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Stimulation of collagen synthesis in fibroblast cultures by the tripeptide-copper complex glycyl-L-histidyl-L-lysine-Cu^{2+}

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Glycyl-L-histidyl-L-lysine (GHK) is a tripeptide with affinity for copper(II) ions and was isolated from human plasma. This peptide appears to play a physiological role in wound healing. We report the stimulating effect of GHK-Cu on collagen synthesis by fibroblasts. The stimulation began between 10^{-12} and 10^{-11} M, maximized at 10^{-9} M, and was independent of any change in cell number. The presence of a GHK triplet in the α_2(I) chain of type I collagen suggests that the tripeptide might be liberated by proteases at the site of a wound and exert in situ healing effects.

Collagen synthesis; Fibroblast culture; Wound healing

1. INTRODUCTION

Glycyl-L-histidyl-L-lysine (GHK) is a tripeptide isolated from human plasma [1], which possesses a high affinity for copper(II) ions, with which it spontaneously forms a complex (GHK-Cu). This peptide was first described as a growth factor for a variety of differentiated cells but recent data suggest a physiological role related to the process of wound healing and tissue repair. The factor directly accelerates wound healing and stimulates biological events in tissue repair such as angiogenesis, nerve outgrowth and chemoattraction of cells critical to healing (e.g., macrophages, monocytes, mast cells, capillary endothelial cells) [2,3]. Herein, we report that GHK-Cu stimulates collagen synthesis by fibroblast cultures, independent of any growth factor activity. The finding of a GHK triplet in the α_2(I) chain of human collagen suggests that the tripeptide might be liberated during the healing process to exert its effects locally.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

GHK and GHK-Cu were provided by Procyte Corporation. L-Ascorbic acid and copper(II) chloride were obtained from Merck, β-aminopropionitrile from Sigma, L-glutamine and L-proline from Calbiochem. Clostridial collagenase (CLSPA grade) was bought from Worthington and purified [4]. Uniformly labelled L-[^{14}C]proline (spec. act. 9.25 TBq·mmol^{-1}) was obtained from NEN. Other reagents were from Prolabo (analytical grade). All the culture media were from Gibco except fetal bovine serum (FBS), which came from Biopro.

2.2. *Cell cultures*

Fibroblasts were cultured from explants of human foreskins, obtained from young children with parental consent and grown in Costar tissue culture flasks by routine techniques [5]. The sterility of the cultures were regularly checked, especially concerning mycoplasms. Cell numbers were performed routinely with a Neubauer cell and trypan blue test was used for checking viability.

2.3. *Incubations*

Cells were grown to confluence in series of four 25 cm^2 flasks. The incubations lasted for 24 h at 37°C, under an at-

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mosphere of 95% air/5% CO_2, in Eagle's minimum essential medium with Earle's salts (MEM) supplemented with 0.2 mM β-aminopropionitrile, 0.28 mM ascorbic acid, 2 mM glutamine, 5% FBS previously dialyzed extensively against phosphate buffered saline, 7.4 KBq·ml^{-1} [^{14}C]proline diluted in 350 μM cold proline and a convenient concentration of GHK-Cu.

2.4. *Measuring the synthesized proteins, the intracellular free proline pool and the intracellular catabolism of the newly synthesized procollagen*

The methods used to measure protein synthesis, intracellular free proline pool and intracellular catabolism of newly synthesized procollagen have been described in detail by Bellon et al. [6]. The culture medium and cell layer were collected separately. In both fractions, the incorporation of [^{14}C]proline into total proteins, collagen and non-collagen proteins was measured by the collagenase digestion technique of Peterkofsky and Diegelman [4]. Measurement of the amount, radioactivity and specific activity of collagen hydroxyproline was also done after acid hydrolysis, derivatization of the amino acids with the fluorophore 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole and separation of the fluorescent derivatives by thin-layer chromatography. The intracellular free amino acids and small peptides were extracted from the cells by 80% ethanol and the extract was divided into 2 aliquots. After evaporation to dryness, the first one was used to measure free proline [6]. The second one was hydrolyzed by 6 M HCl and hydroxyproline was measured as an estimate of the intracellular degradation of newly synthesized procollagen [6,7].

2.5. *Preparation of RNA and hybridization*

Total cellular RNA was extracted [8] from human fibroblasts treated or not with 10^{-9} M of GHK-Cu. 5 or 10 μg of each preparation were electrophoresed and analyzed by Northern hybridization [9]. To ensure that equal amounts of RNA were loaded in each lane and transferred, the gel was stained with ethidium bromide and quantities of 18 S and 28 S were assessed. The transferred mRNAs were then incubated in the presence of labelled cDNA coding for the α_1(I) mouse collagen chain. Hybridization was performed at 42°C for 18 h in 5 × SSC, 50% formamide, 0.1% SDS and 200 μg/ml salmon sperm DNA. Filters were washed in 0.1 × SSC, 0.1% SDS at 50°C and autoradiographed. In some experiments quantifications of α_1(I) mRNA were assessed by dot blot analysis.

