Cosmeceuticals represent a marriage between cosmetics and pharmaceuticals (1). There is an increasing trend towards the use of these agents in skin care regimens. Cosmeceutically active products can be broadly classified into the following categories: antioxidants, oligopeptides, growth factors and pigment lightning agents (2). Much attention has been focused on the tripeptides such as Gly-His-Lys (GHK) and Gly-Gly-His (GGH) and their copper complexes, which have a high activity and good skin tolerance. Recent data suggested their physiological role in process of wound healing, tissue repair and skin inflammation. The mechanism of anti-inflammatory properties of these peptides is not clear. The aim of the study was evaluation of influence of two peptides GGH, GHK and their copper complexes and saccharomyces/copper ferment (Oligolides® Copper) on secretion of pro-inflammatory IL-6 in normal human dermal fibroblasts NHDF cell line. IL-6 was evaluated using the ELISA kit. GGH, GHK, CuCl₂, and their copper complexes decreased TNF-α-dependent IL-6 secretion in fibroblasts. IL-6 is crucial for normal wound healing, skin inflammation and UVB-induced erythema. Because of the anti-inflammatory properties, the copper-peptides could be used on the skin surface instead of corticosteroids or non-steroidal anti-inflammatory drugs, which have more side effects. Our observations provide some new information about the role of these tripeptides in skin inflammation.

**Key words:** normal human dermal fibroblasts, GHK, GGH, copper-peptide complexes, interleukin 6

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study was the evaluation of influence of two peptides Gly-Gly-His, Gly-His-Lys and their copper complexes and saccharomyces/copper ferment (Oligolides® Copper) on secretion of pro-inflammatory IL-6, in normal human dermal fibroblasts NHDF cell line.

EXPERIMENTAL

Cell cultures
Normal human dermal fibroblasts NHDF were obtained from Clonetics™. The cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin G (sodium salt) and 100 mg/mL streptomycin (antibiotic solution, Gibco), and 10 mM HEPES (Gibco). The cell cultures were maintained at 37°C in 5% CO₂ atmosphere.

Modulators supply and their preparation
The solutions of Gly-Gly-His (GGH; copper-binding peptide) (Sigma), Gly-His-Lys acetate salt (GHK; liver cell growth factor) (Sigma) and copper(II) chloride dihydrate (CuCl₂ ◊ 2H₂O) (Sigma) were prepared by dissolving them in sterile-filtered water and were then diluted in a culture medium to the concentration 1 nM or 1 µM. Complexes of GGH or GHK with cooper (GGH-Cu, GHK-Cu) were prepared by mixing equimolar solutions of GGH or GHK and CuCl₂. Oligolides® Copper (Creations Couleurs) – a water solution containing saccharomyces/copper ferment and chlorophenesin was diluted in the medium to the concentrations 1% and 10%. The TNF-α (lyophilisate) was diluted in water to 10 µg/mL.

IL-6 assay
TNF-α stimulated IL-6 secretion by normal human dermal fibroblasts was evaluated using the enzyme-linked immunosorbent assay kit (R&D Systems). Normal human dermal fibroblasts were cultured in 96-well plates for 7–10 days. Cells were grown to the 90–95% confluence. Twenty four hours before initiation of the proper experiment, the medium was replaced with a medium without FBS containing 200 µg/mL bovine serum albumin (BSA). 1 nM or 1 µM solutions of GGH, GGH-Cu, GHK or GHK-Cu and 1 or 10% solutions of Oligolides® Copper were added into each well. Medium contained 100 ng/mL TNF-α. IL-6 concentration was evaluated after 72 h. The absorbance was measured at λ = 450 nm in a microplate reader (Triad LT Multimode Detector, Dynex Technologies).

Statistics
Statistical comparisons were made by the analysis of variance (ANOVA), and post-hoc Tukey’s test. A value of p < 0.05 was considered statistically significant. The results are expressed as the means ± standard deviation from number of experiments (n = 6).

