Stimulation of Growth Factor Communication between Epidermis and Dermis by Crocus chrysanthus Bulb Extract

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Abstract

Chronologically aged skin is characterized by a diminished expression of growth factors. The consequence is a reduced biosynthesis of matrix proteins such as collagen and elastin. These alterations in the extracellular matrix network occur in the dermis, a skin tissue that cannot be easily reached by topically applied cosmetic actives. Our strategy to treat the dermis is to utilize the communication between the cells of the outer skin layer, the epidermis, and the cells of the subjacent dermis. Communication is mediated by growth factors and cytokines released by one sort of cells that reach other cell types by diffusion. In cell culture assays, an extract of crocus bulbs was shown to stimulate epidermal keratinocytes to release growth factors into the medium. The cell-free medium incubated with dermal fibroblast cells was found to enhance the expression of elastin, of the elastin-processing enzyme lysyl oxidase-like 2 and of the connective tissue growth factor (CTGF). Exactly the same expression pattern was obtained by treating fibroblast cells with TGF-β. The crocus bulb extract applied directly to fibroblasts showed no effect. The results clearly demonstrated that the plant extract induced secretion of messenger compounds in the epidermis that could enhance the synthesis of matrix proteins in the dermis. The in vitro results were confirmed in clinical trials with a cream containing the crocus bulb extract. Analysis of skin collagen and elastin with the non-invasive two-photon microscopy demonstrated an increase of both compounds.

Introduction

The vital cells of the skin

Growth factors and cytokines are messenger compounds, in most cases proteins, that allow the communication between cells in our tissues. After binding to specific receptors on cell surfaces, growth factors activate cellular proliferation or differentiation. In the skin, growth factors orchestrate the wound-healing process (1) and also the continuous regeneration and repair. The repair of skin damaged by a wound or after UV exposure takes place in two phases. Firstly there is an inflammatory reaction. Activation of the NF-κB pathway in the cells of the epidermis leads to the formation of inflammatory cytokines such as IL-1, IL-8 and TNF-α (2). These inflammatory messengers activate in the subjacent dermis the synthesis of proteolytic enzymes that start to degrade the extracellular matrix composed of collagen and elastin fibers. After cleaning of the damaged skin area, the inflammatory phase stops and the skin regeneration phase starts with the release of growth factors such as TGF-β. Enhancement of extracellular matrix production is a major effect of TGF-β. In intrinsic skin aging there is no inflammatory phase but the enhanced formation of ROS as consequence of an impaired mitochondrial oxidative metabolism leads to a strong degradation of the extracellular matrix. Thinning and fragility of elderly skin is the result of an imbalance of degradation and regeneration. Enhanced ROS formation favors the degradation in elderly skin. But there is also an age-dependent loss of regeneration capacity because of reduced synthesis of growth factors. A decreased expression

of TGF-beta and CTGF by aged skin fibroblast cells was shown to be responsible for the reduced synthesis of the extracellular matrix proteins (3). In vitro, CTGF exhibits diverse activities such as cell proliferation, cell migration and extracellular matrix production. In skin fibroblasts, CTGF is primarily induced by TGF-β and functions thus as a downstream mediator in the activation of the extracellular matrix formation.

Could aging skin need a supplementation of growth factors for rejuvenation? The use of human growth factors in cosmetic products is forbidden. There are cosmeceutical products on the market based on biotechnologically produced growth factors. Theoretically they could be used for cosmetic products but their efficacy is very limited because of instability and lack of penetration. Growth factors are proteins with a defined spatial conformation and too big to penetrate into the skin. They are not stable in cosmetic emulsions and are normally quickly degraded by protease enzymes after topical application. They most probably just adhere to the outermost skin layers (stratum corneum), because these molecules are too big to penetrate deeper into the skin. Topically applied growth factors cannot reach fibroblast cells of the dermis, where they should bind to membrane receptors to induce the synthesis of matrix proteins.

