

In vivo stimulation of connective tissue accumulation by the tripeptide-copper complex glycyl-L-histidyl-L-lysine-Cu²⁺ in rat experimental wounds.

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Research Article

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In Vivo Stimulation of Connective Tissue Accumulation by the Tripeptide-Copper Complex Glycyl-L-histidyl-L-lysine-Cu²⁺ in Rat Experimental Wounds

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Abstract

The tripeptide-copper complex glycyl-L-histidyl-L-lysine-Cu²⁺ (GHK-Cu) was first described as a growth factor for differentiated cells. Recent in vitro data showed that it possesses several properties of a potential activator of wound repair. We investigated the effects of GHK-Cu in vivo, using the wound chamber model described previously (Schilling, J. A., W. Joel, and M. T. Shurley, 1959. *Surgery [St. Louis]*. 46:702-710). Stainless steel wire mesh cylinders were implanted subcutaneously on the back of rats. The animals were divided into groups that received sequential injections into the wound chamber of either saline (control group) or various concentrations of GHK-Cu. At the end of the experiments, rats were killed, wound chambers were collected, and their content was analyzed for dry weight, total proteins, collagen, DNA, elastin, glycosaminoglycans, and specific mRNAs for collagens and TGFβ. In the GHK-Cu-injected wound chambers, a concentration-dependent increase of dry weight, DNA, total protein, collagen, and glycosaminoglycan contents was found. The stimulation of collagen synthesis was twice that of noncollagen proteins. Type I and type III collagen mRNAs were increased but not TGFβ mRNAs. An increase of the relative amount of dermatan sulfate was also found. A control tripeptide, L-glutamyl-L-histidyl-L-proline, had no significant effect. These results demonstrate that GHK-Cu is able to increase extracellular matrix accumulation in wounds in vivo. (*J. Clin. Invest.* 1993. 92:2368-2376.) Key words: wound healing • collagen • glycosaminoglycans • extracellular matrix • growth factors

Introduction

Glycyl-L-histidyl-L-lysine-Cu²⁺ (GHK-Cu)¹ is a tripeptide-copper complex that was isolated from human plasma by Pick-

art and Thaler in 1973 (1, 2). GHK-Cu was first described as a growth factor for a variety of differentiated cells (3). Subsequent data from various groups indicated that it exhibits several properties of a potential activator of the wound healing process. It was a potent chemotactic agent for monocytes/macrophages and mast cells (4, 5). It stimulated nerve tissue regeneration (6) and was reported to trigger the angiogenesis process in vivo (7). It stimulated collagen synthesis in several fibroblast strains (8). Preliminary in vivo data reported an acceleration of wound closure when GHK-Cu was injected into superficial wounds in animals (9, 10). It also exerted metabolic effects such as inhibition of lipid peroxidation by ferritin (11).

We used the wound chamber model described by Schilling et al. (12) to study the biochemical parameters of wound healing in vivo. As reported by these authors, this model creates a wound with a stable dead space whose content may be easily collected for analysis. We tested the effects of GHK-Cu on this model. Our data demonstrate that GHK-Cu injection into the wound chamber is able to stimulate the accumulation of collagen and dermatan sulfate proteoglycans.

Methods

Animals. Male Sprague-Dawley rats weighing between 250 and 300 g were used in the experiments. They were provided by Iffa-Credo (L'Arbresle, France). They were placed in individual suspended stainless steel cages with food and water ad libitum.

Reagents. Usual reagents (analytical grade) were from Prolabo (Paris, France). Hydroxyproline was from Calbiochem, France-Biochem (Meudon, France), desmosin and isodesmosin was from Elastin Products Co. (St. Louis, MO), fibronectin was from Institut Jacques Boy (Reims, France), antifibronectin antibody was from Institut Pasteur de Lyon (Lyon, France), copper chloride, hyaluronic acid, and glycosaminoglycan standards were from Sigma (La Verpillière, France), and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) was from Aldrich (Strasbourg, France). Cyanogen bromide (CNBr) and thin layer chromatography plates were obtained from Merck (Darmstadt, Germany). GHK-Cu (PC 1020, glycyl-L-histidyl-L-lysine: Cu²⁺, 2:1 molar complex of peptide to copper) was provided by Procyte Corporation (Kirkland, WA). GHK was obtained from Bachem (Basel, Switzerland) and L-glutamyl-L-histidyl-L-proline (EHP) was from Neosystem (Strasbourg, France). Acid-soluble type I collagen was prepared from rat tail tendon in this laboratory. Rat type III collagen was from Institut Jacques Boy (Reims, France). Its purity was checked by SDS-PAGE (13).

cDNA probes. Plasmids used were pH CAL1 with a 670-bp insert specific for the COOH-terminal end of the α1(I) chain of type I collagen (generous gift of Professor E. Vuorio, Turku, Finland) (14) and pBluescript II KS⁺, containing the major coding region of TGFβ1 (generous gift of Professor M. Sporn, Bethesda, MD [15]). Rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA probe (generous gift of Dr. P. Fort, Montpellier, France [16]) was used as reference. Plasmids were labeled to specific activities of > 10⁸ cpm/μg DNA by

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1. *Abbreviations used in this paper:* CNBr, cyanogen bromide; DPBS, Dulbecco's phosphate-buffered saline; EHP, L-glutamyl-L-histidyl-L-proline; GAGs, glycosaminoglycans; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; GHK, glycyl-L-histidyl-L-lysine; GHK-Cu, glycyl-L-histidyl-L-lysine-Cu²⁺; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole.

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random priming using Klenow fragment (Gibco BRL, Gaithersburg, MD) and [32 P]dCTP (Amersham Corp., Arlington Heights, IL). The labeled cDNA-probe was separated from dCTP by chromatography through a G-50 spin column.

Wound chambers. Wound chambers were made of stainless steel wire mesh (C-CX 20; EDMEC Inc., Bellevue, WA) as 1-cm-diameter by 2.5-cm-long cylinders. They were closed at both ends by Teflon caps and sterilized by autoclaving.

Surgical procedures. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg; Clin-Midy, Paris, France). Dorsal hair was clipped widely from scapula to pelvis and the nude area was sterilized with polyvidone iodine (Betadine[®]; Laboratories Sarget, Mérignac, France). Bilateral incisions were made perpendicular to the spine to the skin's full-thickness through the panniculus carnosus to the fascial plane). A space approximately the size of the chamber was opened under the dermis and sterile wound chambers with caps were slipped beneath the skin. The incisions were closed through individual 4.0 nylon sutures. Animals were then returned to their cages until the first injection.

