Collagen Types I and III Propeptides as Markers of Healing in Chronic Leg Ulcers

A Noninvasive Method for the Determination of Procollagen Propeptides in Wound Fluid—Influence of Growth Hormone

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A noninvasive method allowing measurements of the propeptides of collagen type III (PIIINP) and type I (PICP) in ulcer washings was developed. The response to topical human growth hormone was examined. Fourteen patients with venous ulcers were treated sequentially with human growth hormone (0.1, 0.25, and 1 IU/cm²/day), each dose for 1 week, followed by 1 week washout. On alternate days, three and two times during treatment and washout periods, respectively, the ulcers were washed and incubated for 30 minutes with sterile water. No changes in healing rates in relation to growth hormone application were observed. In contrast, PIIINP increased significantly to 168% (154% to 184%) (mean, 95% confidence interval) and 195% (179% to 218%) 5 and 9 days, respectively, after start of treatment, ($p < 0.01$). Propeptides of collagen type I reached a significant increase, to 196% (172% to 232%), in the fourth week, ($p < 0.01$). The areas under the curves of PICP and PIIINP correlated significantly with the healing rates ($r = 0.57$, $p = 0.04$; and $r = 0.64$, $p = 0.01$, respectively). The authors conclude that propeptide measurements may be useful markers of healing in clinical studies.

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THE CLINICAL EVALUATION of new therapies in human wound healing is complicated by ethical concerns and difficulties in performing direct measurements of the quality as well as the speed of healing. Furthermore, in studies of chronic leg ulcers, substantial problems are due to the heterogeneity of patients. As a consequence, controlled clinical trials usually necessitate large series of patients studied for extended periods to demonstrate significant differences in healing rates.[1] The use of biochemical markers in such studies may make them easier to perform.

Proliferation of fibroblasts as well as synthesis and resorption of collagen are key elements in wound healing.[2] This is also true in chronic leg ulcers, in which a substantial granulation tissue formation often has to precede epithelialization. Recently it has been rendered probable that the carboxy terminal propeptide of type I collagen (PICP) and the amino terminal propeptide of type III collagen (PIIINP) in wound fluid and serum reflect local production of collagen types I and III in surgical patients.[3,4] Both collagens, which are formed in large quantities in granulation tissue in the healing wound, are synthesized as procollagens with additional propeptide extensions at both ends. During fibrillogenesis, the complete removal of the carboxy terminal extensions are necessary for correct fiber formation, whereas not all of the amino terminal extensions are cleaved off.[5,6]

Theoretically, an increased healing rate would be reflected by increased collagen synthesis and turnover and augmented PICP and PIIINP levels in the wound. Indeed, measurements of the metabolites in serum and wound fluid have been shown to reflect the healing of surgical wounds in humans.[3,4,7] In addition, serum PIIINP has been shown to correlate quantitatively with the formation of granulation tissue in subcutaneously implanted cellulose sponges in rats.[8] The metabolites have not been analyzed previously in wound fluid collected from chronic leg ulcers.

Topically applied human growth hormone previously has been shown to stimulate the healing of chronic leg ulcers.[9] In addition, subcutaneously administered, the hormone stimulates collagen synthesis in humans, as reflected by increased serum levels of PICP and PIIINP after treatment.[10–13]

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The purpose of our study was to develop a method for noninvasive measurements of extracellular matrix components in chronic leg ulcers and to investigate whether topical growth hormone treatment would influence the metabolite levels.

Materials and Methods

Patients and Treatment

Fourteen consecutive patients with venous ulcers (nine women, five men; mean age, 70 years [44 to 91 years]) referred to the participating centers for treatment were included in the investigation after informed consent. Only a single ulcer in each patient was used for the study. The cause was determined by clinical examination and an arterial blood flow examination with Doppler or Straingauge techniques. Exclusion criteria were diabetes mellitus, malignant or rheumatic disease, allergy to the dressings applied, clinical signs of peripheral arterial disease, cellulitis, anemia (hemoglobin < 7 mmol/L), or severe maceration of the surrounding skin.

