSignalTM West Dura Extended Duration Substrate, Thermo Fisher Scientific, Massachusetts, USA, Cat. No. 34075) and revealed with the ChemiDocTM Touch Imaging System (BIORAD, Hercules, California, USA). Signal intensity was quantitated by the Image-J Software [13].

2.4 Immunofluorescence

The expression pattern of Lumican and αSMA was investigated by immunofluorescence staining of HK grown on coverslips coated with collagen and Fn in 24 well plates at a cell density of 5.000 cells/cm². The next day, cells were starved 4 hours in serum free medium (SFM), and then incubated for 3 hours with Mix1 or left in SFM. TGFβ1 (Sigma-Aldrich) was added at 10 ng/ml, and cells incubated for further 48 hours. At the end of the incubation period, cell monolayers were rinsed three times with PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. Subsequently, cells were incubated for 30 minutes at room temperature in blocking solution (0.1% Triton in PBS with 0.5% bovine serum albumin (BSA)) and incubated overnight with primary polyclonal antibodies diluted 1:500 in blocking solution: anti-αSMA (Abcam, cat. No. ab32575) or anti-lumican (Abcam, cat. No. ab168348). The next day, cells on coverslips were washed 3 times in PBS before adding the secondary antibody diluted 1:1000 in blocking solution (Alexa Fluor 488 AffiPure Goat Anti-Rabbit IgG, cat. No. 111-545-045) for 1 hour at room temperature. After further washings in PBS, nuclei were counterstained with Hoechst 33342 (Invitrogen, cat. No. H3570) diluted 1:5000 in PBS and incubated for 4 minutes at room temperature. Cell fluorescence was detected with a Leica TCS SP 8 AOBS confocal laser scanning microscope.

2.5 Statistics

All experiments have been run in triplicates and repeated at least three times. One-way ANOVA followed by Tukey’s test was used to evaluate the statistical significance of each experimental group. Differences with P value < 0.05 were considered statistically significant. All data are expressed as the mean ± standard deviation. All statistical analyses were conducted with the GraphPad PRISM statistical software package version 5.00 for Windows (GraphPad Software, San Diego, CA USA).

3. Results

3.1 Effects of Different Molecules on the Expression of Lumican and αSMA on Human Keratocytes

Human primary keratocytes kept in cell culture with 2% FBS express more lumican than αSMA, as expected by normal keratocytes in a healthy corneal stroma. However, we observed a variable ratio lumican/αSMA between 1.25 (Figure 1C) and 1.8 (Figure 2C) due to an increased expression of αSMA with senescence. Likely, also the presence of FBS - even though at low concentration - during routinary tissue culture might have contributed toward this progressive shift, as it is known that the keratocyte phenotype is better preserved under serum-free conditions [14]. The effect of the addition to the culture medium of different molecules chosen after a screening of several more, based on their influence on the ratio lumican/αSMA, is also shown in Figure 1. Vitamins A and E, SL and HA at the concentrations used had no effect on cell viability (not shown). Vitamin E at 50 μM and Vitamin A at 10 μM had opposite effects on the lumican/αSMA ratio since it was slightly decreased by Vitamin E and slightly increased by Vitamin A (Figure 1C). SL 2% enhanced lumican expression, almost doubling its ratio to αSMA, while HA had no detectable effect (Figure 1C). The association of all four (Mix 1) or of vitamins only (Mix 2) had the net result of enhancing lumican expression (Figure 1B) and therefore also the ratio lumican/αSMA (Figure 1C).

3.2 Effects of TGFβ on the Ratio Lumican/αSMA is Countered by Each Molecule, either Alone or in Association

Treatment for 48 hours of HK cells with TGFβ (10 ng/ml) triggered a 50% enhanced expression of αSMA...
and a 70% decreased expression of lumican (Figure 2), characteristic of the transition keratocyte-myofibroblast, as expected [7-8]. The simultaneous presence with TGFβ of any of the molecules illustrated above was able to prevent such transition (Figure 2A), as indicated by the reduced changes observed on αSMA and lumican. Every molecule was able to normalize the levels of αSMA and to prevent the decrease of lumican expression (Figure 2B), so that the ratio lumican/αSMA, although still lower than control cells, was significantly higher than TGFβ-only treated cells (Figure 2C). Both associations (Mix 1 and Mix 2) returned a ratio lumican/αSMA clearly in favor of lumican (Figure 2C), although with a different effect on the single components (Figure 2B). In fact, Mix 1 strongly reduced the effects on αSMA (which was even lower than in control) with a lesser effect on the decrease of lumican, while Mix 2 maintained lumican expression at control levels, while slightly decreasing αSMA expression. However, the overall effect on their ratio was comparable (Figure 2C).