Experimental design. A first set of experiments was performed for studying the effects of repeating a single dose of GHK-Cu. Two wound chambers were inserted on the back of each rat. The first one received serial injections of 2.0 mg GHK-Cu dissolved in 0.2 ml Dulbecco's phosphate-buffered saline (DPBS). The first injection was 7 d after implantation, then every 3 d for an additional 2 wk. The other chamber received on the same days injection of the same volume of DPBS. Chambers were collected on day 29, immediately frozen at -20°C , and lyophilized. This day (4 wk after insertion) was initially chosen because of the previous works of Schilling et al. (12), who designed the chamber model and showed that, at this time, the chamber is nearly filled with new connective tissue. The dried chamber content was weighted. It was then dissolved in 0.5 M NaOH and an aliquot was taken for DNA measurement according to Fiszer-Szafarz et al. (17). The remaining solution was neutralized with HCl, and ethanol was added to the final concentration of 80% (wt/vol). After 18 h at 4°C and a centrifugation at 5,000 g for 30 min, supernatant was collected, the pellet resuspended in saline, and the ethanol precipitation repeated once. Both supernatants were pooled, evaporated under nitrogen, and submitted to acid hydrolysis in 6 M HCl at 110°C for 18 h. This fraction was used for fluorometric measurement of hydroxyproline contained in the small peptides formed by collagen degradation, using the fluorophore 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), and a thin layer chromatography as described previously (18). Ethanol precipitation also eliminated some unidentified pigments that interfered in the protein measurement. The ethanol precipitate was redissolved in 0.5 M NaOH and an aliquot was taken for measurement of total proteins by the method of Lowry et al. (19). The remaining fraction was collected and used for measurement of collagen and glycosaminoglycans.

Collagen was measured by its hydroxyproline content. An aliquot of the NaOH-solubilized material was neutralized, and hydrolyzed in 6 M HCl at 110°C for 18 h. Hydroxyproline was quantified in the hydrolyzate by fluorometry (18). For glycosaminoglycan analysis, another aliquot was neutralized with acetic acid and digested with pronase in 0.05 M Tris HCl, pH 8.0, 0.02 M CaCl_2 , for 48 h at 48°C . Trichloroacetic acid was added to the hydrolyzate to 10% (wt/vol) final concentration. The samples were centrifuged at 5,000 g for 15 min and the supernatant was dialyzed exhaustively against distilled water at 4°C . An aliquot of the nondialyzable material was used for the measurement of uronic acid (20) and the remaining part was lyophilized and used for electrophoresis.

The separation of glycosaminoglycans (GAGs) was carried out by cellulose acetate electrophoresis in 0.1 M zinc acetate, pH 5.0, for 80 min at 80 V followed by electrophoresis in 0.1 M HCl for 20 min at 40 V. The GAGs were stained with 0.2% Alcian blue according to Bartold et al. (21). The strips were scanned at 560 nm with a Sebia gel scanning system. The distribution of GAGs was estimated after correction for staining of GAG standards. Specific GAGs were identified by hydroly-

sis with specific enzymes: hyaluronidase (EC 4.2.2.1) for hyaluronic acid, chondroitinase ABC (EC 4.2.2.4) and chondroitinase AC (EC 4.2.2.5) for chondroitin sulfates, and dermatan sulfate, according to Saito et al. (22). Heparan sulfate was identified as the remaining fraction susceptible to HNO_2 degradation (23).

A second set of experiments was used for studying the effects of increasing amounts of GHK-Cu injected into the wound chambers. For that purpose, 18 rats were divided into 6 groups and 2 wound chambers were implanted on the back of each rat. The first group (controls) received the injection of 0.2 ml of DPBS into the wound chambers every 3 d from days 7 to 23 after the chamber implantation. The second group received 0.2 mg GHK-Cu dissolved in 0.2 ml DPBS, the third 0.5 mg, the fourth 1.0 mg, the fifth 2.0 mg, and the sixth 4.0 mg, on the same days as the control group. The chambers were collected on day 29, their contents collected and lyophilized, and their weights measured. An aliquot of the lyophilized material was taken, dissolved in 0.1% SDS, and used for fibronectin measurement by immuno-enzyme assay (24). Another aliquot was used for measuring desmosin and isodesmosin (an estimate of the elastin content) by ion exchange chromatography on a Multichrom B amino-acid analyzer (Beckman Instrs. Inc., Fullerton, CA) after acid hydrolysis (6 M HCl, 18 h at 110°C). The remaining material was dissolved in 0.5 M NaOH and processed as described above for measurement of DNA, total proteins, collagen, and uronic acid.

A third set of experiments was designed for investigating the effects of GHK-Cu on the expression of the genes of type I and type III collagens and TGF β inside the chambers. Two wound chambers per animal were inserted on day 0 on the back of 10 rats. A first series of five rats (controls) received in both wound chambers the injection of 0.2 ml DPBS on day 2, then every 3 d until day 17. The other series received on the same days 2.0 mg of GHK-Cu dissolved in the same volume of DPBS. A control and a GHK-Cu-treated rat were killed on days 3, 7, 10, 14, and 21. Both chambers were collected. The first one was immediately immersed in a formol (10% [vol/vol]) solution in DPBS for histological examination. The tissues inside were formalin fixed, paraffin included, and 4- μm -thick sections were stained with hematoxylin-eosin and safran. The second chamber was immediately frozen in liquid nitrogen before mRNA extraction, according to Chomczynski and Sacchi (25). Once deep frozen, the wound chamber content was carefully collected using a scalpel and homogenized in 4 M guanidine-isothiocyanate containing 25 mM sodium citrate, pH 7.5, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol in order to inactivate ribonucleases. The homogenate was then extracted with phenol, chloroform, isoamyl-alcohol mixture. After centrifugation at 10,000 g for 20 min at 4°C , the aqueous phase was mixed with an equal volume of isopropanol and placed at -20°C for at least 2 h to precipitate RNAs. Centrifugation at 10,000 g for 20 min was again performed, the resulting RNA pellet resuspended in the guanidine-isothiocyanate solution, and precipitated once more with isopropanol. After centrifugation, the RNA pellet was resuspended in 75% ethanol, sedimented, vacuum dried, and finally dissolved in diethylpyrocarbonate-treated water. RNA content was quantitated by its absorbance at 260 nm. Northern blot and slot blot analyses were then carried out to quantify collagen gene expression at the mRNA level.