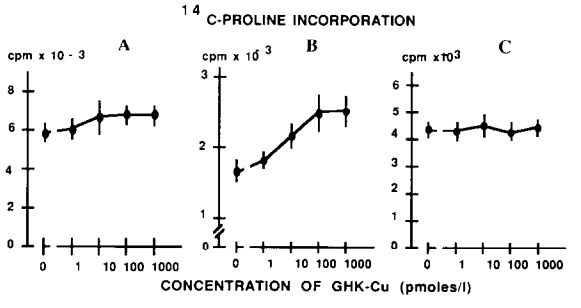

Fig.1. Effects of GHK-Cu on [^{14}C]proline incorporation into total proteins (A), collagen (B) and non-collagen proteins (C) secreted into the culture medium of fibroblasts.

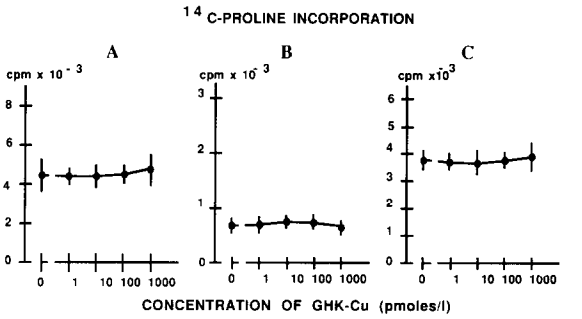

Fig.2. Effects of GHK-Cu on [^{14}C]proline incorporation into total proteins (A), collagen (B) and non-collagen proteins (C) synthesized in the cell layer of fibroblasts.

2.6. *Statistical analysis*

Experiments were done in quadruplicate. The results were calculated per 10 μg DNA [10] and expressed as means ± SD. The Student's *t*-test was used for checking statistical significance [11].

3. RESULTS

When added to the culture medium of confluent fibroblasts, GHK-Cu induced a concentration-dependent increase of the [^{14}C]proline incorporation into collagen secreted into the culture medium (fig.1). The stimulation appeared between 10^{-12} and 10^{-11} M and was maximal for the concentration 10^{-9} M. No significant change of the secretion of non-collagen proteins was found. In the cell layer, the [^{14}C]proline incorporation into collagen and non-collagen proteins was not altered by the tripeptide (fig.2). Thin-layer chromatography

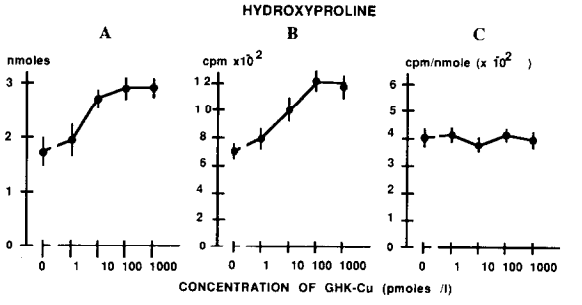

Fig.3. Effects of GHK-Cu on the amount (A), radioactivity (B) and specific radioactivity (C) of collagen hydroxyproline in the culture medium.

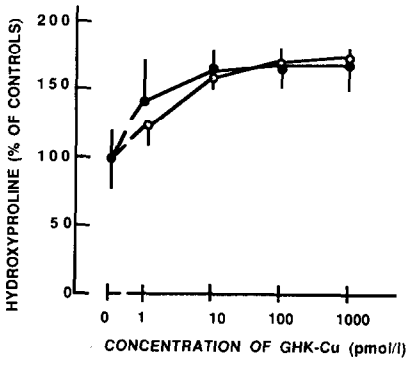

Fig.4. Comparison of the effects of GHK (•) and GHK-Cu (○) on the secretion of collagen in the supernatant of fibroblast cultures. Results are expressed as percentages of the amount of collagen hydroxyproline measured in the supernatant of control cultures incubated without GHK or GHK-Cu.