The total study period was 6 weeks, during which the patients were treated at home by a research nurse. In the three periods with active treatment (weeks 1, 3, and 5), increasing doses of human growth hormone (Norditropin, Novo-Nordisk, Denmark), dissolved in a 2.3% Natrosol (carboxyethyl cellulose) gel preserved with 0.9% benzyl alcohol (0.4, 1, and 4 IU/g, 0.1, 0.25, and 1 IU/cm^2/day), was given 5 days per week (Monday through Friday). In four of the patients, the growth hormone was dissolved in sterile water. The metabolite levels in these patients were not significantly different from the rest. The solution was administered through the connecting piece in a modified hydrocolloid dressing (Comfeel Portal Dressing, Coloplast, Denmark) (Fig. 1). This dressing was placed on top of a standard dressing (Comfeel, Base Plate, Coloplast, Denmark) in which the outlines of the ulcer margins had been cut. Before the administration of drug, wound fluid was removed through the connecting piece, and the ulcer was cleaned by irrigating with sterile saline (1 mL/cm^2). After each week with active treatment, a "washout period" (weeks 2, 4, and 6) followed (Fig. 2). During these periods, the patients were treated with the dressing alone. Cleaning of the ulcer and change of dressings were performed by identical methods in the periods. Throughout the study, all patients wore compression bandages (Comprilan, Beiersdorf, Germany), which were corrected daily. Each week, the ulcer margins were traced onto acetate, and the areas determined by point counting.[14]

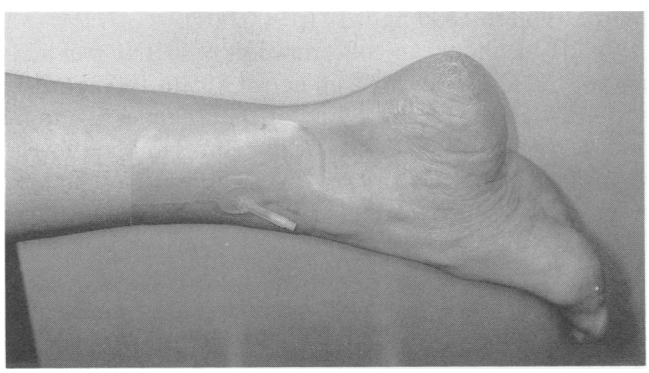

FIG. 1. Hydrocolloid dressing with connecting piece.

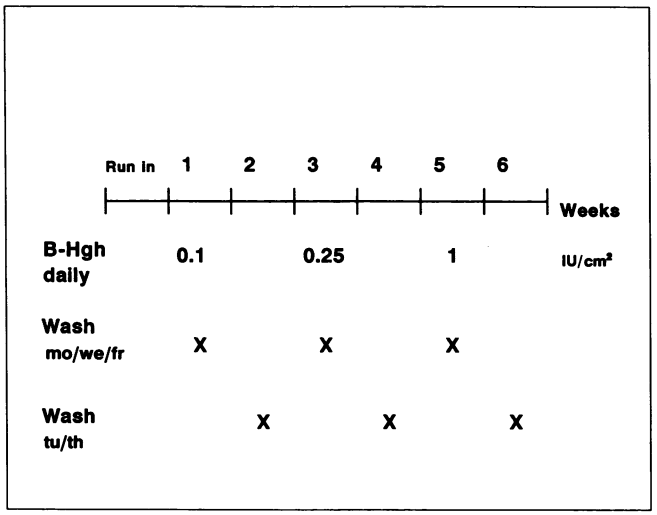

FIG. 2. Timeline of the study. GH, human growth hormone, IU/cm^2, administered 5 days/week.

The study was approved by the regional ethics committee.

Collection of Test Samples

The washings were performed on three alternate days (Mondays, Wednesdays, and Fridays) during the treatment periods, and on two occasions during each washout period (Tuesdays and Thursdays). Each sampling consisted of multiple washing procedures, followed by incubation with the water for 30 minutes. On each day of sampling, the procedures were preceded by primary cleaning of the ulcers by irrigation with sterile water 1 mL/cm^2, to remove most slough and debris macroscopically. Thereafter, the sterile water, 0.1 mL/cm^2, was injected and withdrawn through the connecting piece five times, after which it was collected. This procedure, lasting 20 to 30 seconds, was repeated nine times on each occasion. Finally, the ninth portion was left in the ulcer for 30 minutes before withdrawal. The amount of water used in the washings was adjusted weekly after each area measurement.