RESULTS
The influence of 1 nM or 1 µM solutions of GGH, GHK, GGH-Cu, GHK-Cu and 1% and 10%
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saccharomyces/copper ferment, on IL-6 secretion in NHDF cells was investigated. As shown in Figure 1, the cells, growing in medium without TNF-α, secreted the small amounts of IL-6. The addition of TNF-α caused almost 4-fold increase of IL-6 secretion. The increase of IL-6 secretion was also observed in cells cultured in the medium containing 10% FBS. Also in this case TNF-α intensified this process. According to the requirements of IL-6 assay kit fibroblast during the experiment should be cultured in a medium containing 200 µg/mL BSA. During the experiments the cells were cultured for 72 h in the presence of GGH and GHK. One nM or 1 ìM GGH and GHK decreased TNF-α-dependent IL-6 secretion in fibroblasts (Fig. 2). Copper complexes with GGH and GHK or CuCl₂ gave similar results (Fig. 3). In contrast to the tripeptides and their copper complexes, 1 and 10% solution of Oligolides® Copper had no effect on IL-6 secretion. This tendency, although not significant, was observed in case of 1% solution (p = 0.051) (Fig. 4).

DISCUSSION AND CONCLUSION

The wound healing process involves inflammation, migration, deposition and maturation of skin cells. Cytokines produced by a variety of cells, including fibroblasts, act as mediators in all the phases of wound healing (1). By manipulating various cytokines and growth factors, it may be possible to modify the wound healing process in different clinical states. It was found that copper complex GHK-Cu suppresses inflammation by decreasing the level of acute phase inflammatory cytokines such as transforming growth factor β and TNF-α (11). Cell culture studies showed that GHK-Cu has maximal biological effect at 10⁻⁷ mol/L (5). GHK also reduces oxidative damage by modulating iron levels and by quenching toxic products of fatty acids peroxidation (12). It serves as the chemoattractant for inflammatory and endothelial cells (13) and increases fibroblast proliferation (14). Copper peptides were also used in combination with tretinoin (Retin A) to reduce skin inflammation and after cosmetic procedures such as chemical peels, laser and dermabrasion procedures (15). The mechanism of anti-inflammatory properties of GHK-Cu is not clear. The observed by us influence of GHK, GGH and their copper complexes on TNF-α-dependent IL-6 secretion in fibroblasts provides some new information about role of these peptides in skin inflammation. The fibroblasts and keratinocytes are

Figure 3. Effect of GGH-Cu, GHK-Cu and CuCl₂ on IL-6 secretion by human normal fibroblasts cell line. The results represent the mean ± SD (n = 6); * p < 0.05 (ANOVA, post-hoc Tukey’s test) compared with the control (K)

Figure 4. Effect of Oligolides® Copper, a saccharomyces/copper ferment, on IL-6 secretion by human normal fibroblasts cell line. The results represent the mean ± SD (n = 6); * p < 0.05 (ANOVA, post-hoc Tukey’s test) compared with the control (K)
a crucial source of cytokines in skin tissue, for example, IL-6, which secretion was increased in response to TNF-α. The pleiotropic nature of IL-6 is shown by its actions in both proinflammatory and anti-inflammatory ways. IL-6 is a key mediator in various inflammatory processes by which tissues respond to injury and infection, both in the acute-phase reaction or in chronic inflammatory diseases (16). In the skin, epidermal IL-6 is critical for normal wound healing, which is impaired in IL-6−/− mice (17). Reeve et al. (18) suggested that IL-6 could contribute to the UVB-induced erythema. In contrast to the tripeptides and their copper complexes, Oligolides® Copper – a saccharomyces/copper fermentation had no effect on IL-6 secretion. There are no many publications on the topic of influence of the peptides like GHK or GGH on cytokines secretion at the moment. Fields et al. (19) wrote about palmitoyl tetrapeptide-7, which is an active ingredient of any cosmetics. It is able to down-regulate IL-6 in vitro in both resting and inflamed cells. Marketing materials related to Rigin™ indicate that this reduction in IL-6 can produce increased skin firmness, smoothness, and elasticity (20). One mM GHK significantly inhibited IL-6 expression in SZ95 sebocytes (21).

In the past, several laboratories demonstrated local and systemic anti-inflammatory activity of copper compounds, particularly after subcutaneous administration. Copper-peptides could be used on the skin surface instead of corticosteroids or non-steroidal anti-inflammatory drugs, which have more side effects (22).

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REFERENCES