An ECM-derived Tetrapeptide to Counterbalance ECM Degeneration

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KEY WORDS: *peptides, antiaging, collagen, hyaluronic acid, fibronectin*

ABSTRACT: *Degradation of dermal and epidermal proteins and the reduced proliferation of collagen and hyaluronic acid in the dermis occur during aging. Thus, antiaging technologies must to correct these deficiencies to induce skin regeneration and combat the signs of aging. Data presented here demonstrates that ECM-derived tetrapeptides have the potential to counterbalance ECM degeneration.*

The extracellular matrix (ECM) is the structural backbone of many tissues, especially the skin, and represents a main target for cosmetic applications. ECM proteins are believed to play a pivotal role in cellular migration, proliferation and gene regulation during wound healing. Fragments from ECM constituents have been found capable of stimulating ECM biosynthesis to compensate for tissue destruction.[1] Their mechanisms have been implicated in wound healing, skin aging and skin's response to UV irradiation;[2,3] from this knowledge, new actives have evolved, as the authors describe here.

Building from the concept that ECM constituents stimulate ECM biosynthesis, bioinformatic methods were employed to identify highly repetitive amino acid motifs with inherent antiaging activities. Several dozens of tetrapeptides were found scattered across sequences of the major ECM macromolecules.[4] Ten peptides showed the desired effect of significantly increasing collagen protein in supernatant, thus verifying the underlying assumption that breakdown products of ECM proteins stimulate the ECM neosynthesis.

> Fragments from ECM constituents have been found to stimulate ECM biosynthesis.

Of the ten peptides, the five most promising were subjected to further analysis including concomitant collagen determination in the supernatant, as well as gene expression analysis of the ECM marker genes: collagen (COL1A1), fibronectin (FN1) and hyaluronic acid synthetase (HAS1).

Collagen, being a dermal protein responsible for skin strength and elasticity, was examined since its degradation leads to wrinkles that accompany aging.[5] Hyaluronic acid, one of the main components of the ECM, is a nonsulfated glycosaminoglycan that binds water, also ensuring the elasticity of the skin.[6] Fibronectin, a glycoprotein that helps to create a cross-linked network within the ECM, was also of interest since it provides binding sites for other ECM components such as hyaluronic acid and collagen.[7]

In the end, one tetrapeptide, glycine-glutamic acid-lysine-glycine (GEKG, or INCI: tetrapeptide-21)[a], was evaluated in vivo for effects on these genes.

Material and Methods

Human dermal fibroblasts (HDFs) prepared from neonatal foreskin were cultured for four days in a humidified 5% carbon dioxide atmosphere in Eagle's minimal essential medium[b] supplemented with: 5% fetal calf serum[c], 0.1% l-glutamine, 2.5% sodium bicarbonate, and 1% streptomycin/amphotericin B, until they reached confluence. For the studies described here, only early passage fibroblasts (< 12) were used so as to avoid any changes in their original phenotype during subculture. Cells were kept in 6-well plates for culture.

For isolation of total RNA, kits[d] were used according to manufacturer instructions. The RNA concentration

[a] *Tego Pep 4-17 (INCI: Tetrapeptide-21 (and) Glycerin (and) Butylene Glycol (and) Water (aqua)), is a product of Evonik Goldschmidt GmbH.*
[b] *EMEM is a product of Life Technologies GmbH, Eggenstein, Germany.*
[c] *Fetal Calf Serum is a product of Greiner, Frickenhausen, Germany.*
[d] *The RNeasy Total RNA Mini Kit is a product of Qiagen.*

Table 1. Primer pairs

18S rRNA	5´-GCCGCTAGAGGTGAAATTCTTG-3´
	5´-CATTCTTGGCAAATGCTTTCG´-3´
Collagen 1A1	5´-CCTGCGTGTACCCCACTCA-3´
	5´-ACCAGACATGCCTCTTGTCCTT-3´
HAS-1	5´-GCGGGCTTGTCAGAGCTACT-3´
	5´-AACTGCTGCAAGAGGTTATTCCTATAT-3´
Fibronectin	5´-GAAAGTACACCTGTTGTCATTCAACA-3´
	5´-ACCTTCACGTCTGTCACTTCCA-3´

was determined via photometric measurement[e] at 260/280. To avoid repeated free-thaw cycles for the prepared RNA for multiple experiments, aliquots of total RNA (100 ng) were applied for cDNA synthesis using a synthesis system[f] for the reverse transcription step with random healers. For each gene, a specific primer pair was designed[g] based on the cDNA sequence published as indicated. For each gene expression determination, three independent experiments were performed and the mean value of these was calculated.

