Expression of Glycosaminoglycans and Small Proteoglycans in Wounds: Modulation by the Tripeptide–Copper Complex Glycyl-L-Histidyl-L-Lysine-Cu^{2+}

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Glycyl-histidyl-lysine-Cu^{2+} is a tripeptide–copper complex previously shown to be an activator of wound healing. We have investigated the effects of glycyl-histidyl-lysine-Cu^{2+} on the synthesis of glycosaminoglycans and small proteoglycans in a model of rat experimental wounds and in rat dermal fibroblast cultures. Repeated injections of glycyl-histidyl-lysine-Cu^{2+} (2 mg per injection) stimulated the wound tissue production, as appreciated by dry weight and total protein measurements. This stimulation was accompanied by an increased production of type I collagen and glycosaminoglycans (assessed, respectively, by hydroxyproline and uronic acid contents of the chamber). Electrophoretic analysis of wound tissue glycosaminoglycans showed an accumulation of chondroitin sulfate and dermatan sulfate in control wound chambers, whereas the proportion of hyaluronic acid decreased with time. The accumulation of chondroitin sulfate and dermatan sulfate was enhanced by glycyl-histidyl-lysine-Cu^{2+} treatment. The expression of two small proteoglycans of the dermis, decorin and biglycan, was analyzed by northern blot. The biglycan mRNA steady-state level in the chamber was maximal at day 12, whereas the decorin mRNA increased progressively until the end of the experiment (day 22). Glycyl-histidyl-lysine-Cu^{2+} treatment increased the mRNA level of decorin and decreased those of biglycan. In dermal fibroblast cultures, the stimulation of decorin expression by glycyl-histidyl-lysine-Cu^{2+} was also found. In contrast, biglycan expression was not modified. These results show that the expression of different proteoglycans in wound tissue are regulated in a different manner during wound healing. The glycyl-histidyl-lysine-Cu^{2+} complex is able to modulate the expression of the extracellular matrix macromolecules differently during the wound repair process. ***Key words: glycosaminoglycans/proteoglycans/wound healing. J Invest Dermatol 115:962–968, 2000***

Cutaneous wound healing is a complex and dynamic process, which involves the coordinated and sequential deposition of extracellular matrix molecules, leading to the formation of a resistant new tissue (Clark, 1995). Among these molecules, glycosaminoglycans (GAG) and proteoglycans (PG) are, with collagens and fibronectin, the major components of the connective tissue extracellular matrix (Chen and Abatangelo, 1999). In addition to their structural functions, GAG and PG play a part in several processes in relation to wound healing, such as cellular adhesion, migration, and proliferation (Hocking *et al*, 1998). GAG and PG were also shown to bind growth factors and locally modulate their biologic activities. Among the small PG found in skin, decorin and biglycan have raised considerable attention because they are implicated in collagen fibrils organization. They are composed of a leucine-rich repeats-containing core protein, on which are attached one or two, respectively, chondroitin sulfate (CS) and/or dermatan sulfate (DS) GAG chains. The inactivation of decorin gene by homologous recombination (Danielson *et al*, 1997), but not that of biglycan (Xu *et al*, 1998), was associated with a fragile skin collagen network.

Numerous immunohistochemical studies have shown that GAG and PG are present in granulation tissue and that their distribution is altered in this tissue, in comparison with normal skin. The repartition of GAG in wounds was shown to vary with time: hyaluronic acid (HA) was prominent at the early phases of healing whereas CS and DS accumulated during the later phases (Yeo *et al*, 1991).

Glycyl-L-histidyl-L-lysine (GHK) is a tripeptide that was isolated from human plasma (Pickart and Thaler, 1973). This peptide exhibits a high affinity for copper(II) ions, with which it spontaneously forms a tripeptide–copper complex (GHK-Cu). GHK-Cu was initially described as a growth factor for a variety of differentiated cells (Pickart, 1981). Subsequently, a number of other biologic effects were reported and GHK-Cu appeared as a potent activator of wound healing. For instance, it was described as a chemotactic agent for monocytes/macrophages and mast cells (Poole and Zetter, 1983; Zetter *et al*, 1985). Moreover, GHK-Cu supported nerve tissue regeneration (Grosse and Lindner, 1980) and stimulated angiogenesis *in vivo* (Raju *et al*, 1982; Lane *et al*, 1994). We previously showed that GHK-Cu enhanced the expression of extracellular matrix macromolecules both *in vitro* and *in vivo*

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Abbreviations: CS, chondroitin sulfate; DS, dermatan sulfate; GAG, glycosaminoglycans; GHK-Cu, glycyl-L-histidyl-L-lysine-Cu^{2+}; HA, hyaluronic acid; PG, proteoglycans.

