EFFECT OF GLY-HIS-LYS AND ITS COPPER COMPLEX ON TGF-β1 SECRETION IN NORMAL HUMAN DERMAL FIBROBLASTs

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Abstract : Transforming growth factor β (TGF-β) is a cytokine involved in a wide variety of biological processes such as cell growth, differentiation and proliferation, apoptosis and regulation of the immune response. It has an important role in wound healing process, fibrosis and scar tissue formation. Similarly to TGF-β1, insulin growth factor (IGF) family is expressed locally in response to tissue injury. Treatment of dermal fibroblasts with IGF-1 caused a substantial induction of TGF-β1 mRNA. Not a great deal of research so far has focused on IGF-2. Much attention has been focused on the tripeptides such as Gly-His-Lys (GHK) and their copper complexes, which have a high activity and good skin tolerance. Recent data suggest that their physiological role has been related to the process of wound healing, tissue repair and skin inflammation. In the present study, the influence of 1 nM solutions of GHK, GHK-Cu and CuCl$_2$, on IGF-2-dependent TGF-β1 secretion in normal human dermal fibroblasts cells was investigated. Fibroblasts were cultured in 24-well plates. Total TGF-β1 protein was evaluated using the ELISA kit. The Bradford reagent was used to determine the total quantity of cellular protein. Treatment of fibroblasts with 100 ng/mL IGF-2 resulted in a significant increase in TGF-β1 secretion. GHK and its copper complex and free copper ions decreased IGF-2-dependent TGF-β1 secretion. Our observations provide some new information on the potential use of that peptide contained in cosmetics to treat and prevent the formation of hypertrophic scars.

Keywords: normal human dermal fibroblasts, Gly-His-Lys, GHK-Cu, TGF-β1, IGF-2, fibrosis

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 its copper complex, which have a high activity and good skin tolerance. Recent data suggested that their physiological role have been related to the process of wound healing, tissue repair and skin inflammation (3). GHK was isolated from human plasma, where it is present at concentration of about 200 ng/mL (4). It reveals high affinity to copper(II) ions exhibited in spontaneous formation of complex GHK-Cu. GHK may protect copper(II) ions against redox reaction, modulate copper intake into cell and increase the level of antioxidant enzymes and superoxide dismutase activity (5). It modulated an expression of both matrix metalloproteinases (MMP-1 and MMP-2) and their inhibitors (TIMP-1 and TIMP-2), improving wound healing and facilitating skin remodeling processes (6). GHK-Cu increased mRNA for collagen, dermatan sulfate, chondroitin sulfate, and a small proteoglycan decorin (7). Canapp et al. (8) showed that GHK-Cu improved healing of ischemic wounds and suppresses inflammation by lowering the level of acute-phase inflammatory cytokines such as transforming growth factor β (TGF-β) and tumor necrosis factor α (TNF-α).

TGF-β is a family of growth factors engaged in a number of essential cellular functions. The three isoforms of TGF-β (β1, β2 and β3) are secreted as inactive latent precursors that require activation prior to binding to the TGF-β receptors. TGF-β is a multifunctional cytokine involved in a wide variety of biological processes such as embryonic development, neoplastic growth and differentiation, cell proliferation, apoptosis and regulation of the immune response (9). It is an important regulator of the wound healing process. In particular, its interaction with dermal fibroblasts and the subsequent production of extracel-

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lular products including collagen highlights its central role in abnormal scarring. Wang et al. (10) showed that hypertrophic scar derived fibroblasts produced more mRNA and protein for TGF-β1 than fibroblasts derived from normal skin, suggesting a possible role for TGF-β1 in hypertrophic scar formation. Similarly to TGF-β1, insulin growth factor (IGF) family is expressed locally in response to tissue injury. Expression of IGF-1 increased in parallel to the formation of granulation tissue after injury (11). Ghahary et al. (12) found greater expression of TGF-β1 and IGF-1 in post-burn hypertrophic scar tissues as compared to normal dermis from the same patients. Treatment of human dermal fibroblasts with IGF-1 caused a substantial induction of TGF-β1 mRNA. Although IGF-1 and IGF-2 exert their main effects through the IGF-1 receptor, their biological effects are different (13). According to Musselmann et al. (14), IGF-2 may serve as a mechanism to immediately activate keratocytes upon wounding and to ameliorate the scarring effects of TGF-β.