For Northern blot analysis, 6 μg of total RNA was dissolved in 50% formamide, 2.2 M formaldehyde, 0.1 M 3-[N-morpholino]-propane sulfonic acid, pH 7.0, 40 mM sodium acetate, 5 mM EDTA, pH 8, and denatured by heating at 65°C for 10 min. RNA was then fractionated by electrophoresis in 1% agarose/formaldehyde gel for 4 h at 70 V (26). Duplicate RNA samples were either stained with ethidium bromide or transferred overnight by capillary blotting in 20 $\times$ standard saline citrate solution (1 $\times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7) to nylon filter (Biodyne; Pall Ultrafine Filtration Corp., Glen-Cove, NY). The air-dried nylon filter was UV irradiated for 5 min to bind transferred RNAs.

The nylon filter was prehybridized for 24 h at 42°C in 10% SDS, 5 $\times$ SSC, 5 $\times$ Denhardt's solution, 250 $\mu\text{g}/\text{ml}$ of denatured salmon sperm DNA, and 50% formamide. Specific hybridization was carried out

overnight at 42°C in freshly prepared prehybridization solution with the addition of 10^6 cpm/ml of 32 P-labeled cDNA probe. The filter was washed twice in $2\times$ SSC, 0.1% SDS at room temperature, twice in $0.1\times$ SSC, 0.1% SDS at 68°C, air dried, and exposed to autoradiography at -80°C using hyperfilm MP (Amersham Corp.) and Kodak X-Omat cassette C2 with intensifying screens. Autoradiographic signals were quantitated using a scanning densitometer Desaga (Desaga GmbH, Heidelberg, Germany).

For slot blot analysis, 8 μ g of total RNA was diluted in $6\times$ SSC, 2.2 M formaldehyde, and 50% formamide, denatured at 65°C for 10 min, and applied to a nylon filter in a slot blot apparatus (Bio-Rad Laboratories, Richmond, CA) using gentle aspiration. The subsequent steps were identical to the Northern blot protocol.

Measurement of the ratio of type III to type I collagen. A wound chamber was inserted on day 0 on the back of 18 rats. They were then divided into two groups. The first one (controls) received in the chamber the injection of 0.2 ml DPBS on day 2, then every 3 d until day 17. The other series received on the same days 2.0 mg of GHK-Cu dissolved in the same volume of DPBS. Three control and three GHK-Cu-treated rats were then killed on days 7, 14, and 21. The chambers were collected, immediately frozen in liquid nitrogen, and lyophilized. The chamber content was then collected and submitted to CNBr digestion according to Epstein (27). After dialysis and lyophilization, CNBr peptides were separated by SDS-PAGE (13) using a 5% stacking gel and a 10.0% separating gel. The percentage of type III collagen was then deduced from the ratio of the $\alpha 1$ (III)-CB 5 to $\alpha 1$ (I)-CB 8 peptides, as described by Chan and Cole (28).

Specificity of GHK-Cu effects. For checking the specificity of GHK-Cu effects, wound chambers were inserted on day 0 on the back of 40

rats. 10 rats were used as controls and received serial injections of 0.2 ml DPBS with the usual schedule (every 3 d from days 3 to 17). A second group of 10 rats received serial injections of 2.0 mg GHK-Cu in the same volume of saline. The third group of 10 rats received serial injections of 1.8 mg of GHK alone, which is not complexed with copper. The last group ($n = 10$) received serial injections of 0.4 mg copper chloride. The doses of GHK and of copper chloride were chosen for providing an equimolar amount of product, compared to the chambers injected with the GHK-Cu complex. The chambers were collected on day 21 for measuring collagen content, as described previously.

An additional experiment was performed for comparing the effects of GHK-Cu to those of the tripeptide glu-his-pro (EHP), which is the inactive metabolite of thyroid releasing hormone. The same schedule of injections as above was adopted (every 3 d from days 3 to 17). The first group (controls) received serial injections of 0.2 ml DPBS, the second group 2.0 mg GHK-Cu dissolved in 0.2 ml DPBS, and the third group 2.0 mg EHP in 0.2 ml DPBS. Chambers were collected on day 21 for analysis.

Statistical analysis. Results were expressed as mean $\pm$ 1 SEM. Statistical analysis was done by the Student's *t* test.

Results

Effects of serial injections of a unique (2.0-mg) dose of GHK-Cu into the wound chamber. A series of wound chambers serially injected with 2.0 mg GHK-Cu or with DPBS were collected at various days after insertion and sorted for morphological analysis. A simple macroscopical observation of the