PCR reactions were carried out using a continuous fluorescence detection device[h] and software[i]. Each sample was analyzed twice, employing the universal protocol over 46 cycles. Detailed reaction conditions included: 10 min 94°C of hot-start taq polymerase activation, 20 sec 95°C denaturation, 20 sec 55°C annealing, and 30 sec 72°C extension.

[e]*BioPhotometer is a product of Eppendorf.*
[f]*SuperScript III First-Strand Synthesis System is a product of InVitrogen.*
[g]*Primer Express 2.0 Software is a product of Applied Bio Systems.*
[h]*The Opticon 1 machine is a device from MJ Research, Waltham, MA, USA.*
[i]*The SYBR Green PCR Master Mix software is a product of Applied Biosystems.*

Formula 1. Sample cream used for in vivo tests

Polyglyceryl-3 methylglucose distearate	3.0%
Glyceryl stearate	2.0%
Stearyl alcohol	1.0%
C12-15 alkyl benzoate	9.5%
PPG-3 myristyl ether	9.5%
Glycerin	2.5%
1,2-Butanediol	0.25%
Polysorbate 20	0.025%
Peptide	varied
Preservative	0.8%
Fragrance (*parfum*)	0.1%
Water (*aqua*)	qs to 100.0%

For comparison of relative expression in real time PCR control cells and treated cells, the 2 (-ΔΔC(T)) method was used.

The tested peptides were applied at concentrations of 1 µg/mL for 24 hr to human dermal fibroblast cell cultures, and RNA was extracted to perform gene expression profiling. Induction of hyaluronic acid-synthase-1 and collagen was analyzed by real time

PCR with the primer pairs shown in **Table 1**. Since collagen production depends not only on stimulated gene expression, but also on a complex process of post-translational modification, researchers further quantified the collagen concentration in the fibroblast cell culture supernatants using a collagen assay[k].[8] All samples were incubated in the presence of β-aminopropionitrile (50 µg/mL) to increase the stability of the collagen.

In addition, an in vivo study was conducted with a panel of 60 volunteers divided into four groups. Four variations of a test cream were developed (see **Formula 1**), including a placebo cream without the active, a positive control cream incorporating 10 ppm palmitoyl pentapeptide-4,[2, 3] a cream with 10 ppm GEKG, and a cream with 100 ppm GEKG. The samples were applied to the inner forearms of subjects twice daily for eight weeks. Before and after eight weeks of application, skin volume and roughness were analyzed using a skin surface characterization device[m].

Results

Of all those tested, the most active peptide found had the sequence GEKG (see **Figure 1**). At a concentration of 1 µg/mL, the peptide increased the amount of secreted collagen protein in the supernatant approximately 2.5-fold. On the mRNA level, all three tested ECM marker genes were induced, resulting in a 2.5-fold increase of COL1A1 expression. In addition, HAS1 encoding for the hyaluronic acid was 5.7-fold, and the gene encoding for fibronectin was induced by 10.5-fold. The well-balanced induction of these important ECM constituents by the GEKG peptide suggests strong antiaging effects.