Determinations of PICP and PIIINP

The amino terminal propeptide of type III collagen was measured by a commercially available radioimmunoassay (RIA; PIIINP RIA-kit, Farmos Diagnostica, Oulunsalo,

Finland) modified to a sequential saturation assay.[15] The modifications included 16 hours' preincubation without tracer, followed by 20 hours' incubation with tracer. Antibody-bound radioactivity was separated from unbound radioactivity by a solid-phase second antibody according to the description of the kit. The tracer used in the assay was highly purified human PIIINP (donated by Dr. Juha Risteli, Finland) iodinated by the iodogen method.[16] Irrigations 1 to 8 were diluted 25-fold in buffer (phosphate-buffered saline, pH 7.2, human serum albumin 1 g/L), and irrigation 9 was diluted 200-fold. All procedures were carried out at room temperature. Detection limit expressed as the 80% inhibition of the standard curve was 0.04 μg/L. Intra-assay and interassay variations were 6% and 10%, respectively. The nonspecific binding was less than 4.5%.

The carboxy terminal propeptide of type I collagen was measured by a newly developed (PICP RIA-kit, Farmos Diagnostica, Oulunsalo, Finland).[17] Detection limit expressed as the 80% inhibition of the standard curve was 37 μg/L. All washings were diluted 10 times in RIA-buffer. Intra-assay and interassay coefficients of variation were 8% and 9%, respectively. Nonspecific binding was less than 4.0%.

The samples were analyzed in two periods: All ninth washings were analyzed in the first setup, and all sequential measurements, that is, all washings performed in the first 2 weeks of the study, were analyzed in another setup and used for establishing the "washout curve" for PIIINP. The carboxy terminal propeptide of type I collagen was analyzed in washing 8 as a control for the washout pattern of this metabolite.

Statistical Methods

The statistical analyses were performed using SPSS.[18] The change in ulcer areas were modeled by an individual linear regression of the log ulcer area on time, as earlier described.[9] In addition, the change in ulcer areas was calculated as the percentage change during the study. The influence of varying the dose of growth hormone was investigated by calculating the percentage change in ulcer areas in weeks 0 to 2, 2 to 4, and 4 to 6. Results are expressed as mean and 95% confidence interval. The change in the metabolites and ulcer healing was investigated by analysis of variance after logarithmic transformation of the propeptide concentrations. The tests of Shapiro and Wilk[19] and Liliefors showed the percentage areas and the transformed data to be normally distributed.[18,19]

Results

Clinical Variables

Three patients healed their ulcers during the study, and four patients paused for 1 week because of Christmas. These patients were excluded from the longitudinal analysis of the propeptides and ulcer healing, from the time of dropout or pause and onward. One patient was excluded in period 6 and from the correlation studies because of cellulitis. The ulcer in this patient increased somewhat in size during the study. The levels of PIIINP and PICP in this patient were not significantly different from those in the rest of the group. Consequently, the material included in the longitudinal analysis consists of 14, 14, 13, 10, 8, and 6 patients in periods 1, 2, 3, 4, 5, and 6, respectively.

The mean ulcer area was 11 cm^2 (2.4 to 29.7 cm^2). The mean time since last healed was 26 months (4 to 75 months). The ulcer areas decreased by a mean of 8% (−3.4% to 29%) (mean, 95% confidence interval) per week during the study. Overall, the mean ulcer area decreased to 83% (51% to 114%) of the initial ulcer area in the patients completing the 6 weeks of study without pause. After each new dose of growth hormone, the areas decreased to a mean of 85% (70% to 99%), 80% (64% to 96%), and 86% (61% to 112%) in weeks 0 to 2, 2 to 4, and 4 to 6, respectively. The healing rates (percentage change in area) did not change significantly in response to different doses of growth hormone.

Sequential Washings

In the sequential washings in general, the concentration of PIIINP declined steadily from an initial level of 139 ng/mL (119 to 163 ng/mL) (geometric mean, 95% confidence interval) in the first sample to 64 ng/mL (52 to 79 ng/mL), 66 ng/mL (55 to 79 ng/mL), and 56 ng/mL (46 to 69 ng/mL) in samples 6, 7, and 8, respectively. From sample 6 to sample 8 the change was not significant ($p = 0.7$). After incubation in sample 9, the mean concentration of PIIINP increased significantly to 348 ng/mL (272 to 445 ng/mL) ($p < 0.001$). The same sequence was found in all subsequent days of sampling, and reflected the general "washout" pattern of PIIINP in the ulcers (Fig. 3). The concentrations of PICP in washings 8 and 9 were 190 ng/mL (140 to 259 ng/mL) and 1238 ng/mL (958 to 1600 ng/mL) ($p < 0.001$).