The present study investigated effect of IGF-2 on TGF-β1 secretion in normal human dermal fibroblasts and the influence of GHK and GHK-Cu on this process.

EXPERIMENTAL

Cell cultures

Normal human dermal fibroblasts (NHDF) were obtained from Clonetics™. The cells were cultured in minimum essential medium (MEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 100 IU/mL penicillin G (sodium salt), 100 µg/mL streptomycin (antibiotic solution, Sigma-Aldrich) and 10 mM HEPES (Gibco). The cell cultures were maintained at 37°C in 5% CO_2 atmosphere. In this study, we used cultures at passages 5 and 10. Fresh growth medium was added every 3 to 4 days and cells were kept subconfluent until used for experiments. Because of the significant content of TGF-β1 in FBS, it is recommended to culture cells in a medium without added serum or with its serum content decreased. Ninety percent confluent monolayer was rendered quiescent in media containing 0.2% fetal bovine serum FBS (Life Technologies) or ITS Premix (BD Biosciences) in dilution 1 : 100 for 24 h before use in experiments. ITS Premix contained insulin, human transferrin and selenous acid.

Modulators supply and their preparation

The solutions of GHK (liver cell growth factor, Sigma-Aldrich) and copper(II) chloride dihydrate ($CuCl_2 \times 2H_2O$, Sigma-Aldrich) were prepared by dissolving them in sterile-filtered water and were then diluted in a culture medium to the concentration of 1 nM. Complex of GHK with cooper (GHK-Cu) was prepared by mixing equimolar solutions of GHK and $CuCl_2$. The TNF-α and IGF-2 (lyophilizates) were diluted in water to 100 µg/mL.

TGF-β1 assay

The total TGF-β1 quantity in cultured medium was evaluated using the Legend Max enzyme-linked immunosorbent assay kit with pre-coated plates (BioLegend). Fibroblasts were cultured in 24-well plates (Sarsted). After 24 h, the medium is clarified by centrifugation and samples are stored at –70°C. To activate latent TGF-β1 to the immunoreactive form, the samples were acidified with 1 M HCl. The absorbance was measured at λ = 450 nm in a microplate reader (Triad LT Multimode Detector, Dynex Technologies). TGF-β1 concentration was determined from a standard curve and the resulting values were converted to the total amount of cellular protein.

Total protein assay

The Bradford reagent was used to determine the total quantity of protein in cell lysates. After removal of the culture medium, 24-well plates were placed in –70°C for 24 h, and then 100 µL 0.125% dodecyl sulfate sodium solution was added to each well. The cell lysates were centrifuged at 13000 rpm for 3 min. The absorbance was measured at λ = 595 nm by photometer Epoll-20. Bovine serum albumin was using as the standard protein.

Statistics

Statistical comparisons were made by the analysis of variance (ANOVA, *post hoc* Tukey's test) or by Student's *t*-test. A value of p < 0.05 was considered statistically significant. The results are expressed as the means ± standard deviation from number of experiments. Statistical analysis was performed using data analysis software system STATISTICA (StatSoft, Inc. 2011)