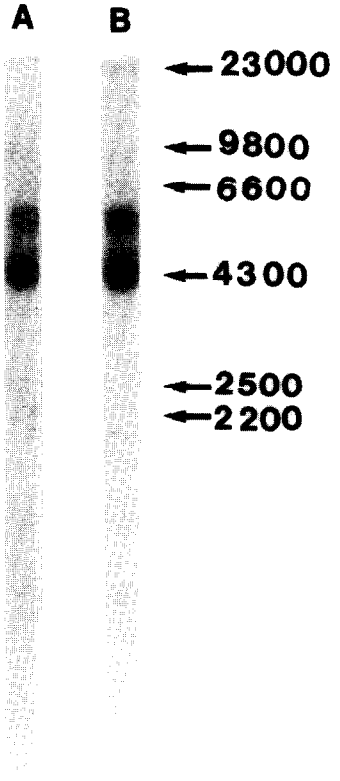

Fig.5. Analysis of total cellular RNA derived from human fibroblasts treated (B) or non-treated (A) with 10^{-9} M GHK, and hybridized with an α_1(I) cDNA probe. The arrows indicate the ^{32}P-labelled *Hin*dIII restriction fragments of lambda DNA.

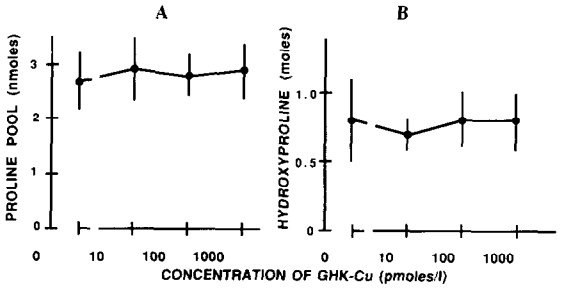

Fig.6. Measurement of the intracellular free proline pool (A) and of hydroxyproline from the intracellular degradation of newly synthesized procollagen (B) in cultures incubated with GHK-Cu.

measurement of the amount (fig.3A) and radioactivity (fig.3B) of collagen hydroxyproline demonstrated that its specific activity was not modified by GHK-Cu (fig.3C). We verified that no change in cell number or viability occurred over the concentrations tested.

When GHK was added to the culture medium instead of GHK-Cu, approximately the same stimulation of collagen synthesis was obtained (fig.4). Copper(II) ions alone had no stimulating effect. Cytotoxic phenomena appeared for concentrations of $CuCl_2$ as low as 100 pM in the absence of FBS (not shown).

Measurement of procollagen mRNAs by dot-blot and Northern blot analysis showed no significant change in cells incubated with GHK-Cu (fig.5). The intracellular free proline pool and the catabolism of newly synthesized procollagen were not modified by the tripeptide (fig.6).

4. DISCUSSION

Our data demonstrate that the addition of GHK-Cu to the culture medium of fibroblasts induced a specific, concentration-dependent, stimulation of the secretion of collagen into the supernatant of cultures. This phenomenon was not a general enhancement of protein synthesis since the incorporation of [^{14}C]proline into non-collagen proteins was not altered. The lack of increase of the cell layer collagen might correspond to the existence of a steady state between the rates of collagen synthesis and excretion into the extracellular medium.

Since the tripeptide was first described as a

growth factor [1], we checked its effect on the cell number and viability in our fibroblast cultures. The absence of any change of these 2 parameters might be explained by the short time of our incubations (24 h) and by the use of confluent cultures.

The techniques that we used for measuring protein synthesis combined the classical method of [^{14}C]proline incorporation into collagen and non-collagen proteins [4] and a specific measurement of the amount and radioactivity of collagen hydroxyproline [6]. The latter permitted verification that GHK-Cu induced no change in the specific radioactivity of the synthesized collagen and demonstrated that the amount of collagen synthesized by the cells (and not only its radioactivity) was actually increased.

The finding that GHK induced exactly the same enhancement of collagen synthesis as GHK-Cu, and the lack of enhancement of collagen synthesis by copper(II) ions, indicated that the tripeptide alone might be responsible for the effects of GHK-Cu. However, we cannot exclude the fact that some copper ions might be present in the culture medium and reform GHK-Cu complexes.

The mechanism of action of GHK-Cu is still under study. The lack of alteration of the procollagen mRNA levels in cells incubated with GHK-Cu made it unlikely that the tripeptide acted at the transcriptional level. Measurements of the intracellular free proline pool and catabolism of the newly synthesized procollagen indicated that GHK-Cu did not increase the rate of collagen synthesis by changing the size of the proline pool or by decreasing the intracellular degradation of procollagen. Further studies will be necessary to elucidate the exact step of collagen synthesis which might be concerned. The increased copper uptake into cells incubated with GHK-Cu [12] might be involved in the effects of the tripeptide.

The sequence GHK is not frequently found in the proteins. Consequently, it was of great interest to find a GHK triplet in the position 853-854-855 of the triple helical part of the α_2 chain of the human type I collagen [13]. It could be hypothesized that the tripeptide might be liberated by some proteases on the site of a wound or inflammation and exert locally its healing effects.

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