(Maquart *et al*, 1993). When injected in superficial wounds, GHK-Cu accelerated wound closure (Downey *et al*, 1985; Pickart, 1987). Clinical trials showed that GHK-Cu treatment might significantly improve the healing of skin ulcers in diabetic patients (Mulder *et al*, 1994). We also recently showed that GHK-Cu is able to modulate tissue remodeling by selectively increasing the expression and activation of gelatinase A (matrix metalloproteinase-2) (Simeon *et al*, 1999).

In this study, we investigated the effect of GHK-Cu on the accumulation and the distribution of GAG during the different phases of wound healing *in vivo*. The expression of decorin and biglycan, two small PG of the dermis, were especially studied. For that purpose, we used the *in vivo* wound chamber model described by Schilling *et al* (1953) and *in vitro* rat dermal fibroblast cultures. Our results demonstrate that GHK-Cu stimulates CS and DS production, and decorin but not biglycan expression *in vivo*. *In vitro* experiments demonstrate that fibroblasts were the main target cells implicated in this effect.

MATERIALS AND METHODS

Animals Male Sprague–Dawley rats weighing between 250 and 300 g were used in the experiments. They were provided by the Centre d'Elevage Dépré (St Doulchard, France). They were placed in individual suspended stainless steel cages with food and water *ad libitum*.

Wound chambers Wound chambers were made of stainless steel wire mesh cylinders (C-CX 20; EDMEC, Bellevue, WA) measuring 1 cm × 2.5 cm long. 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 per kg; Clin-Midy, Paris, France). Dorsal hair was clipped widely from scapula to pelvis and the nude area was sterilized with polyvidone iodine (Betadine; Laboratoire Sarget, Merignac, France). Full-thickness skin incisions were made perpendicular to the spine through the panniculus carnosus to the fascial plane. A space that was approximately the same size as the chamber was opened under the dermis and the sterile wound chambers with caps were slipped beneath the skin. The incisions were closed through individual 4.0 nylon sutures.

Experimental design After separation at random, a group of rats was used as controls and received the injections, directly into the chamber, of 0.2 ml of Dulbecco's phosphate-buffered saline on day 0, then twice a week for 3 wk. The other group received the injections on the same days of the same volume of Dulbecco's phosphate-buffered saline containing 2 mg GHK-Cu (Procyte Corporation, Kirkland, WA) per injection. Groups of six to eight rats depending on the experiments (three to four controls and three to four GHK-Cu injected) were killed on days 3, 7, 12, 18, or 22 after chamber implantation. Wound fluid was immediately collected by aspiration with a 1 ml sterile syringe and discarded. The material deposited in the chambers was collected with a scalpel and frozen at –80°C until analysis.

Biochemical analysis of the chamber content Deposited material was lyophilized and weighted. It was then dissolved in 0.5 M NaOH for 30 min at 60°C. After neutralization, ethanol was added to the final concentration of 80% (mass/vol). After 18 h at 4°C and centrifugation at 5000 × *g* for 30 min, supernatant was collected, the pellet resuspended in saline, and the ethanol precipitation repeated once for elimination of the pigments that could interfere in subsequent assays. The ethanol precipitate was redissolved in 0.5 M NaOH and an aliquot was taken for measurements of total proteins by the method of Lowry *et al* (1951). The remaining fraction was used for the measurement of collagen and GAG.

Collagen was measured by its hydroxyproline content. An aliquot of the NaOH-solubilized material was hydrolyzed in 6 M HCl at 110°C for 18 h. Hydroxyproline was then quantitated in the hydrolysate (Szymanowicz and Laurain, 1981).

For GAG 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 24 h at 48°C to release the GAG chains. Pronase was precipitated by the addition of trichloroacetic acid to the hydrolysate to 10% (mass/vol) final concentration. The samples were centrifuged at 5000 × *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 according to Bitter and Muir (1962). The separation of the GAG was carried out on cellulose acetate gel (Chemetron, Milano, Italy). An aliquot containing 2 μg of total uronic acid for each sample was applied on the membrane and electrophoresis was performed 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. Standards were deposited together with the sample in order to identify the position of the separated bands after migration. The membrane was then fixed in absolute ethanol and stained with 0.2% Alcian blue in buffer ($MgCl_2$ 50 mM, sodium acetate 25 mM, pH 5.8, 50% ethanol) for 20 min and destained three times in the same buffer. The GAG distribution was determined after scanning the strips with a Vilber–Lourmat image analyzer.

GAG identification was done on samples collected at day 18. Aliquots containing 6 μg of total uronic acid were hydrolyzed with either 10 units streptococcal hyaluronidase per ml (Seikagaku Kogyo, Tokyo, Japan) in 0.1 M sodium acetate, pH 5.4 at 60°C for 1 h or with 1 unit chondroitinase AC per ml in 0.2 M Tris, 0.2 M sodium acetate, pH 7.3 at 37°C for 5 h (Lagunoff and Warren, 1962). Heparan sulfate was identified after deaminative cleavage with 1.8% $NaNO_2$ in 10% acetic acid for 90 min at room temperature (Saito *et al*, 1968).