RESULTS AND DISCUSSION

Wound healing is a multi-stage process leading to the reconstruction of damaged tissue. It may be divided into inflammation, proliferation and maturation or remodeling. TGF-β1 is a well known cytokine to initiate the sequent wound healing phases of inflammation, angiogenesis, reepithelialization, and connective tissue regeneration. It is close-
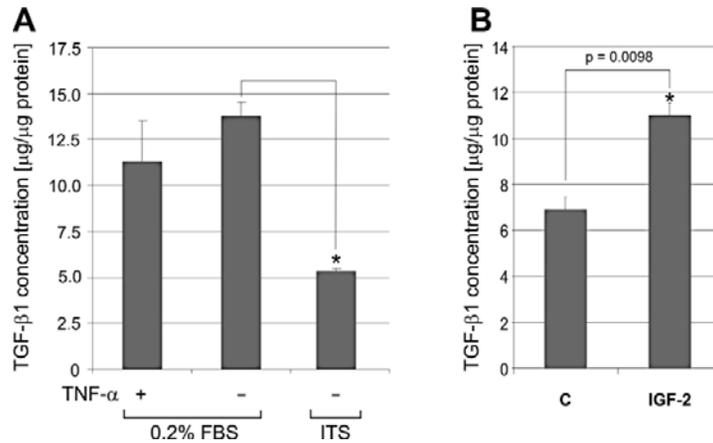
Figure 1. Influence of 100 ng/mL TNF-α, 100 ng/mL IGF-2 and type of medium on TGF-β1 secretion by NHDF cell line. (**A**) The cells were cultured for 24 h in medium containing 0.2% FBS and its Premix dilution 1 : 100. The results represent the mean ± SD (n = 3); * p < 0.05 (*t*-Student's test). (**B**) The cells were cultured for 24 h in serum-free medium containing ITS Premix in dilution 1 : 100. The results represent the mean ± SD (n = 4); * p < 0.05 (*t*-Student's test) compared with the control (**C**)
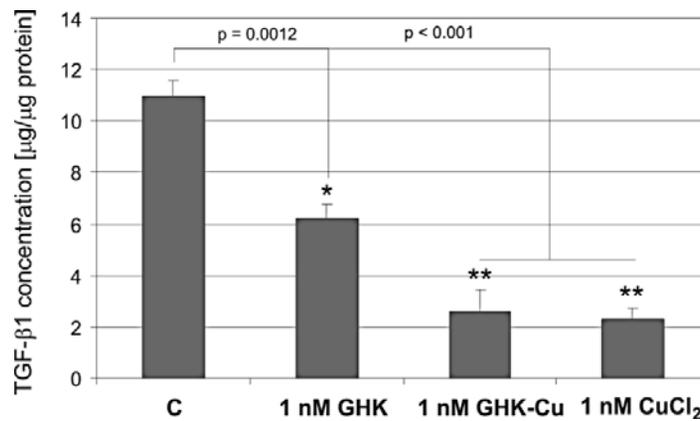

Figure 2. Influence of 1 nM GHK, GHK-Cu and CuCl₂ on IGF-2-dependent TGF-β1 secretion by NHDF cell line. The cells were cultured for 24 h in serum-free medium containing ITS Premix in dilution 1 : 100. The results represent the mean ± SD (n = 4); * p < 0.05 (ANOVA, *post hoc* Tukey's test) compared with the control (**C**)
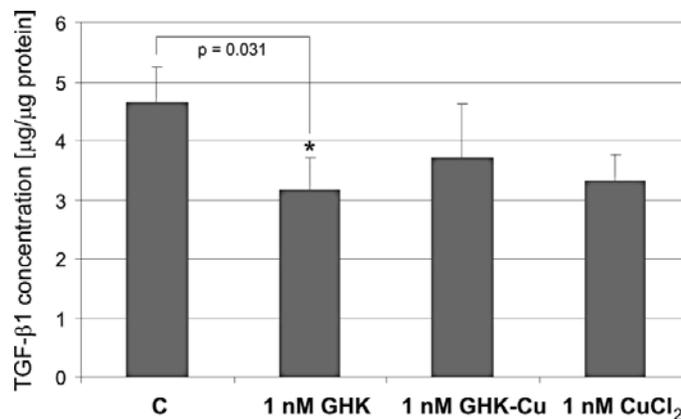

Figure 3. Influence of 1 nM GHK, GHK-Cu and CuCl₂ on TGF-β1 secretion by NHDF cell line. The cells were cultured for 24 h in medium containing 0.2% FBS. The results represent the mean ± SD (n = 4); * p < 0.05 (ANOVA, *post hoc* Tukey's test) compared with the control (**C**)

ly involved in the remodeling phase with function of stimulating the collagen synthesis and disposition by the sustained activation of fibroblasts. TGF-β1 can up-regulate the angiogenic growth factor like vascular endothelial growth factor (VEGF) and is also a potent inhibitor of MMP-1, MMP-3, and MMP-9 and a promoter of tissue inhibitor of MMP-1 synthesis (15). The action of TGF-β1 depended on its concentration and amount of active latent form. The proliferation of mink lung fibroblasts is stimulated by low concentrations of TGF-β1 (5–10 ng) and inhibited by higher concentrations (16). The chronic overexpression of TGF-β1 or one of its fellow family members β2 and β3 drives the formation of keloids and hypertrophic scars (17).