On day 1, the mean concentration of PIIINP was 124 ng/mL (88 to 176 ng/mL), 60 ng/mL (35 to 100 ng/mL), and 232 ng/mL (122 to 439 ng/mL) in samples 1, 6 to 8, and 9, respectively. On day 5, the corresponding levels were 124 ng/mL (83 to 185 ng/mL), 78 ng/mL (48 to 128 ng/mL), and 461 ng/mL (247 to 863 ng/mL) ($p = 0.96$, 0.02, and 0.002, respectively) (Fig. 4), showing the relative values. The increase was sustained in the start of the following week, when no growth hormone was administered, and reached 160 ng/mL (119 to 216 ng/mL), 85 ng/mL (53 to 136 ng/mL), and 511 ng/mL (280 to 933 ng/mL), respectively, on the ninth day after initiation of growth

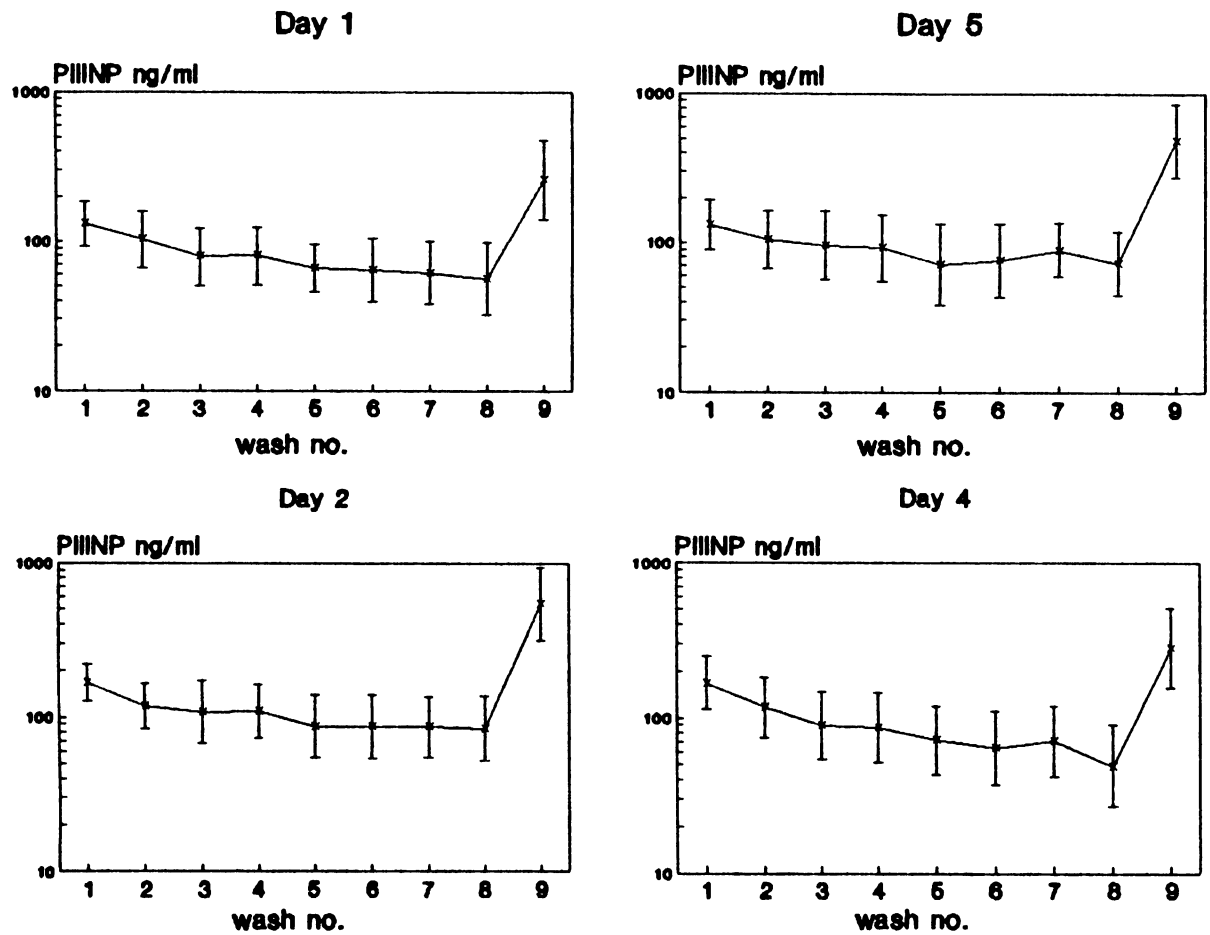

FIG. 3. Sequential washout pattern of PIIINP during the first two periods of the study. Two days from each period are shown. Days 1 and 5 represent period 1 (growth hormone treatment, 0.1 IU/cm^2/day), whereas days 2 and 4 represent period 2 (no growth hormone). Log scale, mean, 95% confidence interval.

hormone treatment ($p = 0.09$, 0.002, and 0.0005, respectively). Thereafter, the values declined toward baseline levels 6 days after termination of the growth hormone treatment. The concentrations of PIIINP in washing 9 correlated significantly with PICP in the same washing ($r = 0.75$, $p < 0.001$).