***In vitro* experiments** Dermal fibroblasts from explants of rat skin were grown to confluence in plastic 75 cm^2 flasks (Life Technologies, Cergy-Pontoise, France) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. After three washes with phosphate buffered saline, the cells were incubated for 24 h in Dulbecco's modified Eagle's medium supplemented with 1.5% fetal bovine serum with or without 10^{-9} M GHK-Cu. Cultured cells between the third and the sixth passage were used in this study.

RNA extraction The wound chambers were frozen in liquid nitrogen immediately after collection. Once deep frozen, the wound chamber content was collected using a scalpel and homogenized in a 4 M guanidinium–thiocyanate/phenol mixture (Tri Reagent, Euromedex, Souffelweyersheim, France). Total RNA was extracted according to the instructions of the manufacturer using the single-step method of Chomczynski and Sacchi (1987).

Northern blotting Ten micrograms of RNA were 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 ethylenediamine tetraacetic acid, pH 8.0, and denatured by heating at 65°C for 15 min. RNA was then fractionated by electrophoresis in 1% agarose/formaldehyde gel for 6 h at 70 V (Sambrook *et al*, 1989) and transferred overnight by capillary blotting in 20 × standard saline citrate solution (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) to nylon filter (Genescreen plus, NEN, Boston, MA). The air-dried nylon filter was ultraviolet-irradiated for 5 min to bind transferred RNA.

The filter was prehybridized for 18 h at 42°C in a 10% sodium dodecyl sulfate (SDS), 5 × SSC, 5 × Denhardt's solution, 250 μg per ml of denatured salmon sperm DNA, and 50% formamide. Specific hybridization was carried out overnight in the same solution with addition of 10^6 cpm per ml of ^{32}P-labeled cDNA probe. The filter was washed twice in 2 × SSC, 0.1% SDS at 42°C, once in 0.1 × SSC, 0.1% SDS at 65°C and exposed to autoradiography at –80°C using hyperfilm MP (Amersham Life Science, Amersham, Bucks, U.K.) and Kodak X-Omat cassette C2 with intensifying screens.

Autoradiographic signals were scanned on a Molecular Imager System GS-63 (Bio-Rad Laboratories, Richmond, CA) and quantitated with Molecular Analyst software (Bio-Rad Laboratories).

Decorin immunoprecipitation Rat fibroblasts were cultured in 25 cm^2 flasks to confluency and incubated for 24 h in Dulbecco's modified Eagle's medium supplemented with 1.5% fetal bovine serum with or without 10^{-9} M GHK-Cu. After three washes with phosphate-buffered saline, the cells were incubated for 7 h with 740 kBq [^{35}S]sulfate in sulfate–low minimum essential medium with or without 10^{-9} M GHK-Cu. At the end of the incubation period, culture media were supplemented with proteinase inhibitors, 0.1% (wt/vol) Na_2SO_4. A 700 μl aliquot of each sample was precipitated with 66% acetone and analyzed by SDS–polyacrylamide gel electrophoresis (7.5% acrylamide). The PG decorin was immunoprecipitated from 1 ml of culture medium using a specific anti-decorin antibody (LF-113, generous gift of Dr. Larry Fisher, NIDR, Bethesda) according to previously described methods (Wegrowski *et al*, 1995a). The immune complex was collected by adsorption on protein A–protein G–Sepharose beads and analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography.

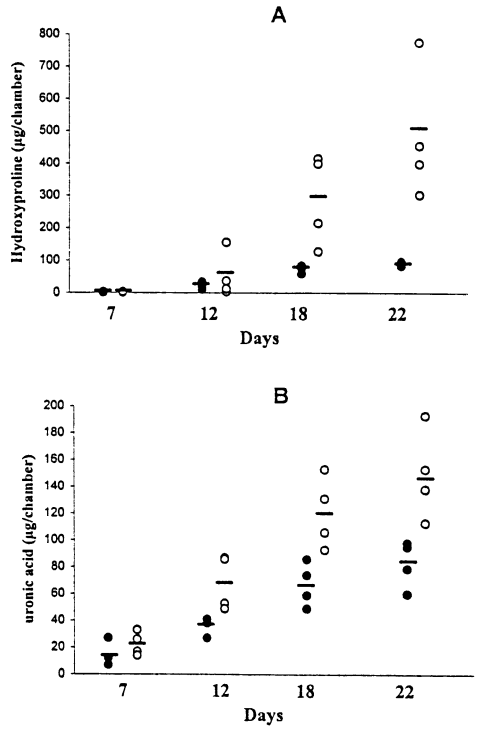

Figure 1. GHK-Cu increases the accumulation of collagen and GAG in wound chamber. Chambers were injected twice a week with (○) or without (●) GHK-Cu, 2.0 mg per injection. Rats were killed and chambers collected to measure hydroxyproline (*A*) and uronic acid (*B*) contents at days 7, 12, 18, or 22 as described in *Materials and Methods*. Symbols represent individual results for the four rats. Means are shown by horizontal bars.