3.3 Immunofluorescence Staining of Human Keratocytes for Lumican and αSMA Indicates a Phenotypic Shift, Countered by the Association of All Four Molecules (Mix1)

Figure 3A shows by immunofluorescence labeling of HK the decreased intensity of lumican after TGFβ treatment, and how Mix 1 (the most effective among the two, including all 4 components) was able to preserve lumican expression. On the contrary, αSMA expression was enhanced by TGFβ (Figure 3B), with HK showing a more elongated phenotype, with long stress fibers of αSMA. The presence of Mix 1 prevented the increase of αSMA and the morphological shift.

3.4 Enhancement of Fn Expression by TGFβ is Prevented by Each Molecule either Alone or in Association

Among the changes induced in keratocytes by TGFβ
Figure 2. Effect of different molecules on the expression of lumican and αSMA in HK (passage 3) treated with TGFβ1. A: HK were pre-treated for 3 hours with each molecule at the indicated concentration; then TGFβ1 (10 ng/ml) was added, and incubation continued for further 48 hours. Thirty μg of proteins were loaded on a 4-20% SDS PAGE, and specific bands revealed by the respective antibodies. B: Densitometry analysis of each band (lumican or αSMA) is reported with respect to GAPDH as arbitrary units (a.u.). C: Ratio of lumican vs. αSMA derived from the densitometric analysis shown in B (actual numbers are shown at the bottom of each bar).

* p < 0.05 vs. control; § p < 0.05 vs. TGFβ

HK: Human Keratocytes; SMA: Smooth Muscle Actin; SFM: Serum Free Medium; SDS-PAGE: Sodium Dodecylsulphate-Polyacrylamide-Gel-Electrophoresis; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; TGF: Transforming Growth Factor.

Figure 3. Immunofluorescence analysis of lumican and αSMA in HK (passage 3) treated with TGFβ1 (10 ng/ml) in the presence or absence of the association of the four compounds (Mix 1). A: lumican expression is decreased by TGFβ treatment, and conserved in the presence of Mix 1; B: αSMA expression is increased by TGFβ1 treatment, where also a morphological change is apparent, while Mix 1 blunted both effects of TGFβ1.

HK: Human Keratocytes; SMA: Smooth Muscle Actin; SFM: Serum Free Medium; TGF: Transforming Growth Factor.
treatment, Fn expression is known to be enhanced \[14\]. Therefore, we evaluated how Fn expression was modulated in this human keratocyte cell line by the different molecules in the presence or in the absence of TGFβ (Figure 4). In the absence of TGFβ, Fn expression was doubled by HA treatment, while the association of the four components (Mix 1) resulted in a 50% decrease (Figure 4A-C). Addition of TGFβ (10 ng/ml) to the cell culture resulted in a 100% increase of Fn expression (Figure 4B-D). The simultaneous presence of each single molecule was already enough to prevent such increase. The presence of the Mix 1 further decreased Fn expression by 50% despite the presence of TGFβ, while the association of the vitamins A and E alone (Mix 2) only partially prevented the increase of Fn expression, which however remained at levels higher than the control.

4. Discussion

We have shown in this paper a basic proof of concept that an association of natural molecules at nontoxic concentrations may have the ability to counteract the consequences of an inflammatory state of the cornea which, through the action of TGFβ, leads to keratocytes transdifferentiation, alterations of corneal stroma, and finally to corneal opacification (corneal haze).