Because the analyses showed the ninth samples to be the most sensitive (Fig. 4), the longitudinal changes in the metabolite concentrations were described by analysis of all ninth samples.

Longitudinal Washings

The longitudinal changes in the relative concentrations of PICP and PIIINP during the study are shown in Figure 5. The amino terminal propeptide of type III collagen increased significantly to 168% (154% to 184%) and 195% (179% to 218%) 5 and 9 days, respectively, after initiation of growth hormone treatment ($p < 0.01$). The carboxy-terminal propeptide of type I collagen reached a significant increase to 196% (172% to 233%) in the fourth week after the second period of growth hormone treatment ($p < 0.01$). The concentration of both components had declined toward baseline levels in the measurements performed 6 to 9 days after withdrawal of growth hormone. The highest dose of growth hormone administered in period 5, however, failed to induce an increase in the metabolite levels during the study period (Figs. 5 and 6). The areas under the curves of PICP as well as PIIINP (in washing 9) correlated significantly with the healing rates, $r = 0.57$, $p = 0.04$, and $r = 0.64$, $p = 0.01$, respectively.

There was no significant change in the relation PIIINP/PICP during the study.

Discussion

In the current study, a new, noninvasive method of measuring tissue metabolites in chronic leg ulcers was established. The method allows frequent sampling because the procedure is harmless and not associated with pain or discomfort.

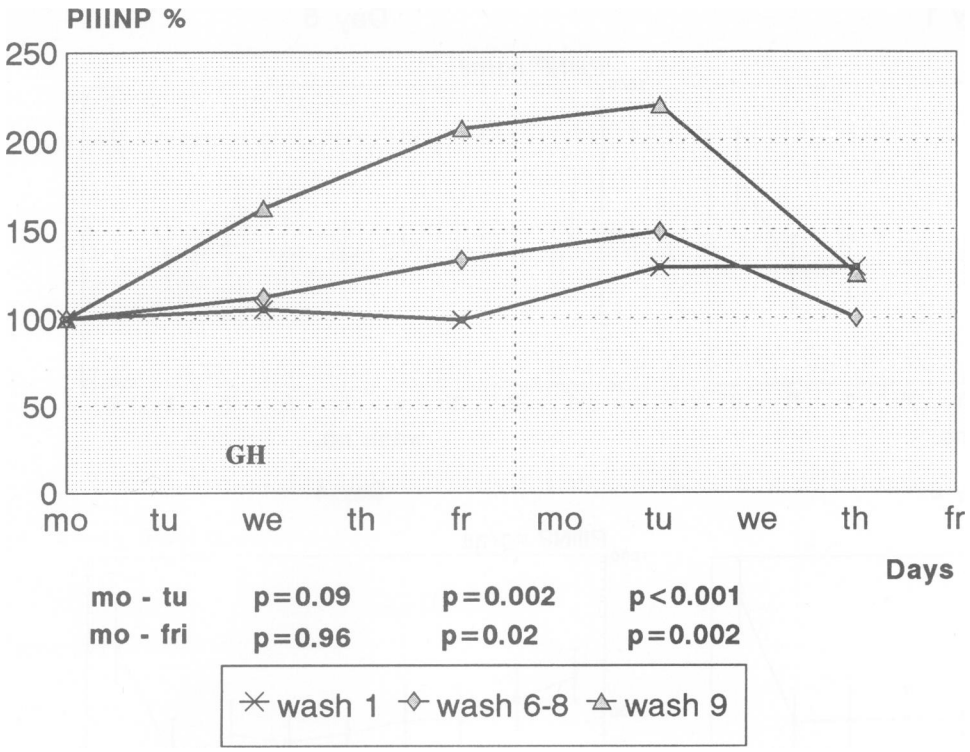

FIG. 4. Sequential washings. Effect of growth hormone (GH) on the concentration of PIIINP in washings 1, 6 through 8, and 9, during the first 2 weeks of the study. Values in percentage of the first measurement performed. Mean.