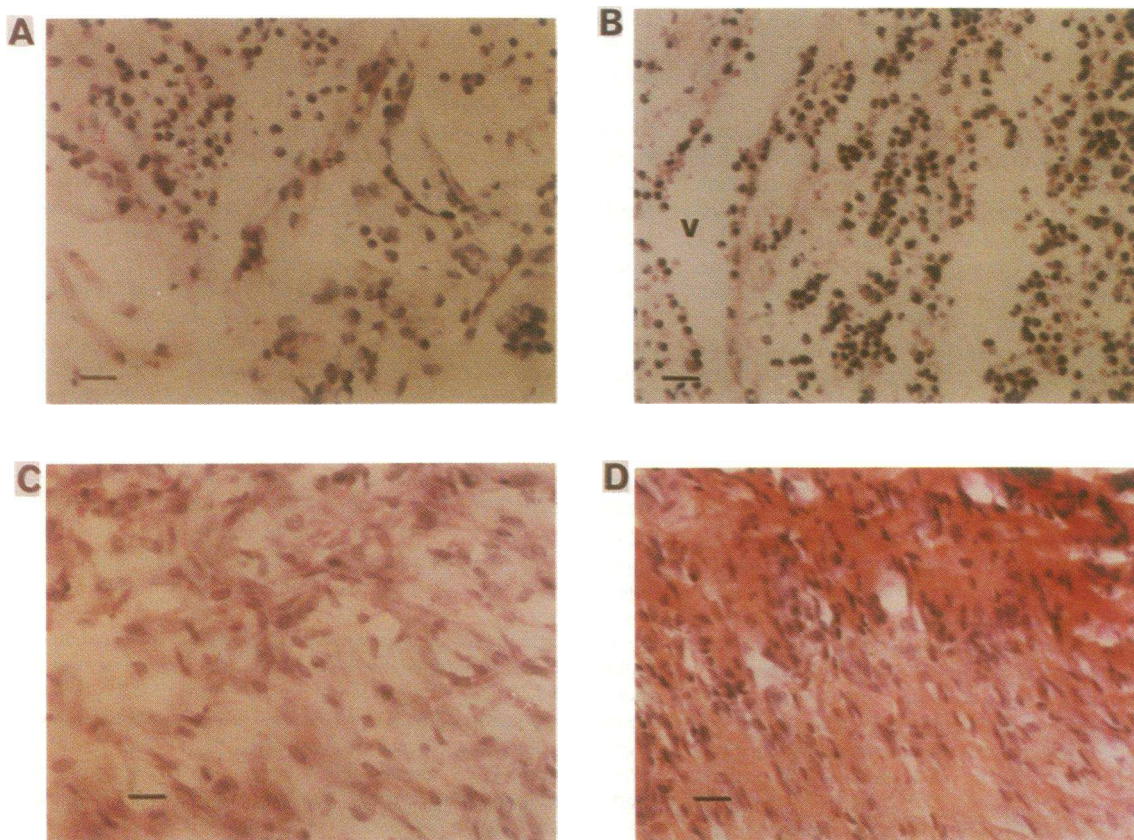


Figure 1. Histological examination of the chamber content at days 3 (A and B) and 14 (C and D) after insertion. At day 3, more inflammatory cells (polymorphonuclear cells and macrophages) were present in the treated chambers (B) than in the controls (A) and some neovascularization (v) was already visible. At day 14, large areas of well-organized fibrosis with elongated fibroblasts were seen in the GHK-Cu-injected chambers (D), whereas the control ones (C) were characterized by a cellular accumulation, mainly fibroblastic, with oedematous areas and few extracellular matrix deposition (bar = 50 μ m).

chambers clearly showed that more connective tissue accumulated in the treated than in the controls (data not shown). Histological examination at day 3 (Fig. 1, *A* and *B*) showed a more intense inflammatory cellular infiltrate with some neovessels clearly visible in the GHK-Cu-injected chambers. Later examination showed a more rapid appearance of collagen with large, dense, and well-organized fibrosis areas clearly visible in the treated chambers as soon as day 14 (Fig. 1, *C* and *D*).

Biochemical analysis of the chamber content at day 29 (Fig. 2) demonstrated a significant increase of dry weight (223% of the controls, $P < 0.01$; Fig. 2 *A*), total proteins (230% of the controls, $P < 0.01$; Fig. 2 *B*), glycosaminoglycans (208% of the controls, $P < 0.01$; Fig. 2 *C*), and collagen (344% of the controls, $P < 0.01$; Fig. 2 *D*) in wound chambers injected with 2.0 mg GHK-Cu. Small collagenous peptide hydroxyproline (Fig. 2 *E*) was also increased in wound chambers (188% of the controls, $P < 0.01$), showing that collagen degradation was enhanced as well as its synthesis but to a lesser extent. DNA content was not significantly altered (Fig. 2 *F*).

The increase of collagen in the treated chambers was approximately twice that of total proteins, demonstrating a preferential effect of GHK-Cu on collagen synthesis. By contrast,

glycosaminoglycans were increased approximately to the same extent as total proteins. Their distribution, however, was markedly altered (Fig. 3). In control wound chambers, the dermatan sulfate/heparan sulfate/hyaluronic acid ratio was 53:18:29 (the total content being considered as 100), whereas in chambers serially injected with 2.0 mg GHK-Cu, it was 70:13:17.

Effects of increasing doses of GHK-Cu injected into the wound chambers. The injection of increasing doses of GHK-Cu demonstrated a dose-effect relationship between the amount of GHK-Cu injected into the wound chamber and the dry weight (Fig. 4 *A*), total protein (Fig. 4 *B*), collagen (Fig. 4 *C*), and glycosaminoglycan content (Fig. 4 *D*) at day 29. In every case, the effect was significant from doses of 0.5 mg GHK-Cu per injection. Elastin, as detected by its isodesmosin and desmosin content, was present in low amounts, compared with collagen, but was also increased after GHK-Cu injections (Fig. 4 *E*). Fibronectin measurements showed no significant alteration (Fig. 4 *F*).

Calculation of the ratio of collagen to total protein confirmed the preferential effect of GHK-Cu on collagen synthesis (Fig. 5 *A*), with a significant stimulation at 0.5 mg GHK-Cu per injection, maximal at 2.0 mg. The increases in uronic acid and elastin were nearly parallel to that of total proteins (data not shown). A dose-dependent increase of the collagen content was also found when the results were expressed per milligram DNA (Fig. 5 *B*).

Effects of GHK-Cu on collagen gene expression in the wound chambers. Northern blot and slot blot analysis of pro $\alpha 1(I)$ and TGF- β mRNAs (Fig. 6) showed that the collagen gene was expressed in both control and treated wound chambers. However, measurement of the ratio of pro $\alpha 1(I)$ to GAPDH mRNAs at different times after chamber insertion showed a large increase in collagen gene expression in the GHK-Cu-treated vs. control wound chambers (Fig. 7 *A*). The increase was clearly seen even in the first set of wound chambers collected at day 3 and persisted until day 14. By day 21, collagen mRNAs ceased to increase in the treated wound