cDNA probes Plasmid used was pBluescript SK containing an insert for mouse decorin core protein, or an insert for mouse biglycan core protein cDNA (generous gifts of Dr. Larry Fisher, NIH, Bethesda, MD) (Fisher *et al*, 1995). The GPDN5 plasmid containing the full-length rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA was used as reference (generous gift of Dr. P. Fort, Montpellier, France) (Fort *et al*, 1985). cDNA were excised from their plasmids with appropriate restriction enzymes, and labeled with random priming using Prime It II kit (Stratagene, La Jolla, CA). The labeled cDNA probe was separated from ^{32}P-deoxycytidine triphosphate by chromatography through a G-50 spin column.

Statistical analysis All the experiments were performed in triplicate or quadruplicate, depending on the experiment. Quantitative data were expressed as mean ± 1 SEM. Statistical analysis was done by the Student's t test or the Mann–Whitney *U* test.

RESULTS

GHK-Cu increases collagen and GAG accumulation in wound chambers Wound chambers injected with 2.0 mg GHK-Cu or with Dulbecco's phosphate-buffered saline were collected at different intervals after implantation and wound tissues were studied by biochemical analysis. Hydroxyproline and uronic acid were used as an index of collagen and GAG contents, respectively. Collagen content of the wound tissue increased progressively throughout the experiment in the control rats (**Fig 1*A***). GHK-Cu injections significantly increased the collagen content of the wound chambers at days 18 and 22 (396% and 538% of the corresponding controls, respectively, $p < 0.05$).

In parallel to collagen, there was a progressive accumulation of GAG in wound chambers, as measured by total uronic acid content (**Fig 1*B***). The increase was statistically significant at days 12, 18, and 22 (191%, 180%, and 179% of the corresponding controls, respectively, $p < 0.05$).

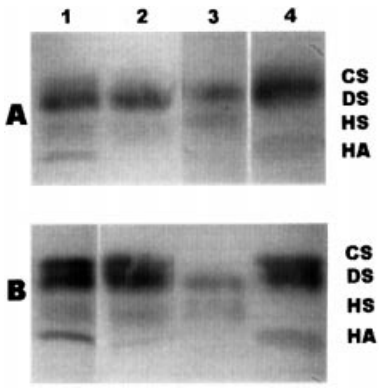

Figure 2. Chondroitin-sulfate, dermatan-sulfate, and hyaluronic acid are the major GAG present in the wound tissue. CS, chondroitin-sulfate; DS, dermatan-sulfate; HS, heparan-sulfate; HA, hyaluronic acid. Identification of the wound tissue GAG collected from wound chambers at day 18. (*A*) Chambers injected with saline solution. (*B*) Chambers injected with 2 mg GHK-Cu. Samples were analyzed by cellulose acetate electrophoresis without prior treatment (*lane 1*) or after digestion with streptococcal hyaluronidase (*lane 2*), chondroitinase AC (*lane 3*), and $NaNO_2$ (*lane 4*).

GHK-Cu modulates the distribution of GAG in wound tissue As GHK-Cu increased accumulation of GAG in the wound tissue, we investigated whether its effect was undifferentiated on all the GAG chains or if it specifically modulated their relative repartition. GAG species in wound tissue were identified by cellulose acetate electrophoresis (**Fig 2**). Enzymatic and chemical degradations permitted us to identify three mains bands corresponding to HA, DS, and CS. A minor band corresponding to heparan sulfate was also detected (**Fig 2**, *lane 1*). Streptococcal hyaluronidase specifically hydrolyzed HA (**Fig 2**, *lane 2*) and chondroitinase AC hydrolyzed CS and HA (*lane 3*). The weak, diffuse band was completely degraded after $NaNO_2$ treatment (**Fig 2**, *lane 4*), which shows that it corresponds to heparan sulfate. This fraction, representing less than 2% of total GAG, was not studied further.

The relative distribution of GAG varied with time. In the wound tissue extracted from control rats, the HA part decreased (25% of total GAG at day 7 *versus* 6% at day 22) whereas the CS/DS part increased with a predominance of DS (70% of total GAG at day 22). GHK-Cu injections modified this distribution by increasing the relative CS content. Quantitation by image analysis showed that, at day 18, the percentages of CS/DS/HA were 26%/68%/6% in the control wound tissue compared with 44%/49%/7% in the GHK-Cu treated one. At day 22, the percentages of CS/DS/HA were 22%/68%/10% in the control group *versus* 37%/53%/10% in the treated group (**Fig 3**).