This paper describes an approach to stimulate the growth factor-dependent synthesis of matrix proteins in the dermis by using epidermis / dermis communication processes. In a screening assay, human primary keratinocyte cells were incubated with a series of extracts of different plant bulbs. After incubation, the keratinocyte cells were separated and the culture supernatants (conditioned medium) were used to culture fibroblast cells in them. At the end of the incubation, the expression of growth factors and matrix proteins in the fibroblast cells was analyzed. Bulbs are plant parts specialized for the storage of nutrients used to grow a new plant after a low-temperature-induced dormancy period. There are structurally two different forms of bulbs, real bulbs (e.g. tulips) and corms (e.g. crocus) that are both modified stem segments. There is already a cosmetic ingredient based on narcissus bulbs on the market. It is reported to have general anti-aging effects and to reduce hair growth. In the screening assay for growth-factor-signaling bulb extracts of plants of the Amaryllidaceae, Asparagaceae, Iridaceae, Liliaceae and Myrsinaceae were tested. Clearly the best results were found for the Crocus chrysanthus bulb extract from the Iridaceae family. There are cosmetic ingredients based on Crocus sativus available. But these are extracted from the petals and have anti-aging and whitening effects.

MATERIALS AND METHODS

Preparation of extracts of plant bulbs

A mixture of 12% of plant bulbs w/w was homogenized in a 15% ethanol solution and incubated at 50°C for 4 hours. The extraction was done in a DIG-MAZ 50 system (samtech Extraktionstechnik GmbH). The extraction process was controlled by chromatographic fingerprint analysis (Waters, ACQUITY UPLC system, C18 column).

Screening assay with conditioned media

Primary human keratinocytes from a 50 year old donor were cultured in culture medium for 24 hours. The medium was then replaced with the assay medium (Epilife medium) containing or not (control) the bulb extracts at non-toxic concentrations based on a preliminary cytotoxicity assay (MTT). The keratinocytes were then incubated for 72 hours. At the end of the incubation, the cell cultures were centrifuged to prepare cell pellets and culture supernatants (conditioned media). The cells were used to analyze the expression of 30 genes of growth factors and cytokines typically expressed in keratinocytes. The expression analysis was done by quantitative PCR (LightCycler® system; Roche Molecular System Inc.). The conditioned media were used to treat normal human dermal fibroblasts (pool of donors over 50 years old). Beforehand, the fibroblasts were cultured for 24 hours in a normal culture medium and then for 72 hours in an assay medium (Epilife medium). The medium was then removed and replaced by either the conditioned media, or the assay medium with TGF-beta (10 ng/ml), or the assay medium alone or the assay medium containing the bulb extracts at non-toxic concentrations based on a preliminary cytotoxicity assay (MTT). The fibroblasts were then incubated for 24 or 72 hours. At the end of the incubation, the fibroblasts were isolated and used for expression analysis of 61 genes known for their central role in fibroblast biology and cutaneous aging. All experimental conditions were performed in triplicate.

Clinical pilot study using two-photon microscopy

A cream with 0.4% crocus bulb extract was tested in a study over 4 weeks on a 53 year old Caucasian woman. The cream was applied twice a day to the inner forearm skin. The other forearm was treated with the placebo cream. Two-photon microscopy pictures were taken at baseline conditions, after two and after four weeks. Image stacks were acquired using Olympus Fluoview 1000 MP two-photon microscopy setup customized for human skin imaging in vivo. The volunteer's forearm was placed under the microscope objective (25x water immersion) and the skin was gently adhered to the

proprietary holder using double-sided adhesive tape. For repeated imaging sessions, the same skin spot was targeted every time. Two-photon images were obtained simultaneously in two modes: autofluorescence (AF) and second harmonic generation (SHG).
Image acquisition setup
Illumination: 800 nm
Emission filters:
Channel 1 – band pass 397-412 nm (SHG, color-coded red)
Channel 2 – band pass 455-490 nm (AF, color-coded green)

Clinical anti-aging study
A cream with 2% crocus bulb extract was tested in a study over 4 weeks on 20 women aged between 36 and 65. The cream was applied twice daily on the inner side of the forearm and in the crow's feet area. The other forearm and other half side of the face were treated with the placebo cream. On days 14 and 28, 8 – 12 hours after the last product application, skin firmness and elasticity on the forearm (Cutometer MPA 580, Courage & Khazaka GmbH) and wrinkle depth in the crow's feet area (PRIMOS®, GFMesstechnik GmbH) were measured.