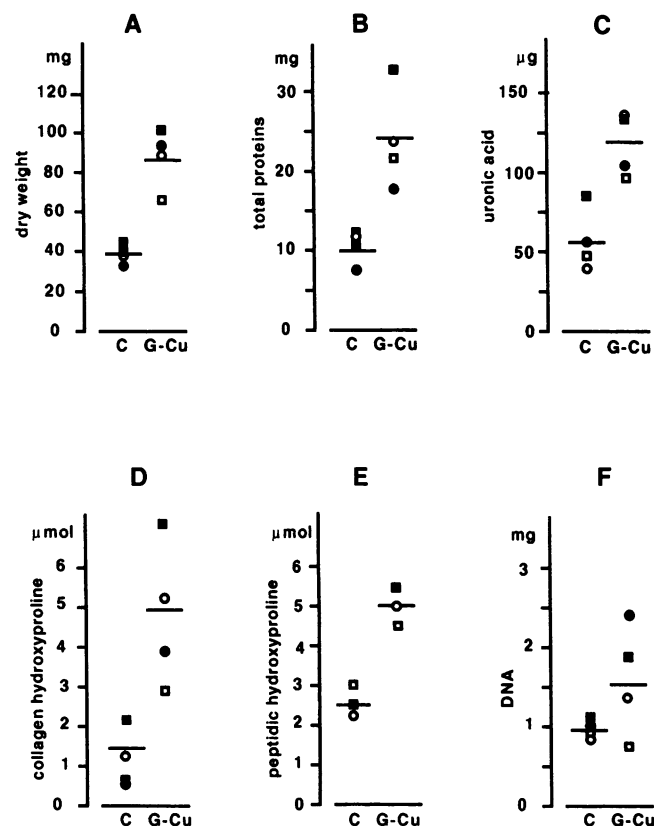


Figure 2. Effects of serial injections of a unique dose (2.0 mg) of GHK-Cu on wound chamber content. *A*, dry weight; *B*, total proteins; *C*, uronic acid; *D*, collagen hydroxyproline; *E*, hydroxyproline from the small collagenous peptides; *F*, DNA. Each rat received one wound chamber on both sides of the back. The first chamber was used as control injected with saline. The second chamber received GHK-Cu injections. Symbols represent individual results (quantity per wound chamber) for the four rats. Means are shown by horizontal bars. (*C*) Control chambers injected with DPBS; (*G-Cu*) chambers injected with GHK-Cu.

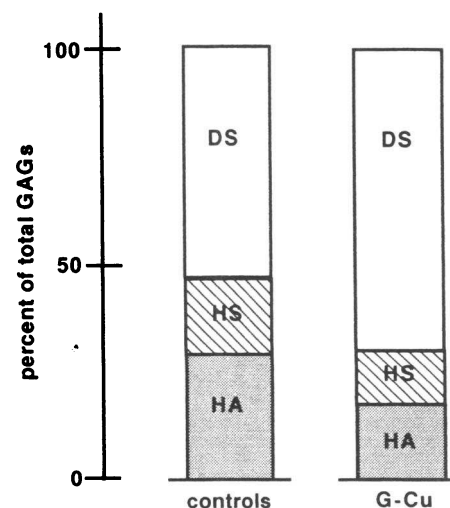


Figure 3. Distribution of the dermatan sulfate (DS), heparan sulfate (HS), and hyaluronic acid (HA) in the wound chambers. Results are expressed as percentages of total glycosaminoglycans. (*C*) Control chambers; (*G-Cu*) chambers injected with GHK-Cu.

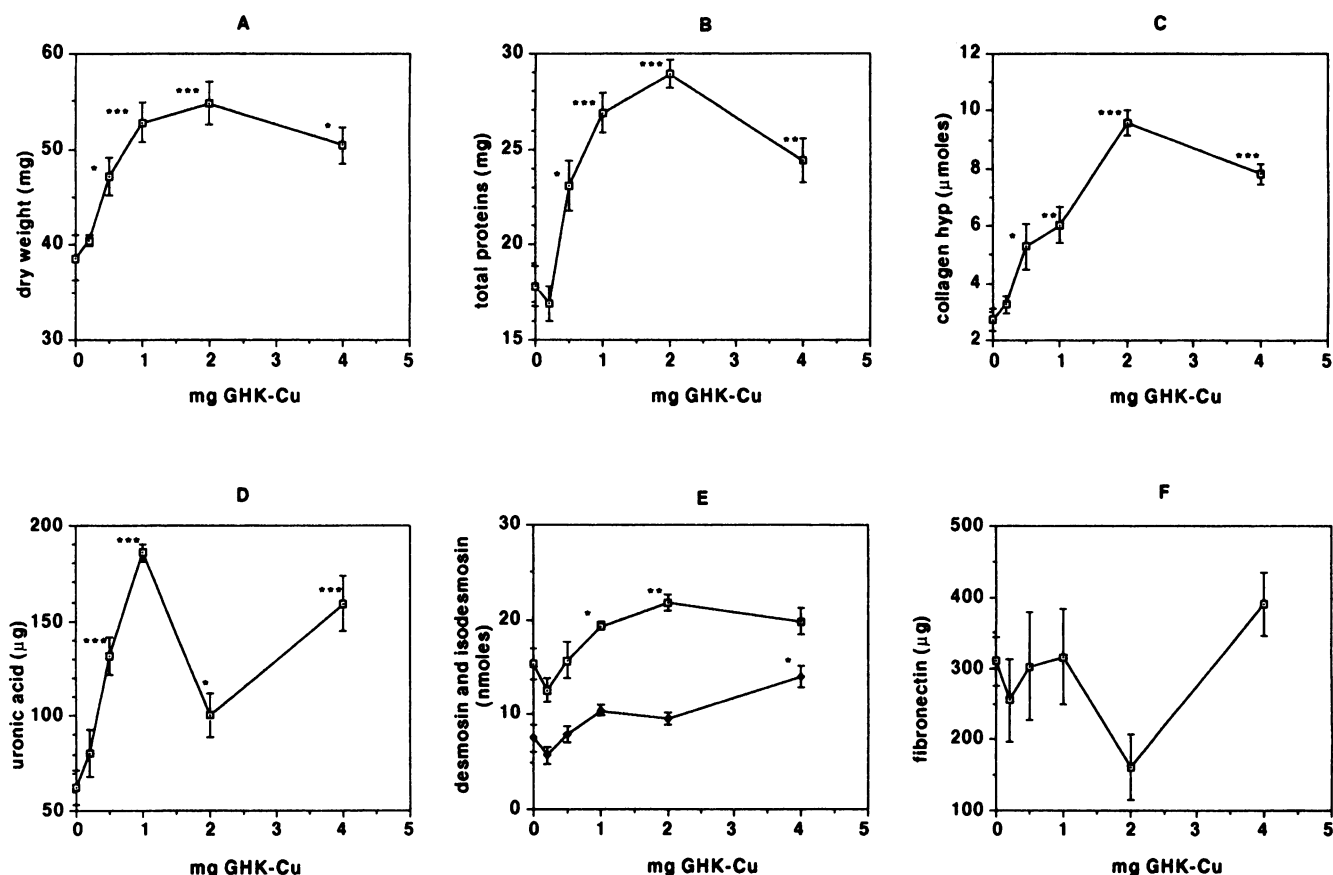


Figure 4. Effects of serial injections of increasing doses of GHK-Cu on the wound chamber content. Data represent the mean of six wound chambers $\pm$ 1 SEM. A, dry weight; B, total proteins; C, collagen; D, glycosaminoglycans; E, elastin (squares, isodesmosin; circles, desmosin); F, fibronectin. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

chambers while they continued to increase in the controls. TGF β mRNAs were not increased in the treated chambers compared with the controls (Fig. 7 B).