Kinetic measurement of the amount of each GAG species in the wound chambers showed a relative stability of the HA content from day 7 to day 22 in the control group (**Fig 4*A***). GHK-Cu injections enhanced HA content at days 12 and 22, as compared with the control. On the other hand, DS and CS contents increased progressively in control wound chambers throughout the experiment (**Fig 4*B*, *C***). GHK-Cu injections did not significantly affect the accumulation of DS but increased that of CS. At days 18 and 22, the CS content in the GHK-Cu injected wound chamber was 2.8 ($p < 0.01$) and 3.1-fold ($p < 0.05$) higher than in the corresponding controls.

Decorin and biglycan expression are differentially modulated by GHK-Cu *in vivo* The galactosaminoglycans, DS and CS, exist in the tissues in PG form. In skin, two major species of DS/CS-containing small PG are present: decorin and biglycan. We studied, using northern blot, the expression of these PG in the wound chambers. GAPDH expression was studied in parallel as a housekeeping gene. Autoradiographic analysis showed the expression of only one transcript for each gene, migrating at 1.9 kb,

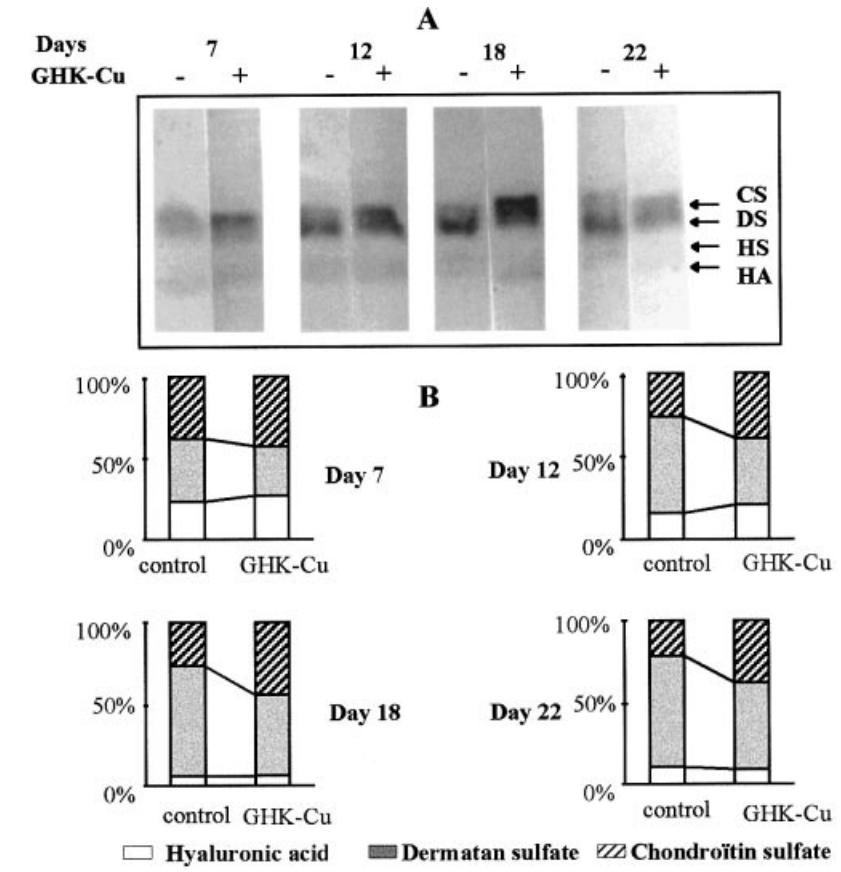

Figure 3. GAG distribution in the wound tissue is altered by GHK-Cu. (*A*) Separation and identification of GAG by cellulose acetate electrophoresis. Two micrograms of uronic acid were deposited on each lane. The day of sample collection for analysis is indicated above the picture. The migration position of reference GAG are indicated by arrows: (HA) hyaluronic acid (HS) heparan sulfate (DS) dermatan sulfate (CS) chondroitin sulfate. (*B*) Distribution of GAG in wound tissue. Signals from (*A*) were scanned on a Vilber-Lourmat Image Analyzer and quantitated with Bio1D software.

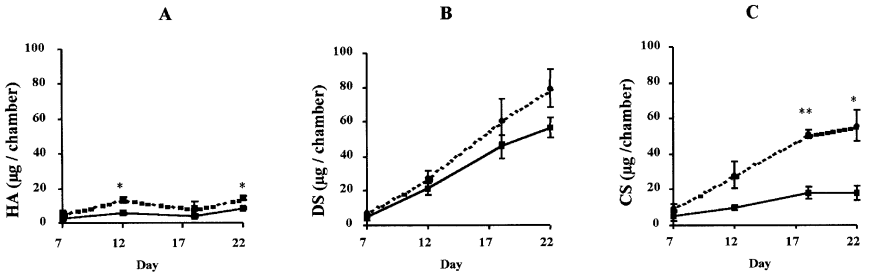

Figure 4. GAG deposition in the wound chambers is increased by GHK-Cu. The same amount of total GAG (2 µg of uronic acid) were deposited on each lane and separated by electrophoresis as described in **Fig 2**. Hyaluronic acid (*A*), dermatan sulfate (*B*), chondroitin sulfate (*C*) contents were determined by densitometric scanning after correction for the uronic acid content of the wound chamber. Results are mean of three determinations ± 1 SEM