RESULTS AND DISCUSSION

Normal human fibroblast cells were incubated in a conditioned medium derived from keratinocyte cultures that had been treated with extracts of plant bulbs. Gene expression analysis in the fibroblast cells showed that the conditioned medium of keratinocytes treated with an extract of *Crocus chrysanthus* bulbs strongly stimulated the expression of genes involved in the synthesis of matrix proteins (figure 1).

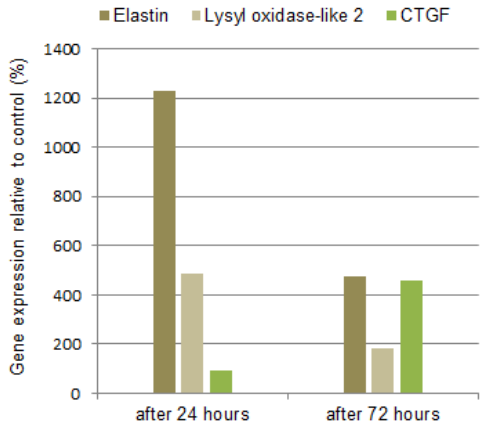

Figure 1. Effect on gene expression in fibroblast cells after incubation for 24 and 72 hours with a keratinocyte conditioned medium. The keratinocytes were treated with the crocus bulb extract. The values are compared to gene expression values of fibroblasts after incubation with the conditioned medium of untreated keratinocytes (100%).

Compared to the conditioned medium of untreated keratinocytes, the expression of elastin was enhanced 12-fold and of lysyl oxidase-like 2, 5-fold after 24 hours' incubation. After 72 hours' incubation, the expression of CTGF was also enhanced 5-fold. The lysyl-oxidase-like enzymes are important for elastic fiber formation by cross-linking elastin. The expression of lysyl-oxidase-like enzymes is reported to be reduced in aged skin (4). The role of CTGF in adult tissues is linked to fibrosis, a group of diseases with excessive formation of fibrous connective tissue. But reduced collagen and elastin levels in aging skin might be well caused by limiting CTGF levels as shown by Quan et al. (3). Treatment of fibroblast cells with TGF-β induced a very similar expression pattern (figure 2).

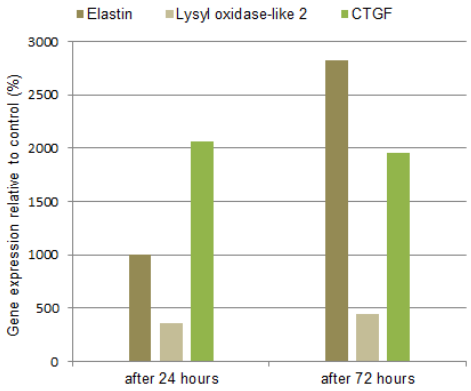

Figure 2. Effect of TGF-β on gene expression in fibroblast cells.

TGF-β was used as a positive control because it is a known promoter of the synthesis of extracellular matrix proteins (5). Incubation of fibroblast cells with the extract of crocus bulbs did not affect gene expression, indicating that the effect in fact depends on compounds secreted by keratinocytes. Treatment of keratinocytes with the extract of crocus bulbs was found to up regulate the expression of several growth factors known to play important roles in epidermis / dermis communication (figure 3).