Analysis of type III collagen gene expression (Fig. 8) showed that pro $\alpha 1$ (III) mRNA was also increased in the GHK-Cu-injected chambers as soon as day 3. Maximal differences between control and treated chambers occurred at days 7 and 14.

Kinetic measurements of the collagen content of the chamber showed an increased deposition of collagen in the GHK-Cu-injected chambers. The maximal difference was observed at day 21 after insertion (Table I).

Percentage of type III collagen. The percentage of type III collagen was calculated after CNBr digestion of the chamber content at days 7, 14, and 21 after insertion and SDS-PAGE analysis of the collagen CB peptides (Fig. 9). At day 21, type III collagen was $12.9 \pm 1.3\%$ of total collagen in the control chambers vs. $10.8 \pm 0.4\%$ in the GHK-Cu-injected chambers (not significant). Not enough type III collagen was present at days 7 and 14 for calculation of the type III to (types I + III) ratio.

Specificity of GHK-Cu effects. Wound chambers were serially injected with either GHK-Cu, GHK alone, or CuCl₂ at equimolar concentrations and collected at day 21 for analysis.

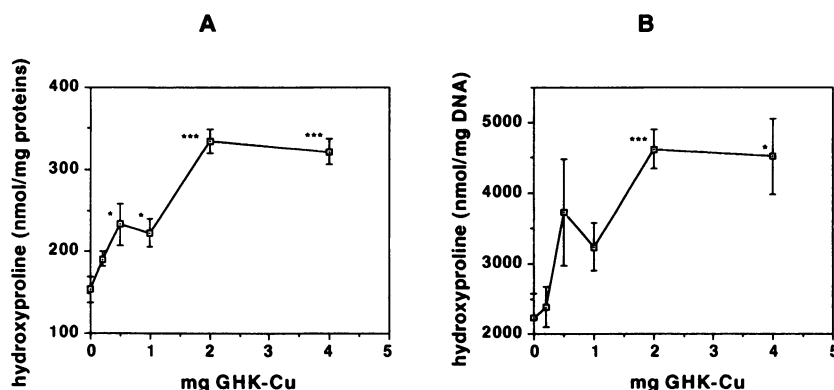


Figure 5. Ratio of collagen to total proteins (A) and to DNA (B) in the wound chambers receiving serial injections of increasing doses of GHK-Cu. Results are mean of six wound chambers $\pm$ 1 SEM. * $P < 0.05$; *** $P < 0.001$.

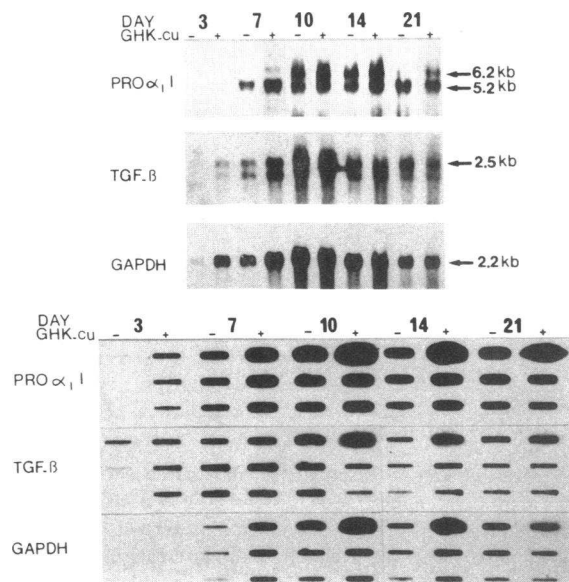


Figure 6. Northern blot (A) and slot blot (B) analysis of pro α_1 (I) collagen, TGF- β 1, and GAPDH mRNAs in wound chambers injected with 2.0 mg GHK-Cu (+) or with saline (controls, -) every 3 d from days 2 to 17. The day of chamber collection for analysis is indicated.

A significant increase of collagen accumulation was found in the case of GHK-Cu only (Fig. 10). GHK not complexed with copper induced a slight decrease of collagen content. Copper chloride injection had no significant effect.

A complementary experiment was done in which three groups of rats received serial injections into the wound chamber of either DPBS (controls), 2.0 mg GHK-Cu, or the tripeptide EHP at the same concentration. Chambers were collected at day 21 for analysis. GHK-Cu only induced a statistically significant increase of collagen accumulation in the chamber (Table II).

Discussion

GHK-Cu is a naturally occurring peptide-copper complex that was shown to possess potentially interesting properties in the wound healing process. It was a potent chemoattractant for inflammatory (4, 5) and endothelial cells (6), promoted nerve tissue regeneration (6), and stimulated collagen synthesis (8) in several fibroblast strains. Preliminary data suggested that it is able to accelerate tissue repair in animal (9, 10) and human models (29-31).