Although the palmitoyl pentapeptide-4 positive control showed a stronger induction in COL1A1 gene expression, the effect was not completely translated into collagen protein. This could be due to different gene induction kinetics. For instance, GEKG may cause a faster response on expression

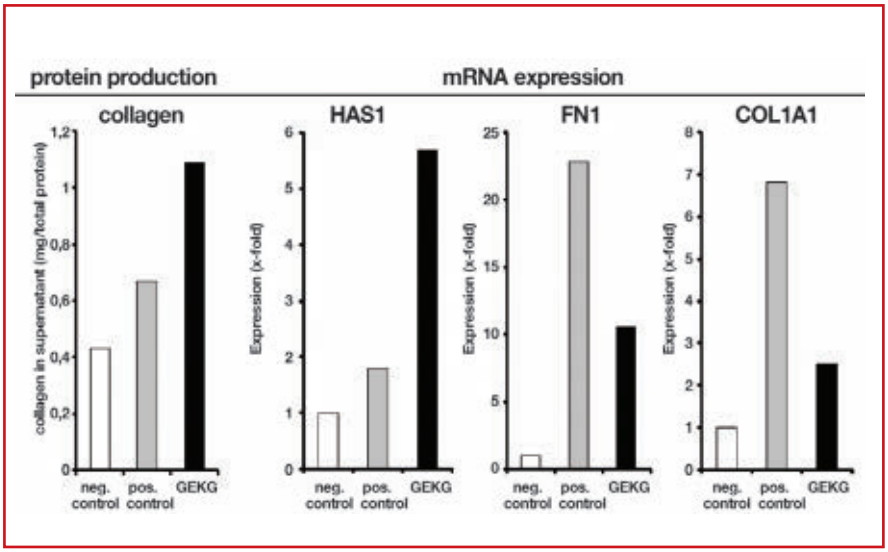

Figure 1. In vitro effects of GEKG on secreted collagen levels and ECM marker gene expression in human dermal fibroblasts

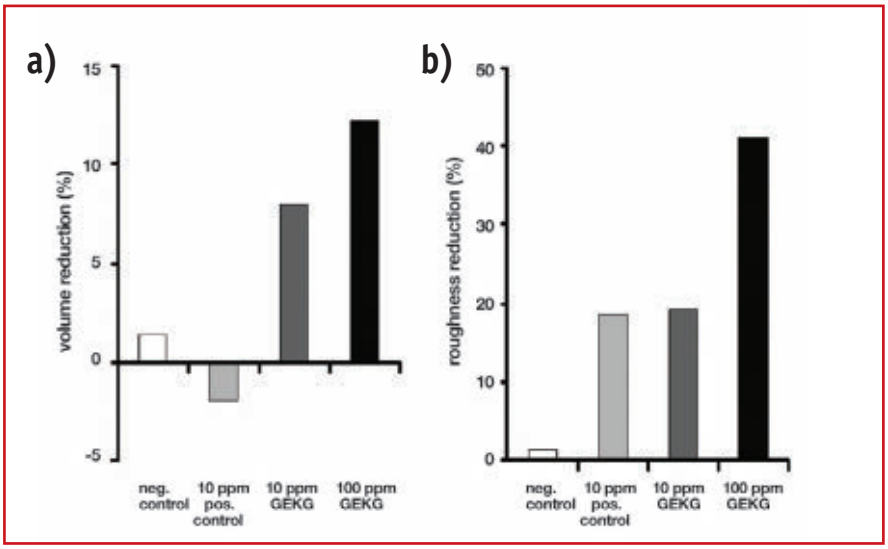

Figure 2. Topical application of GEKG reduced: a) the parameter volume, and b) skin roughness in vivo