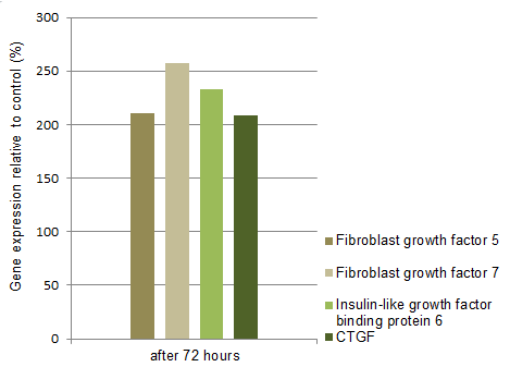

Figure 3. Effect of an extract of crocus chrysanthus bulbs on gene expression in keratinocytes.

Members of the fibroblast growth factor family are involved in a variety of biological processes including cell growth, morphogenesis and tissue repair. Insulin-like growth factors promote the accumulation of the extracellular matrix in various cell types, including fibroblasts (6). Insulin-like growth factor binding proteins modulate the activity of the growth factors.

The cell culture assays with all the relevant control conditions showed in fact that the stimulatory effect of an extract of crocus bulbs on the synthesis of matrix proteins is mediated by growth factor signals released by keratinocytes. Thus, there is a realistic chance for in vivo efficacy because keratinocytes can be reached by cosmetic actives.

A first clinical pilot study was performed using two-photon microscopy for non-invasive analysis of the skin tissue. Compared to confocal microscopy, multiphoton microscopy such as two-photon microscopy makes skin imaging possible at greater depths. Near infrared wavelengths are used to build up a tissue contrast based either on autofluorescence generated for example by elastin and NADH or based on second harmonic generation induced by collagen proteins. Already after two weeks' application, there was a clear improvement seen in the skin zone treated with the cream containing the crocus bulb extract at 0.4 % (verum) compared to the placebo cream treated skin (figure 4). The area in blue (figure 4), corresponding to the papillary and reticular layers of the upper dermis, was integrated to define collagen and elastin content.

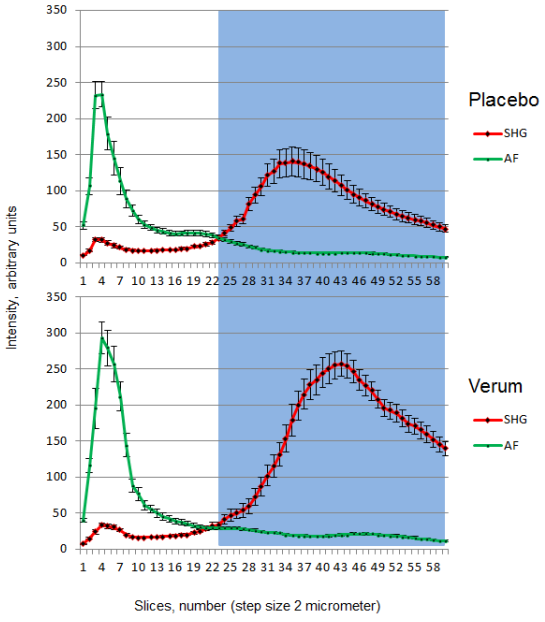

Figure 4. Autofluorescence (AF) and second harmonic generation (SHG) signal of the skin after two weeks' application.

The amount of collagen was significantly increased over the baseline by as much as 115% for the verum (figure 5). For the placebo, an increase of 30% was found. The difference between the two forearms was highly significant ($p<0.001$). The amount of elastin was increased by 25% for the verum and slightly decreased by 7% for the placebo (figure 6). The difference between the two forearms was again significant ($p<0.01$).

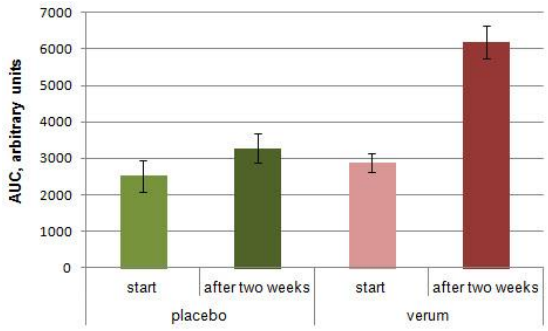

Figure 5. Increase of collagen amount in the upper dermis after two weeks' application.