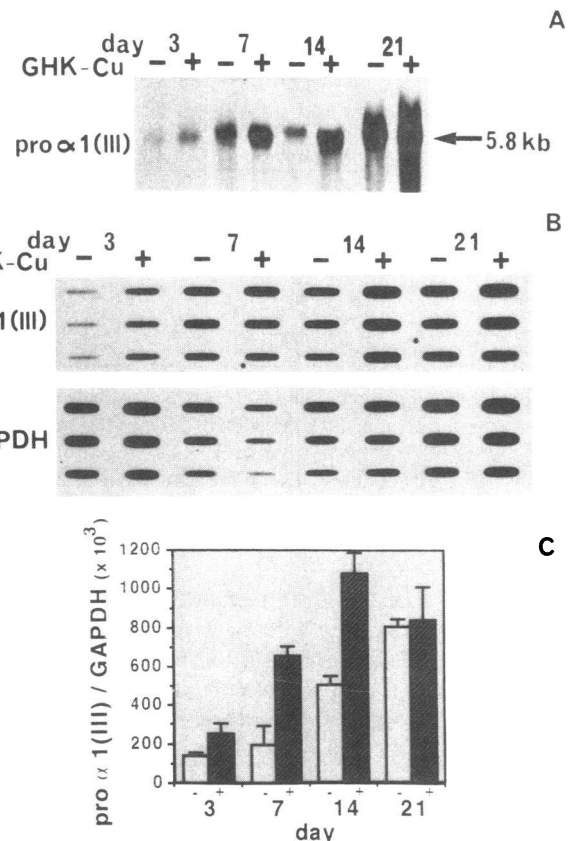


Figure 8. Expression of type III collagen mRNA in wound chambers injected with 2.0 mg GHK-Cu (+) or with saline (controls, -) every 3 d from days 2 to 17. The day of chamber collection for analysis is indicated. (A) Northern blot; (B) slot blot; (C) ratio of pro α_1 (III) to GAPDH mRNAs. Results are mean of three determinations $\pm$ SEM. The Northern blot for GAPDH is not shown.

In this study, we used the wound chamber model described by Schilling et al. (12) for studying the effects of GHK-Cu on connective tissue accumulation in vivo. As reported by these authors (12), the implanted cylinder is rapidly invaded by inflammatory cells, then fibroblasts, and is nearly filled by new connective tissue after 3-4 wk. This simple model permits us to obtain granulation tissue at a wound site in large amounts and perform static and kinetic studies of the healing process in the absence or presence of pharmacological agents. Sporn et al. (32) used it successfully for demonstrating the healing properties of TGF β in vivo.

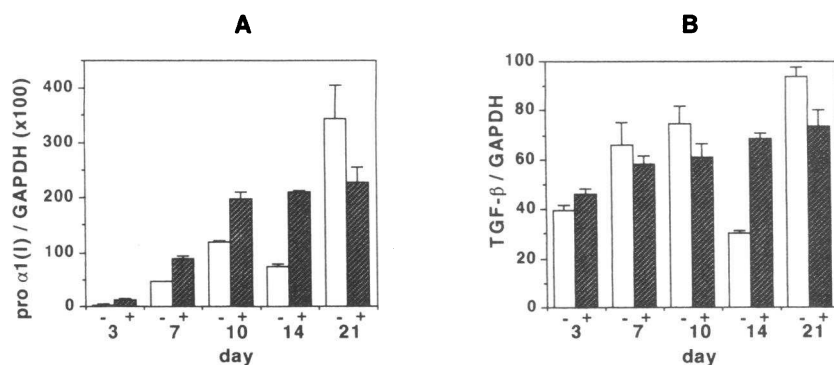


Figure 7. Ratio of pro α_1 (I) (A) and TGF- β 1 (B) to GAPDH mRNAs in wound chambers injected (hatched bars) or not (controls, open bars) with 2.0 mg GHK-Cu every 3 d from days 2 to 17. The day of chamber collection is indicated. Results are means of three determinations $\pm$ SEM.

Table I. Kinetic Measurement of Collagen Accumulation in the Wound Chambers

Day	Collagen hydroxyproline	
	Controls	GHK-Cu
	$\mu\text{mol}/\text{chamber}$	
0	0	0
7	0.49 ± 0.11	0.70 ± 0.36
14	3.33 ± 0.22	4.00 ± 1.08
21	9.45 ± 2.20	$20.28 \pm 2.28^*$

Results are mean of three rats $\pm$ 1 SEM. GHK-Cu-treated rats received, into the wound chamber, the injection of 2.0 mg GHK-Cu dissolved in 0.2 ml DPBS every 3 d, from days 2 to 17. Control rats received 0.2 ml DPBS without GHK-Cu on the same days. * Significantly different of controls, $P < 0.05$.

The first series of experiments were done by placing one chamber on each side of the back of the rats and injecting GHK-Cu in one side and DPBS in the other side. However, since GHK-Cu is a small molecule, it might diffuse from the chambers to the whole organism and exert systemic effects that would decrease the sensitivity of the method. For that reason, in the following experiments, we preferred to use different groups of rats as controls and GHK-Cu-treated animals.

Repeated injections of GHK-Cu induced an acceleration of healing and a concentration-dependent increase of connective tissue components in the chambers. After 4 wk, which is near the end of the repair process, total proteins, collagen, and glycosaminoglycans were simultaneously increased but the stimu-

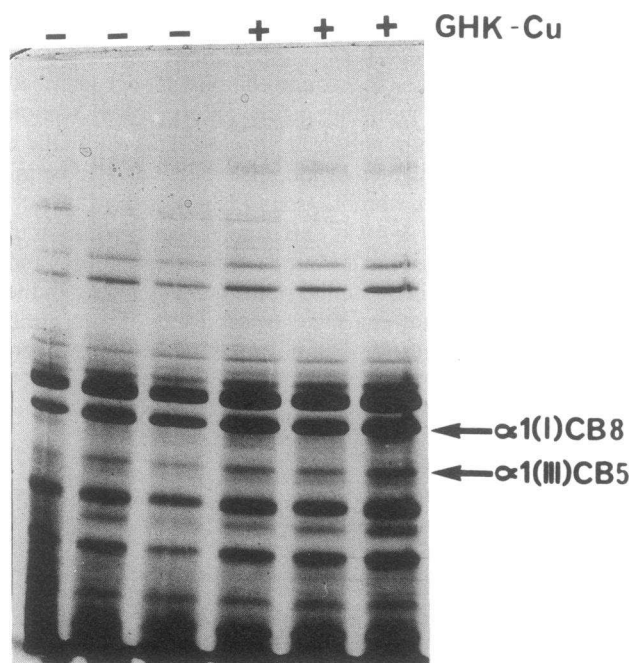


Figure 9. SDS-PAGE of the collagen CNBr peptides from wound chambers collected at day 21. Chambers were inserted on the back of six rats and injected every 3 d with either saline (controls, -) or 2.0 mg GHK-Cu (+), from days 2 to day 17. Locations of the $\alpha 1(\text{III})$ -CB 5 and $\alpha 1(\text{I})$ -CB 8 peptides are indicated.