Corneal stroma keratocytes produce a specific and balanced population of proteoglycans (lumican, keratocan, mimecan, decorin), the arrangement of which is essential for corneal transparency \[15\]. Among these, lumican seems to be the main orchestrator in order to produce and maintain a regular structure of corneal stroma, which is an essential requisite for transparency. Lumican is a keratan sulfate (KS) proteoglycan belonging to the small leucine rich proteoglycan family (SLRP). SLRPs contain a protein core modified by glycosaminoglycans side chains, which confer them their specific properties in the regulation of tissue-specific matrix assembly, and mediating cell-matrix interactions \[16\]. Preclinical studies have indicated a role for lumican in the pathogenesis of different ocular diseases such as glaucoma, myopia, inflammatory eye diseases and wound healing \[17\]. A prominent role of lumican in the corneal stroma is the regulation of collagen fibrils assembly \[18\]. Mice made homozygous for a null mutation in lumican show collagen fibril abnormalities in

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**Figure 4.** Effect of different molecules on the expression of fibronectin in human keratocytes (passage 5) with or without TGFβ1 treatment. A: HK were grown in SFM for 48 hours in the presence of each compound at the indicated concentration. Thirty μg of proteins were loaded on a 4-20% SDS PAGE, and specific bands (Fn or GAPDH) revealed by the respective antibodies. B: HK were pre-treated for 3 hours with each molecule at the indicated concentration; then TGFβ1 (10 ng/ml) was added, and incubation continued for further 48 hours. Thirty μg of proteins were loaded on a 4-20% SDS PAGE, and specific bands (Fn or GAPDH) revealed by the respective antibodies. C and D: Densitometry analysis of Fn is reported with respect to GAPDH as arbitrary units (a.u.) (actual numbers are shown at the bottom of each bar).

* p < 0.05 vs. control; § p < 0.05 vs. TGFβ

HK: Human Keratocytes; SMA: Smooth Muscle Actin; SFM: Serum Free Medium; SDS-PAGE: Sodium Dodecylsulphate-Polyacrylamide-Gel-Electrophoresis; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; TGF: Transforming Growth Factor; Fn: Fibronectin.
the posterior stroma, where collagen is more abundant, finally resulting in a 25% reduction in KS content, a 40% reduction of stromal thickness and bilateral corneal opacification [19]. Differently, null mice for keratocan (another cornea specific KS proteoglycan) show a less severe corneal phenotype, with a thinner, but still transparent cornea [20,21]. Indeed, it is known that mutated keratocan may result in changes of corneal curvature, and thus its refraction ability, but no significant collagen fibril structural defects [22]. On the other hand, lumican overexpression in corneas of wild type mice also resulted in no alteration of collagen organization and corneal transparency, however it decreased corneal keratocan expression, thus indicating a regulatory role of lumican for keratocan expression [23]. Moreover, lumican contains an aminoacid sequence endowed with self-assembling properties, and able to stimulate collagen biosynthesis [24,25].

αSMA is a cytoskeletal protein typical of muscle cells. Expression of αSMA in fibroblasts or keratocytes (during their transition to myofibroblasts) may happen during wound healing since wound closure is facilitated by the contractile properties and higher motility of these myofibroblasts [26,27]. The production of αSMA is linked to the presence of Fn in the basement membrane because its synthesis is enhanced by the interaction of Fn with its integrin receptor [28].

Therefore, in keratocytes, the ratio lumican/αSMA and the amount of Fn produced are indicative of the differentiation state of the cells: a predominance of lumican over αSMA, and low levels of Fn production suggest a well differentiated state, whereas a decrease of lumican/αSMA ratio because of an increased production of αSMA and Fn indicate a degree of transdifferentiation. We report here that primary human keratocytes in tissue culture can indeed maintain a differentiated state with a ratio lumican/αSMA > 1 (Figure 1 and Figure 2), and low levels of Fn production (Figure 4), which can be basically modulated by the addition of some natural compounds, either isolated or mixed together.

Transforming growth factor-β (TGFβ) is a pleiotropic cytokine that regulates a myriad of cellular processes and has important roles in morphogenesis, embryonic development, adult stem cell differentiation, immune regulation, wound healing and inflammation [29]. In the eye, it has been found to be induced after PRK to regulate wound healing and keratocytes transdifferentiation, thus triggering corneal opacification [30]. TGFβ was found to regulate cell phenotype and induce epithelial-mesenchymal transition in cancer cells, thus enhancing their invasive and metastatic ability, through the mechanistic target of rapamycin (mTOR) pathway [30]. The same trigger and pathway is also involved in the transdifferentiation keratocytes-myofibroblasts after PRK [31]. Rapamycin, a known inhibitor of the mTOR pathway [31,32], can thus prevent the transdifferentiation and also the consequent corneal haze [31].

