# A New Method to Measure Type I and III Collagen Synthesis in Human Skin In Vivo: Demonstration of Decreased Collagen Synthesis After Topical Glucocorticoid Treatment

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Collagen is synthesized as procollagen and large extra domains known as propeptides are cleaved off enzymatically. In the present study we have measured the carboxyterminal propeptide of type I collagen (PICP) and the aminoterminal propeptide of type III collagen (PIIINP) in blister fluids of human skin. High concentrations of PICP were found in the spontaneous blisters of patients with bullous pemphigoid, erysipelas, or erythema multiforme. Detectable amounts were also found in suction blisters induced on healthy skin. Because the concentrations in suction blisters were several times higher than in corresponding serum, most of PICP and PIIINP was derived from the underlying dermis.

This method was used for assessing type I and type III collagen synthesis after topical glucocorticoid treatment. Clobetasol-17-propionate (CP) decreased the concentrations of PICP by 75% after 1 d of treatment, the maximum inhibition (92%) being found after 2 d treatment. PIIINP was also affected. Hydrocortisone and hydrocortisone-17-butyrate also decreased the concentrations of PICP and PIIINP, but less markedly than CP. Partial recovery was seen 3 d after stopping the treatment. Thus measurement of collagen type specific propeptides in suction blisters can be used as an estimate of collagen synthesis in vivo, avoiding both local anesthesia and skin biopsing. With radioimmunoassays for PICP and PIIINP a large number of samples can also be processed simultaneously. *J Invest Dermatol 98:220–225, 1992*

Collagen, the most abundant protein of the skin [1], undergoes marked changes both in amount and biosynthetic activity during aging, in several diseases and upon treatment with various drugs [1–5]. For decades, fibroblasts have been cultured from skin biopsies. However, measurement of collagen synthesis rate in vitro in cell cultures may not reflect actual collagen synthesis in vivo, because in cell culture conditions the effects of any known and unknown mediators may be lost. Another approach to study collagen synthesis rate has been to incubate skin slices with radioactive proline in vitro and to measure the hydroxylation of proline [6]. A third method has been to measure the activity of the enzyme prolyl 4-hydroxylase in skin biopsies [7]. This activity is generally increased in the skin if the collagen synthesis rate is high. The disadvantages of this method are partly identical with those of cell culture.

Recently, quantitive measurement of collagen specific mRNA [8] and the in situ hybridization techniques have given new tools to study the actual collagen synthesis of the skin [9]. The quantitive measurement of collagen-specific mRNA by northern blotting has the same disadvantages as the methods explained above. The RNA must be isolated [10], and is easily degraded. RNA from all the cell types present in the skin may disturb the results. In situ hybridization elegantly visualizes the cells actively synthesizing collagen [9]. This method requires both skin biopsies and a sophisticated technique. In situ hybridization cannot be used for a direct quantitative analysis and to study a large number of samples.

For the reasons above we have adapted a new method for estimating collagen synthesis in human skin in vivo. This method is based on assessment of propeptides of type I and type III procollagens in suction-induced interstitial fluid and on subsequent estimation of the changes in collagen synthesis rate in vivo. In suction blister fluid the propeptides are cleaved off from procollagen in a stoichiometric ratio and thus form a reliable index of collagen synthesis. The method is further applied to show how topical glucocorticoids repress the synthesis of type I and type III collagen in human skin.

## PATIENTS AND METHODS

In the first part of the study, blister fluid samples were collected from spontaneous blisters of patients with bullous pemphigoid, erysipelas, erythema multiforme, and pompholyx; from liquid nitrogen–induced blisters following treatment of warts; and from suction blisters induced on the abdominal skin of voluntary male subjects (ages 19–22) [11].

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Abbreviations:
BP: bullous pemphigoid
CP: clobetasol-17-propionate
ER: erysipelas
HB: hydrocortisone-17-butyrate
HC: hydrocortisone
PICP: carboxyterminal propeptide of type I procollagen
PIIINP: aminoterminal propeptide of type III procollagen
SB: suction blister
SBF: suction blister fluid

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In the second part clobetasol-17-propionate was applied three times daily for 3 or 4 d on healthy abdominal skin of four voluntary male subjects. A vehicle was similarly applied on the opposite side of the abdominal skin. Blister fluid samples were taken immediately after induction and 24 and 72 h later. The time course of clobetasol-17-propionate–induced decrease in the concentration of PICP was studied by treating abdominal skin for 4, 2, or 1 d.

In the third part of the study, glucocorticoids having different anti-inflammatory potency (hydrocortisone, hydrocortisone-17-butyrate, and clobetasol-17-propionate) were compared. The abdominal skin was treated three times daily for 2 d before blister induction. Otherwise the study was carried out as in the second part.

All the samples were taken in accordance with the provisions of the declaration of Helsinki.

**Assays for the Procollagen Propeptides** The concentration of the carboxyterminal propeptide of type I procollagen (PICP) was determined with a recently developed radioimmunoassay for the human protein [12], obtained from Farmos Diagnostica (SF-90460 Oulunsalo, Finland). The assay was used in a sequential saturation fashion in order to increase the sensitivity because of the small volume of sample available, using duplicate 5–20 $\mu$l aliquots of blister fluid diluted with phosphate-buffered saline containing 0.04% Tween 20. The sensitivity of this method in its original form, performed under equilibrium conditions, is 1.2 $\mu$g/l, when defined as the mass equivalent to twice the standard deviation of the zero binding value. Use of sequential saturation makes the assay approximately six- to eightfold more sensitive. Correspondingly the original standards were diluted 1:8. The small sample volumes prevented the assessment of reproducibility parameters for blister fluid samples, but for serum samples this method has intra- and interassay coefficients of variation of about 3 and 5%, respectively.