After a previous pilot study, we abandoned the collection of "undiluted" wound fluid because of problems with leakage from the dressings and large variations in the wound fluid secretion rate. The principle of sequentially washing "down" to baseline, followed by incubation, seems to overcome these problems. The uniform washout curves in all days of sampling, as shown in Figure 2, indicate good reproducibility of the method. The ninth

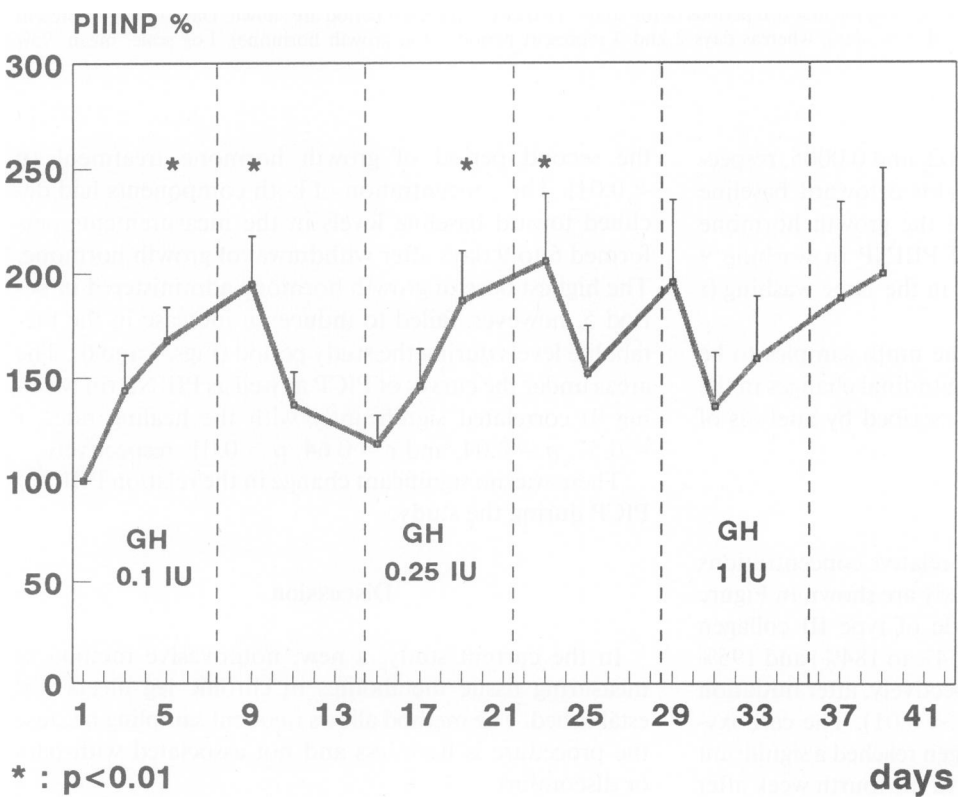

FIG. 5. Longitudinal washings. Effect of increasing doses of growth hormone (GH) (0.1, 0.25, and 1 IU/cm^2/day) on the concentration of PIIINP in washings 9. Values in percentage of the first measurement performed. Mean, 95% confidence interval. *$p < 0.01$.