Our data show indeed that TGFβ treatment resulted in decreased lumican expression and a parallel increase of both αSMA and Fn, so that the ratio lumican/αSMA was dramatically decreased (Figure 2).

Different strategies have been published aimed at preventing the keratocyte/myofibroblast transition. The only clinical intervention allowed nowadays consists in topical application of mitomycin C, which, by its genotoxic activity, inhibits the proliferation of myofibroblast progenitor cells, and thereby inhibits mature myofibroblasts generation [31]. It is an effective treatment [33], even though there is no unanimous consensus on the treatment protocol [34]. Among alternative treatments under investigation, rapamycin has been shown to work efficiently in a preclinical setting [32], also after topical application as eye drops [35], but no clinical data have been reported so far. Onion extract, due to its flavonoid content, can also reduce scar formation by inhibiting fibroblasts metabolism [36], and has shown its efficacy also in a dermatologic clinical setting [37]. Topical application as eye drops on canine eyes subjected to PRK were shown to prevent αSMA increase in keratocytes, and significantly reduced corneal haze formation [38].

We now show evidence that an association of vitamins A and E with SL and HA can also prevent TGFβ-induced expression changes of lumican, αSMA and Fn, typical of the transdifferentiation of keratocytes. Vitamin A deficiency in humans (and in mammals in general) can lead to alterations of the corneal epithelium (designated as xerophthalmia) characterized by opacification and keratinization [39] and dependent on the inactivation of the Notch1 pathway [40]. The efficiency of corneal proteinases also depends on a correct supply of vitamin A, the deficiency of which can lead to decreased exfoliation of epithelial cells, increased levels of keratofibrils in corneal keratocytes, increased stromal keratocyte degradation and increased susceptibility towards ulceration [41]. However, a clinical study designed to evaluate the efficacy of a topical perioperative supplementation of vitamin A (250 IU per gram of ointment) in patients undergoing PRK found no significative effects on re-epithelialization time, postoperative pain, corneal haze formation, or visual outcomes after PRK [42]. On the contrary, a high dose oral supplementation of vitamin A and E (25 000 IU retinol palmitate and 230 mg alpha tocopheryl nicotinate) to patients scheduled to receive PRK treatment resulted in
accelerated re-epithelialization time and reduced corneal haze formation \(^{[4]}\). These latter results are consistent with our results shown in Figures 2 and 3 in which treatment of HK with vitamins A and E either isolated or in association (Mix 2) in the presence of TGFβ was already able to counteract its effects on the expression of lumican, αSMA and Fn, and likely on the transdifferentiation of keratocytes.

We had already shown that topical instillation of SL, either alone or in association with HA, is able to exert an antiinflammatory effect on the cornea, preventing the increase of TGFβ and matrix metalloproteinase 9 (MMP9) induced by inflammatory agents \(^{[44]}\). Consistently, in a rabbit model of PRK the instillation of HA eye drops inhibited subepithelial haze by favoring a more physiologic wound healing \(^{[45]}\), likely due to the interaction of HA with its cellular receptor CD44, shown to be involved in rabbit corneal epithelial wound healing \(^{[46]}\). Our data here illustrated further support a role for HA in corneal healing after the wound caused by the PRK procedure, because just by itself it can limit the changes in lumican, αSMA and Fn expression induced by TGFβ (Figure 2 and 4), and in association with the other compounds shows an even better conservative ability (Figure 2 B). Similar results are also obtained with SL (Figure 2 and 4), showing for the first time its ability to influence keratocyte transdifferentiation and their response to inflammatory events.

In conclusion, we have shown here the respective efficacy of vitamins A and E, HA and SL either alone or in association to contrast the shift in the expression of keratocyte transdifferentiation markers such as lumican, αSMA and Fn induced by the pro-inflammatory cytokine TGFβ, likely the main responsible of keratocyte transdifferentiation and corneal opacification after PRK. Morphological changes induced by TGFβ could also be prevented by the association of the 4 compounds (Figure 3). We are now investigating the molecular pathways involved in this shift, and how the different molecules here tested can interfere with them, and prevent the transdifferentiation and the corneal haze. Also, the efficacy of such formulation in a rabbit model system of corneal haze will be verified.

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