2.8 kb, and 1.2 kb for decorin, biglycan, and GAPDH, respectively. These sizes are those usually described in the literature. In our experimental conditions, decorin and biglycan mRNA were detected from day 7 to 22 (**Fig 5*A***). We observed strong differences in their expressions during the wound repair process. Decorin mRNA increased progressively throughout the experiment (**Fig 5*B***), whereas biglycan mRNA increased during the first days to reach a plateau at day 12 (**Fig 5*C***).

GHK-Cu injections significantly increased the decorin mRNA steady-state level (+73%, $p < 0.05$ at day 22) and decreased that of biglycan (–45%, $p < 0.05$ at day 18 and –26%, $p < 0.05$ at day 22).

Decorin and biglycan expressions in cultured fibroblasts are differentially affected by GHK-Cu Confluent dermal rat fibroblasts were cultured during 24 h in Dulbecco's modified Eagle's medium containing 1.5% fetal bovine serum, with or without GHK-Cu (10^{-9} M). Northern blots were then performed to study the mRNA steady-state levels of decorin and biglycan (**Fig 6*A***). The same transcripts as those observed in the wound tissue were found in the fibroblast cultures. Compared with GAPDH, decorin mRNA expression was increased with GHK-Cu (302% of the controls, $p < 0.05$) (**Fig 6*B***), whereas biglycan expression was not significantly affected (**Fig 6*C***).

Immunoprecipitation of the culture medium with a specific anti-decorin antibody confirmed the enhancement of decorin production by the fibroblasts incubated with GHK-Cu (**Fig 7**).

DISCUSSION

GAG and PG are constituents of the granulation tissue of normal or pathologic wounds (Yeo *et al*, 1991). In this study we studied the accumulation of GAG and small PG in the wound chamber model described by Schilling *et al* (1959) and their modulation by the tripeptide–copper complex, GHK-Cu. The wound chamber represents a model of dermal healing. After 4 wk of healing, the chambers are nearly filled with newly formed connective tissue. We used this model successfully to demonstrate the stimulating effect of GHK-Cu on connective tissue accumulation and remodeling in rat wounds (Maquart *et al*, 1993; Simeon *et al*, 1999). The expression pattern of small PG during dermal wound healing has never been studied before.

Biochemical analysis of the wound chambers confirmed that GHK-Cu injections increased GAG and collagen deposition. Interestingly, we noticed that glucuronic acid deposition occurred rapidly in the wound, whereas collagen deposition, appreciated by its hydroxyproline content, was slower. This suggests that GAG

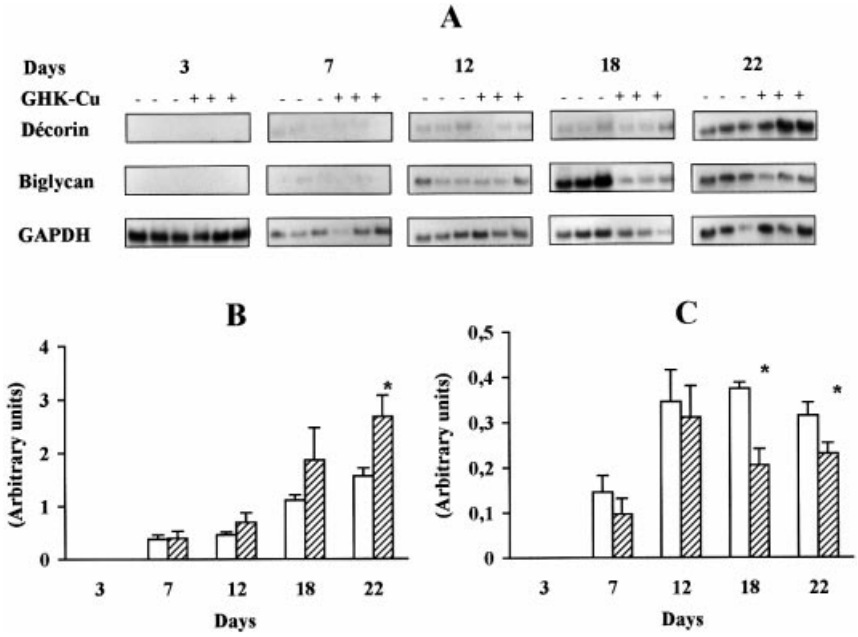

Figure 5. Decorin and biglycan expression are differentially regulated during wound healing. (*A*) Northern blot analysis of decorin, biglycan, and GAPDH mRNA in the wound chambers injected with 2.0 mg GHK(+) or with saline (controls, –) twice a week from days 0 to 21. The day of sample collection for analysis is indicated. (*B*, *C*) mRNA steady-state levels were expressed by the ratio of decorin (*B*) or biglycan (*C*) to GAPDH mRNA in wound chambers injected (hatched bars) or not (controls, open bars) with GHK-Cu. Results are mean of three determinations ± 1 SEM.

may play an important part in the initial phases of the wound tissue formation.