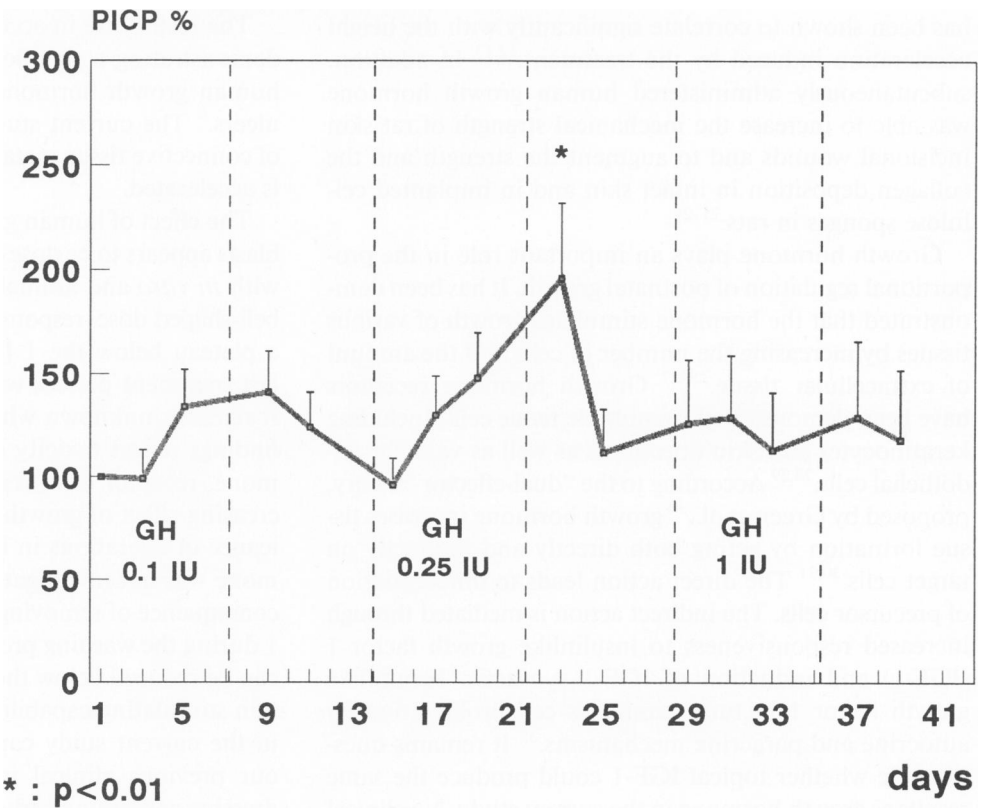

FIG. 6. Longitudinal washings. Effect of increasing doses of growth hormone (GH) (0.1, 0.25, and 1 IU/cm^2/day) on the concentration of PICP in washings 9. Values in percentage of the first measurement performed. Mean, 95% confidence interval. *p < 0.01.

washing was clearly the most sensitive sample for detecting changes in PICP and PIIINP in response to growth hormone. However, PIIINP also fluctuated systematically in samples 6 through 8, although with less amplitude and with larger intraindividual and interindividual variation. Accordingly, the incubation after establishing baseline levels has been shown to be a suitable method to obtaining reproducible measurements. The metabolites measured in the ninth sample may reflect synthesis during the incubation period as well as diffusion of propeptides, previously produced or locally metabolized, but still not cleared from the tissue, that is, free propeptides or propeptides released during remodulation or degeneration of tissue collagen fibers.

Our findings are in accordance with earlier studies of PIIINP and PICP in wound fluid from surgical patients. These studies were based on the collection of wound fluid through implanted silicone tubes.[3,4] Because our washing procedures implies dilution of the wound fluid, the overall concentrations of the metabolites in our washings were somewhat lower than those found in the wound fluid. The ninth washing, however, seemed to be equally sensitive for detecting day-to-day changes in PIIINP and PICP in our patients, as were wound fluid propeptides from the surgical patients.

In the current investigation, growth hormone induced an increase in the concentrations of PICP and PIIINP in the washings. Because all PICP is cleaved off in relation to the synthesis of new type I collagen, our findings probably reflect increased collagen type I synthesis in the ulcers.[6] Not all PIIINP is released during collagen type III synthesis, however. Some propeptide is retained as pN collagen type III on the surface of the collagen fiber. It has been demonstrated that collagen type I and III coexist in the same fibers and subsequently hypothesized that propeptides of pN collagen type III need to be cleaved off the collagen fiber to allow its further growth.[5] This hypothesis of sequential growth of the collagen fibrils may explain the high degree of correlation observed between PICP and PIIINP. Accordingly, the increase in PICP and PIIINP during growth hormone treatment reflect an overall increased collagen production in response to the treatment. This theory is supported by our findings of a significant positive correlation between PIIINP and PICP with the healing rates. Some propeptide may be degraded locally. No information indicating such local degradation is currently available, however. The propeptides leave the tissue through the lymphatics and are cleared by the liver and kidneys[20] (unpublished observations).