TGF-β1 production may be controlled at the levels of transcription, translation, secretion of preformed protein and activation of the latent protein to its active form (18). IGF-1 may directly increase secretion of TGF-β1 into extracellular matrix (12). IGF-1 and IGF-2 mainly exert their effects through the IGF-I receptor but their biological action are different (13). In contrast to IGF-1, poorly understood is the role of IGF-2 in this process. Two potential secretion inductors of TGF-β1, TNF-α and IGF-2 were used in this study. Similarly to IGFs family, TNF-α rapidly (at 4–6 h) upregulated expression of TGF-β1 mRNA resulting in increased production of TGF-β1 protein (18), while there are several studies that reported no increase in (or reduced) TGF-β1 expression consequent to TNF-α treatment (19, 20). We recorded almost twofold increase in TGF-β1 secretion in response to 100 ng/mL IGF-2 in contrast to 100 ng/mL TNF-α. NHDF cells released small amounts of TGF-β1 regardless of the inductor used (Fig. 1). All marked TGF-β1 most likely was coming from a latent complex. Its active form remained undetected in the studied samples or its concentration was below detection threshold. No significant effect of TNF-α on TGF-β secretion may be associated with the use of high concentrations of TNF-α (100 ng/mL). These conflicting data may be explained by differences in the experimental protocol or in the cell types used, regulation of TGF-β1 may be cell type-specific.

Much attention has been focused on the tripeptides such as GHK and its copper complex, 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. Matalka et al. (21) demonstrated that GHK or its pegylated form at a very low, nontoxic concentration (1–10 nM) stimulated fibroblast proliferation. Cangula et al. (22) reported that open wounds in

rabbits treated with GHK-Cu tend to heal faster than similar wounds treated with zinc oxide or kept untreated. They observed more granulation tissue formation in the GHK-Cu-treated wounds. According to Borkow et al. (23), this effect may be associated with the supply of copper to the wound tissue. This mechanism is poorly understood. In the present study, we investigated an effect not only GHK-Cu but also GHK and free copper (II) ions on IGF-2-dependent TGF-β1 secretion in NHDF cells. We observed that 1 nM GHK inhibited IGF-2-dependent (Fig. 2) and IGF-2-independent TGF-β1 secretion (Fig. 3), although in the second case we can say about a trend (p = 0.31). The addition of copper in the ions form (CuCl$_2$) and in the GHK complex form inhibited IGF-2-dependent TGF-β1 secretion more strongly than the use of the same peptide. The copper (II) ions effect was similar to the GHK-Cu action (Fig. 2). One nanomole of GHK-Cu and CuCl$_2$ have no effect on IGF-2-independent TGF-β1 secretion when the fibroblasts were treated with medium containing only 0.2% FBS (Fig. 3). McCormack et al. (24) obtained similar results. GHK-Cu reduced the secretion of TGF-β1 by normal fibroblasts and keloid producing fibroblasts. Although both cell types show sensitivity to GHK-Cu treatment, only keloid fibroblasts demonstrated statistically significant reductions (24). The simplest form of copper, an inorganic salt, cannot be a possible source of delivery of the metal ions to the low layers of skin because of its low bioavailability and its general toxicity to the organism. One of the widely used methods of delivering metal ions into the skin is their complexation with different ligands, among which peptides play a main role (25). Altering the availability of these peptides and its copper complexes at their target sites within the skin may offer new therapeutic approaches in skin disease and dermatology, wound healing and for cosmetic applications. Copper tripeptide therapy seems to suppress secretion of "fibrogenic" growth factor (TGF-β1) in fibroblasts and it may have application in decreasing excess scar formation.

Acknowledgment

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