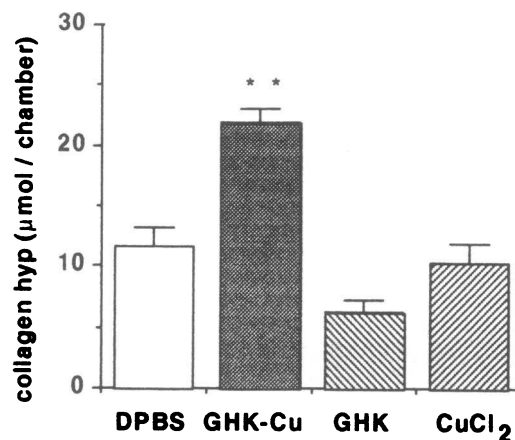


Figure 10. Measurement of collagen in wound chambers injected every 3 d for 18 d with either DPBS, 2.0 mg GHK-Cu, or an equimolar amount of the tripeptide GHK not complexed with copper, or CuCl_2 . Chambers were collected on day 21 for analysis. Results are mean of 10 wound chambers $\pm$ 1 SEM. ** Significantly higher than DPBS-injected chambers, $P < 0.01$.

lation of collagen accumulation was approximately twice that of the other parameters measured, showing a preferential effect of GHK-Cu on collagen synthesis. The increased collagen accumulation was also found when the results were expressed per milligram DNA, showing that collagen synthesis was increased on a per cell basis. Kinetic measurement of collagen accumulation showed only small differences between controls and GHK-Cu-injected chambers after 7 and 14 d. By contrast, collagen content was largely increased after 21 and 29 d. This delay might be due to the time necessary for fibroblasts to invade the chambers and synthesize new extracellular matrix.

Measurement of the percentage of type III collagen was done after CNBr digestion of the chamber content, electrophoretic separation of the CB peptides, and calculation of the ratio of $\alpha 1(\text{III})$ -CB 5 to $\alpha 1(\text{I})$ -CB 8 peptides. As pointed out by Light (33), CNBr digestion is the method of choice for solubilizing soft tissue samples for collagen quantitation because it is the only one that permits a total solubilization of the collagens originally present. Our results show that type I and III collagens were both increased in the GHK-Cu-injected chambers and that the percentage of type III was not altered. The amount of type III was too low at days 7 and 14 to be evaluated.

Table II. Collagen Measurement in Wound Chambers Serially Injected with either Saline (controls), GHK-Cu, or EHP

	Controls	GHK-Cu	EHP
	$\mu\text{mol}/\text{chamber}$		
Collagen hydroxyproline	11.63 ± 0.43	$20.27 \pm 1.32^*$	$14.62 \pm 0.48^\dagger$

Results are mean of three rats $\pm$ 1 SEM. GHK-Cu-treated rats received, into the wound chamber, the injection of 2.0 mg GHK-Cu dissolved in 0.2 ml DPBS every 3 d for 18 d. EHP-treated rats received on the same days the injection of 2.0 mg EHP in DPBS. Control rats received injections of DPBS only. Chambers were collected on day 21 for analysis. * Significantly different of controls, $P < 0.02$. † Not significantly different.

Peptidic hydroxyproline was increased in the treated chambers, which demonstrated that collagen turnover was accelerated, compared with the control ones. Since collagen content was always higher in the treated chambers, the activation of collagen synthesis was globally more efficient than catabolism.

Extraction of mRNAs followed by Northern blot and slot blot analyses demonstrated an increase of type I procollagen mRNAs in the treated chambers, compared with the controls, from days 3 to 14. This difference did not exist at day 21. Type III collagen mRNA followed a similar kinetics. These data indicated that the effects of GHK-Cu occurred very early and accelerated the repair process. It was not possible, in such an *in vivo* model, to specify if the effects of GHK-Cu on collagen mRNAs were related to an increased transcription of collagen genes or to an increased stability of the corresponding transcripts. Further *in vitro* studies will be necessary to solve this question. Northern blot hybridization with the rat TGF β 1 cDNA probe revealed the presence of two species of transcripts, the first one 2.5 kb and the second $\sim$ 1.9 kb. These results are consistent with previous findings of Qian et al. (15), who reported the appearance of a 1.9-kb TGF- β 1 mRNA in infarcted rat heart. As discussed by these authors, this mRNA has been observed also in a variety of other mouse and rat tissues and was most often elevated after tissue injury. The injection of GHK-Cu in the treated chambers did not increase TGF- β 1 mRNA level, showing that GHK-Cu effects are not mediated through an increase of TGF- β 1 gene expression.

The distribution of glycosaminoglycans was markedly altered in GHK-Cu-injected chambers, with a large increase of the relative amount of dermatan sulfate. Dermatan sulfate-containing proteoglycan is known to interact with collagen (34) and to contribute to the organization and strength of the fibrillar network (35). Its specific accumulation with GHK-Cu treatment might improve the formation of a fully resistant scar. We reported previously a similar increase of dermatan sulfate-containing proteoglycan in human dermis fibroblast cultures incubated with GHK-Cu (36).

The mechanism of action of GHK-Cu is still under discussion. Since GHK-Cu was shown *in vitro* to possess activating properties on a variety of cells implicated in the wound repair process, it is likely that, *in vivo*, it is also able to trigger a large number of events such as increased angiogenesis, increased recruitment of inflammatory cells, and fibroblast activation. As pointed out by Raju et al. (7) and by Odedra and Weiss (37), the presence of the copper ion in the GHK-Cu molecule might be of paramount importance for explaining its effects. Several copper-containing low molecular mass substances have been shown to possess angiogenic properties (37). It is the same for the copper-containing serum protein, ceruloplasmin (7). Copper alone is highly cytotoxic in cell cultures and was devoid of effect in our *in vivo* study. On the other hand, an increased copper uptake into cells incubated with GHK-Cu was reported previously (38). It seems likely that copper delivery to the cells is involved in the effects of the tripeptide. In this regard, it was of great interest to note that GHK alone, not previously complexed with copper, was devoid of any stimulating effects on collagen synthesis in our model.

Our results demonstrate that GHK-Cu may promote wound repair *in vivo*. The origin of naturally occurring GHK-Cu and its exact role in physiological tissue repair remain to be determined.

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