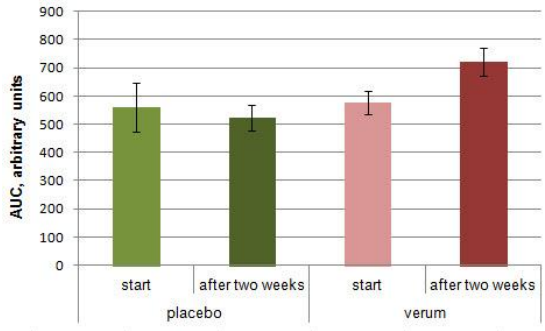

Figure 6. Increase of elastin amount in the upper dermis after two weeks' application.

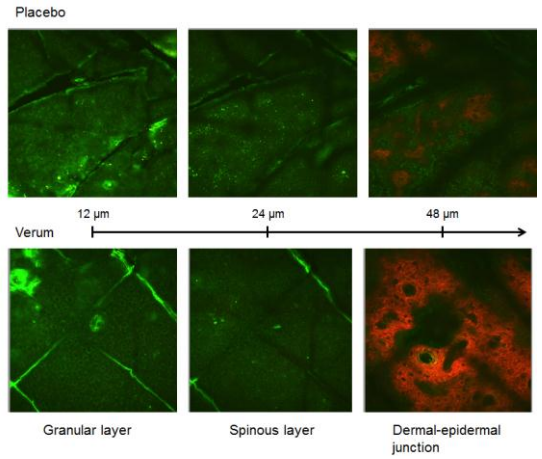

Figure 7. Two-photon microscopy images of the epidermis after two weeks' application. Autofluorescence signal in green, second harmonic generation signal in red.

In addition to these quantitative changes in collagen and elastin amounts, the two-photon microscopy pictures showed also qualitative differences between verum and placebo. The verum was found to clearly reduce the size of wrinkles. The wrinkles in the placebo treated skin zone are visible as dark, crack-like openings that are wide at the depth of 12 microns and become narrower at 24 and 48 micron depths (figure 7). By contrast, there was a complete absence of such dark openings, even in the superficial layers, in the verum treated skin. The tendency to increase the amount of collagen and elastin observed at week 2 persisted also at week 4. However, the difference between the two forearms was less pronounced after 4 weeks.

A second clinical study was performed with 20 women aged between 36 and 65. A cream with 2% of an extract of crocus bulbs and the control cream without extract (placebo cream) were applied twice daily over four weeks. Already after two weeks' application, skin firmness was found to improve significantly compared to the value of placebo treated skin (figure 8).

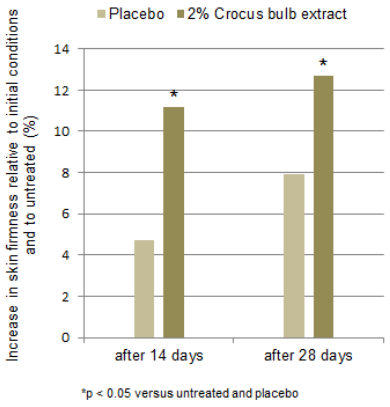

Figure 8. Increase in skin firmness compared to the beginning of the study and the untreated area.

Skin elasticity improved to a similar extent. At the end of the study, wrinkle depth in the crow's feet area was reduced by 9% (significant against initial conditions).

CONCLUSIONS

The age-related decline in the synthesis of growth factors is the chief cause for chronologically aged skin. Wrinkles which are the main manifestation of aging are formed in the dermis, a skin layer that cannot be easily reached by cosmetic actives. An extract of crocus bulbs was shown to solve both problems, the missing growth factors and the inaccessible problem zone. The crocus bulb extract was found to stimulate the release of keratinocyte-derived growth factor messengers that induced the neo-synthesis of matrix proteins in the dermis.

References

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