Electrophoretic analysis of the GAG chains showed that HA, DS, and CS were the major GAG of the wound chamber throughout the analysis. Heparan sulfate was not quantitated in our study because this GAG was present in too small an amount (less than 2% of total GAG) in the wound chambers. HA represented between 25% at day 7 and 6% at day 22 of the total GAG content. The raw amount of HA per chamber remained nearly constant throughout the experiment. The relative reduction of HA content in comparison with sulfated GAG confirmed previous studies that showed early hyaluronan deposition was followed by sulfated GAG accumulation (Clark, 1995). In normal dermis, HA and DS are the most abundant GAG. In the wound tissue, CS, a minor component of the normal dermis, was increased up to 45% of the total GAG. Similar data were reported by Yeo *et al* (1991) and Lamme *et al* (1996) in full-thickness wounds performed in guinea pigs or Yorkshire pigs, respectively. It may reflect a decreased activity of glucuronate epimerase in healing tissue and/or the preferential accumulation of other type(s) of PG in healing tissue, for example versican. GHK-Cu injections altered the GAG distribution in the wound tissue. We noted a strong stimulation by GHK-Cu of the CS accumulation and, to a lesser extent, of DS. Previous works from our laboratory showed that GHK-Cu stimulated DS synthesis by cultured human dermal fibroblasts, but had no effect on the CS synthesis (Wegrowski *et al*, 1992a). Taken together, these differences between *in vitro* and *in vivo* data reflect the crucial part of the cellular environment on the cellular behavior during wound healing.

Quantitation of the different GAG extracted from the wound tissue was based on quantitation of the Alcian blue staining after electrophoretic separation. Alcian blue staining may depend on the charge density along the GAG chain. This study, however, was comparative and designed to follow GAG deposition in the wounds under different conditions. For that reason, we used a method able to screen a large number of samples. The small variations of staining that may be caused by differences of charge density cannot explain the large differences in the relative amounts of the different varieties of GAG that we observed.

The significant increase of CS in wounds treated with GHK-Cu prompted us to explore the CS/DS-containing PG. In the dermal extracellular matrix, galactosaminoglycans, i.e., DS and CS, are present in the large, aggregating PG versican, but also in two small, nonaggregant PG: biglycan and decorin. This study focused on

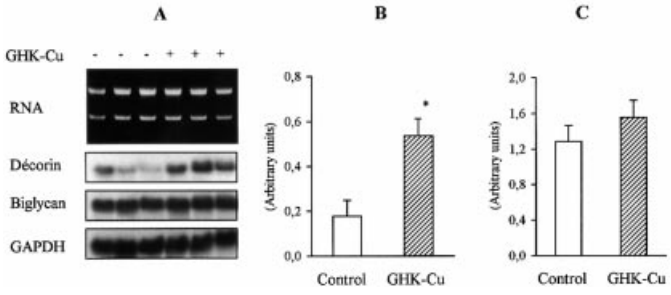

Figure 6. Decorin but not biglycan expression by cultured rat fibroblasts are stimulated by GHK-Cu. (*A*) Northern blot analysis of decorin, biglycan, and GAPDH mRNA in rat skin fibroblasts cultured with or without 10^{-9} M GHK-Cu for 24 h (*B*, *C*): Expression of steady-state levels of decorin (*B*) or biglycan (*C*) to GAPDH mRNA in fibroblasts cultured with (hatched bars) or without (controls, open bars) 10^{-9} M GHK-Cu. Results are mean of three determinations ± 1 SEM.

these small PG only, because they are highly expressed in normal wound tissue. Their mRNA was not detected in the early stages of wound healing (day 3), but appeared at day 7. They had different expression patterns during wound tissue formation. Biglycan mRNA increased rapidly to reach a plateau at day 12, whereas decorin mRNA increased throughout the experiment. This difference in their kinetics of expression might be explained by the cellular types responsible for their synthesis. Decorin is synthetized exclusively by the cells from mesenchymal origin (Wegrowski *et al*, 1995b), whereas biglycan, in addition to fibroblasts, may be synthesized by epithelial and endothelial cells (Bianco *et al*, 1990). The increase of decorin expression may reflect the accumulation of fibroblasts in the chambers. Our results are in agreement with those reported by Oksala *et al* (1995) in experimental wounds of the human palatal mucosa, who found a strong positive signal for decorin but no signal for biglycan at day 7. Similar to our results, the biglycan mRNA expression in the infarcted zone was enhanced at days 2 and 7 in experimentally induced myocardial infarction in rats (Yamamoto *et al*, 1998).