The changes in the concentrations of PICP and PIIINP were detectable 5 to 9 days after initiation of the growth hormone treatment. Such findings are in accordance with studies in short children with growth hormone deficiency, in which replacement therapy with human growth hormone increased the levels of PICP as well as PIIINP in serum.[10-13] Furthermore, the increase in PIIINP in serum

has been shown to correlate significantly with the height acceleration induced by the treatment.[12,13] In addition, subcutaneously administered human growth hormone was able to increase the mechanical strength of rat skin incisional wounds and to augment the strength and the collagen deposition in intact skin and in implanted cellulose sponges in rats.[21-24]

Growth hormone plays an important role in the proportional regulation of postnatal growth. It has been demonstrated that the hormone stimulates growth of various tissues by increasing the number of cells and the amount of extracellular tissue.[25-27] Growth hormone receptors have been demonstrated in multiple tissue cells, including keratinocytes and skin fibroblasts as well as vascular endothelial cells.[28,29] According to the "dual-effector" theory, proposed by Green et al.,[30] growth hormone increases tissue formation by acting both directly and indirectly on target cells.[30,31] The direct action leads to differentiation of precursor cells. The indirect action is mediated through increased responsiveness to insulinlike growth factor 1 (IGF-1) and induction of IGF-1 synthesis. Insulinlike growth factor 1 in turn stimulates cell proliferation by autocrine and paracrine mechanisms.[32] It remains questionable whether topical IGF-1 could produce the same results as growth hormone in the current study. No clinical wound healing studies of IGF-1 have been reported. After topical application, IGF-1 has been shown to increase rat sciatic nerve regeneration after injury, whereas the growth factor did not influence dermal regeneration in the pig when administered alone.[33,34]

A local action of growth hormone in the context of experimental wound healing was demonstrated by Steenfos and Jansson,[35] who found increased granulation tissue formation, DNA, and IGF-1 mRNA in subcutaneously implanted wire-mesh cylinders in rats, in response to human growth hormone.[35] By contrast, IGF-1 application, did not influence the ingrowth of granulation tissue (H. Steenfos, personal communication). In the current study, local action of growth hormone was demonstrated biochemically for the first time in humans. The findings indicate a capability of the hormone to influence the metabolism of wound fibroblasts, leading to an increased matrix synthesis, including collagen types I and III. The effect seems to be reversible, and relatively short-lasting, because the concentrations of the metabolites in most of the study periods reapproached baseline levels 1 week after withdrawal of growth hormone. The fact that the highest dose of growth hormone, administered in the last treatment period, failed to increase the propeptide levels, renders it highly unlikely that our findings can be explained as nonspecific effects of the protein content in the test substance. The gel and preservative alone did not affect the release of the metabolites in a similar setup (unpublished observations).

The results are in accordance with our previous study demonstrating a significant stimulating effect of topical human growth hormone on the healing of chronic leg ulcers.[9] The current study suggests an increased release of connective tissue metabolites locally when ulcer healing is accelerated.

The effect of human growth hormone on wound fibroblasts appears to be dose dependent. This is in accordance with *in vitro* and animal studies that seem to indicate a bell-shaped dose–response curve.[36,37] Our findings suggest a plateau below the 1 IU/cm^2/day administered in the last treatment period, with respect to chronic leg ulcers. It remains unknown whether the present dose–response findings reflect toxicity of the high dose of growth hormone, receptor downregulation, or are caused by a decreasing effect of growth hormone over time. The significance of alterations in local degradation of growth hormone was not investigated in our study. Neither was the consequence of removing growth-hormone–induced IGF-1 during the washing procedures. It is likely that the dose can be reduced below the 0.1 IU/cm^2/day and still maintain stimulating capabilities. The dose–response findings in the current study cannot be compared directly with our previous clinical investigation, in which a portal dressing containing carboxymethylcellulose was used, because this hydrocolloid binds human growth hormone. The current results with respect to the effect of growth hormone, and dose–response relationship should be further elucidated in a placebo-controlled study with parallel study groups.

The significant correlation between the propeptides and healing rates suggests applicability of our method in wound healing research, particularly in relation to new drug development. Such work implies extensive pharmacologic investigations with screening of different formulations, including bioavailability and dose–response studies. A substantial part of the investigations will have to be performed in patients, because no satisfactory animal model of chronic leg ulcers exists. Clinical studies based on measurements of healing are tedious, however, and require large series of patients studied for extended periods, because of the substantial heterogeneity of the ulcers.[1] Indeed, varying the dose of growth hormone did not lead to any detectable difference in healing rates in the current short-term study.

In conclusion, our method may be valuable in the evaluation of connective tissue turnover in chronic leg ulcers. Using wound fluid PIIINP and PICP as markers may allow important information with respect to healing and evaluation of the treatment to be obtained in shorter-term studies with a relatively low number of patients in the study groups. The degree of correlation between wound fluid propeptide concentrations and the healing rates indicates that other factors influence the propeptide levels

and calls for further studies to investigate the limitations in using the wound fluid propeptide concentrations as markers of wound healing.

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