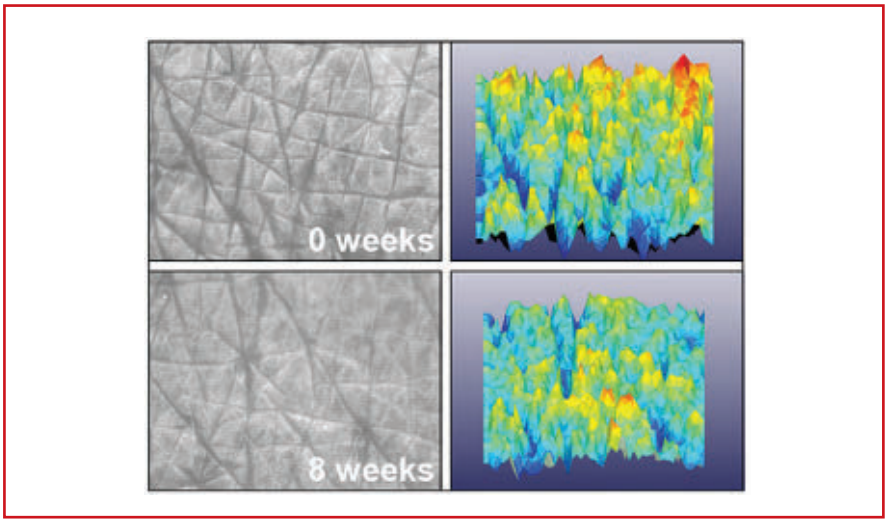

Figure 3. Photographs of one volunteer's skin taken before and after eight weeks of topical application of 100 ppm GEKG; for software-supported analysis, each photograph was digitalized and the differences were calculated

[k]*The Sircol Collagen Assay is a product of Biocolor.*
[m]*The Visioscan VC 98 is a device of Courage & Khazaka Electronic GmbH.*

levels that is immediately translated into protein production, and that afterward diminishes to minor levels; whereas the positive control peptide could take longer to react to the stimulus, thus delaying the response.

The in vivo relevance of GEKG activity was additionally tested by a vehicle-controlled biopsy and elasticity study. After eight weeks' application of a cream formulation containing 50 ppm GEKG, both collagen gene expression and skin elasticity significantly increased, compared with the vehicle and untreated skin (data not shown but available).

Thus, to demonstrate the proposed antiaging effect of GEKG in vivo, a parameter "volume" measurement was taken of the previously mentioned 60 volunteers. This software-based method compares the distribution of gray scales in photographs taken of the volunteers' skin before and after the eight-week application period. It calculates the theoretical amount of liquid that would be necessary to fill the wrinkles and generate a plain surface. A reduction of the parameter volume is interpreted as an overall improvement in skin structure resulting from the reduction of skin wrinkles in number and depth.

With an increasing concentration of GEKG, an increased reduction of the volume was observed. Compared with the positive control, a significant increase was obtained with 100 ppm GEKG (see **Figure 2a**).

Besides parameter volume measurements, parameter roughness was assessed via the same skin texture analysis device[m].[9,10] These roughness parameters originate from the DIN-parameters Ra–Rz, and describe the depth of fine and coarse wrinkles. R1–R5, for instance, describe the maximum and average amplitude of a surface structure, as well as the mean height level. In the case of GEKG in skin, **Figure 2b** again demonstrates a dose-dependent effect; increasing concentrations of GEKG increased the reduction of skin roughness. Compared with the positive control, 10 ppm GEKG showed comparable efficacy whereas 100 ppm GEKG doubled the effect.

Only 10 ppm of palmitoyl pentapeptide was tested, which is closer to its maximum suggested use level. GEKG at 10 ppm and 100 ppm translates to approximately 0.5–5.0% of the tetrapeptide-21 use concentration, which contains 2,000 ppm peptide.

Figure 3 shows the skin structure of one volunteer who applied the formulation containing 100 ppm GEKG for eight weeks. The pictures demonstrate an overall improvement of the skin structure; the wrinkles are less deep and less pronounced and the skin roughness is decreased.

Conclusion

Aging is associated with changes in the skin at all levels. For instance, degradation of key dermal-epidermal and dermal proteins occur, together with reduced epidermal proliferation and collagen and hyaluronic acid synthesis in the papillary dermis. Antiaging technologies must correct these deficiencies in order to induce skin regeneration to combat the resulting signs of aging.

The data presented in this study demonstrates that ECM-derived tetrapeptides have the potential to counterbalance the ECM degeneration observed during skin aging. In silico analysis identified approximately 30 abundant tetrapeptide motifs in ECM proteins, and in vitro analysis showed that 10 of these motifs can stimulate collagen synthesis.

GEKG was identified as a highly active tetrapeptide that is able to stimulate dermal repair and renewal mechanisms. An increased expression of ECM-synthesizing enzymes like hyaluronic acid synthethase, as well as expression and production of ECM proteins like collagen and fibronectin, was observed. Finally, in vivo data confirmed the efficacy of GEKG, leading to an improved skin structure and reduced wrinkles.

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