It must be noted that modifications of the GAG distribution pattern, on the one hand, and of core proteins of biglycan and decorin, on the other, do not exclude the fact that structural alterations of the GAG moieties of the molecules may occur. Further studies will be necessary to compare the fine structure of

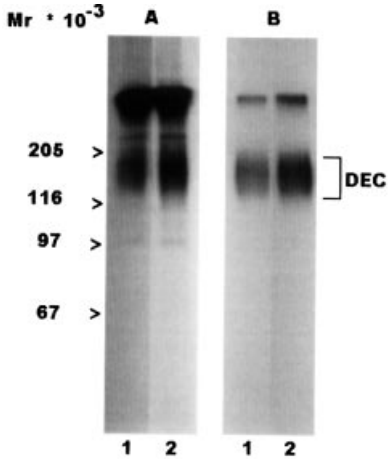

Figure 7. Decorin synthesis is increased in cultured rat fibroblasts incubated with GHK-Cu. After a 24 h incubation with or without GHK-Cu, fibroblasts were incubated for 7 h in sulfate–low minimum essential medium containing or not 10^{-9} M GHK-Cu and with [^{35}S]sulfate. The culture medium of fibroblasts was then collected. An aliquot was precipitated with 66% acetone and another aliquot was immunoprecipitated with specific anti-decorin antibody as described in *Materials and Methods*. Finally, proteins were denatured at 100°C and analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. (*A*) Culture medium. (*B*) Immunoprecipitate of the culture medium after incubation with anti-decorin antibody. *Lane 1*, control fibroblasts; *lane 2*, fibroblasts cultured with 10^{-9} M GHK-Cu. The migration positions of the Mr markers are indicated on the left margin and that of decorin (DEC) on the right margin.

decorin and biglycan GAG chains during the early and late phases of wound healing. Our study shows for the first time a differential regulation of the two small PG, decorin and biglycan, mRNA in wounds. GHK-Cu was able to modulate the steady-state mRNA levels of both PG. In the later stages of wound healing (days 18 and 22), it increased the decorin mRNA, whereas it decreased biglycan. A differential regulation of biglycan and decorin was also observed in bleomycin-induced pulmonary fibrosis in rats (Westergren-Thorsson *et al*, 1993). *In vitro* studies showed that transforming growth factor (TGF) -β upregulates biglycan, whereas it downregulates decorin synthesis (Westergren-Thorsson *et al*, 1992b); on the contrary, glucocorticoids downregulate biglycan and upregulate decorin synthesis (Kimoto *et al*, 1994).

To study a direct effect of GHK-Cu on fibroblast PG synthesis, we used cultured rat dermal fibroblasts and showed that GHK-Cu increased the decorin mRNA steady-state levels in these cultures, reproducing the effect observed *in vivo*. On the contrary, the inhibiting effect of GHK-Cu on biglycan gene expression *in vivo* was not reproduced in the cell culture model. These results show that the effect GHK-Cu has on decorin mRNA *in vivo* concerns mainly the fibroblasts whereas different types of cells may contribute to biglycan synthesis. Further studies are needed to elucidate the regulation of biglycan synthesis completely.

The increased decorin expression in wounds injected with GHK-Cu is interesting in the context of wound healing. This PG is involved in skin architecture, particularly by controlling collagen fibrillogenesis. Decorin interacts noncovalently with type I collagen fibrils (Scott *et al*, 1995) and contributes to the organization and strength of the fibrillar network (Hocking *et al*, 1998). Recently, Danielson *et al* (1997) observed that decorin gene invalidation leads to skin fragility with markedly reduced tensile strength.

Decorin and biglycan may also act on wound tissue formation by interactions with growth factors such as TGF-β (Scott *et al*, 1995). The affinity of both PG for TGF-β is, however, similar, and both of them may be involved in the regulation of the TGF-β effects. A recent report showed that dermatopontin, a 22 kDa extracellular matrix protein, increases the biologic activity of TGF-β. Decorin, by increasing the dermatopontin ability to bind TGF-β might also increase the TGF-β activity (Okamoto *et al*, 1999). Another study showed that DS released after injury is a potent promoter of fibroblast growth factor-2 function (Penc *et al*, 1998). By its ability to increase GAG and/or PG accumulation in the wound tissue, GHK-Cu may then act on the biodisponibility of different growth factors and might facilitate some essential events of wound healing such as cell proliferation, migration, and angiogenesis.

The increased decorin expression in the GHK-Cu-injected wounds, with the increased collagen deposition, might contribute to the formation of a mature extracellular matrix and may explain the increased wound strength observed in the GHK-Cu-treated wounds.

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