The concentration of the aminoterminal propeptide of type III procollagen (PIIINP) was assessed similarly using duplicate 10–40 $\mu$l aliquots of diluted blister fluid and a radioimmunoassay for the intact human protein [13]. This method was also obtained from Farmos Diagnostica. The sensitivity of the original assay is 0.2 $\mu$g/l and sequential saturation increases it by about three- to fourfold. As above the original standards were also diluted (1:4). Its coefficients of variation are similar to those of the assay for PICP.

**Gel Filtration Analysis of Suction Blister Fluid** Two-hundred microliter samples of suction blister fluid were gel filtrated on a column (170 × 1.5 cm) of Sephacryl S-300 (Pharmacia, Uppsala, Sweden) equilibrated in radioimmunoassay assay buffer (phosphate buffered saline, pH 7.4, containing 0.04% of Tween 20). The flow rate was 7 ml/h and fractions of 20 min were collected. The PICP and PIIINP analyses were performed directly in the fractions using the above radioimmunoassays with sequential saturation.

**Other Assays** For statistical analyses, Student t test and linear regression were used.

## RESULTS

**Demonstration of PICP in Blister Fluids** PICP was assayed in the fluid of spontaneous blisters and the concentrations were compared with those obtained in SBF and in serum. High levels were found in the spontaneous blisters of five patients with bullous pemphigoid, two with erysipelas and one with erythema multiforme (Fig 1). Measurable levels were also seen in the spontaneous blisters of patients with pompholyx and in suction blisters of healthy subjects. In contrast, only small concentrations of PICP were present in kryoblisters. The levels of PICP in spontaneous or suction blisters were clearly higher than the corresponding serum values (see Fig 1). The concentration of PICP in the sera of the bullous pemphigoid, erysipelas, and pompholyx patients studied was 108 ± 30 (mean ± SD). It should be noted that the concentration of PICP in the sera of the young male volunteers studied was 180 ± 30 $\mu$g/l. However, the levels of PICP in suction blister fluids in these subjects were several times higher than corresponding serum levels (Fig 1).

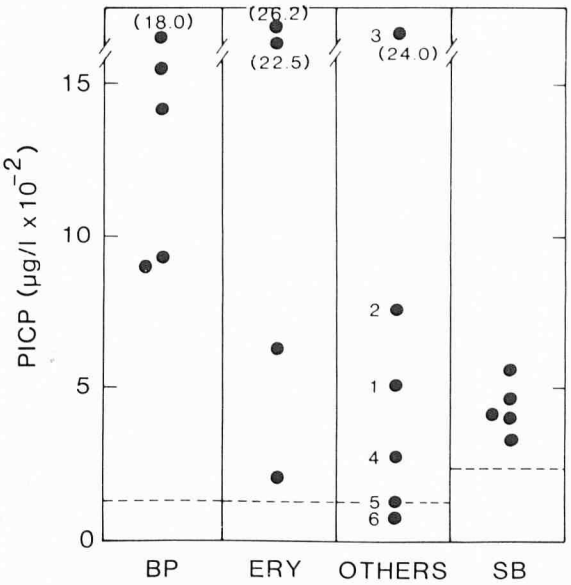


**Figure 1.** Concentration of the carboxyterminal propeptide of type I procollagen (PICP) in spontaneous blisters of bullous pemphigoid (BP), erysipelas (ERY), pompholyx (1–2), and erythema multiforme (3–4) and kryoblisters (5–6). For comparison, PICP was also measured in suction blisters (SB) of healthy male volunteers. The concentration of PICP in the sera of the patients studied was 108 ± 28 $\mu$g/l (the mean + 1 SD is marked with a *broken line*). The concentration of PICP in the sera of the young male volunteers studied was 180 ± 30 $\mu$g/l (mean + 1 SD is also marked with a *broken line* in the section to the right).

**Characterization of PICP and PIIINP in SBF** Because detectable levels of PICP were observed in suction blisters, the concentration and the molecular weight of the PICP antigen were characterized in detail in newly induced suction blisters. PICP was also compared with PIIINP, the latter reflecting the synthesis of type III collagen. Both antigens were measured in subjects without any treatment.

There was a highly significant correlation between the suction blister fluid concentrations of PICP and PIIINP ($r = 0.850$, $p < 0.001$) (Fig 2). Because the molecular weight of PICP is 100,000 and that of PIIINP 42,000, the relative synthesis of type III collagen calculated as percent of total collagen synthesis (type I plus type III collagen) was 37.7 ± 11.1%. By measuring the concentrations of PICP and PIIINP in serum and blister fluid it was possible to estimate the proportion of the antigens that could be derived from serum and the proportion released locally from the surrounding dermis into the blister compartment [14–16]. This estimation, however, is complicated: PIIINP has a peculiar molecular conformation and behaves in gel filtration as a molecule with a molecular weight of about 100,000 [17]. The calculations revealed that maximally about 7% of the PICP and about 0.6% of PIIINP in blister fluid could be derived from serum (Table I). Thus the PICP and PIIINP in blister compartment mainly reflect local synthesis of type I and type III collagens.

The PICP and PIIINP antigens were further analyzed by gel filtration of SBF (Fig 7*A*). Both were eluted as one major peak, though PIIINP appeared slightly earlier than PICP.

**Effect of Blister Aging on Its PICP Concentration** The concentration of PICP was followed during aging of suction blisters up to 72 h. The mean level of PICP in the fluid of 24-h suction blisters was 469 ± 56 $\mu$g/l (mean ± SD) and it increased in 72-h blisters to 1100 ± 63 $\mu$g/l correspondingly (Fig 3). This increase was highly significant ($p < 0.001$). During regeneration of the blisters the relative proportion of type III collagen remained constant: immediately

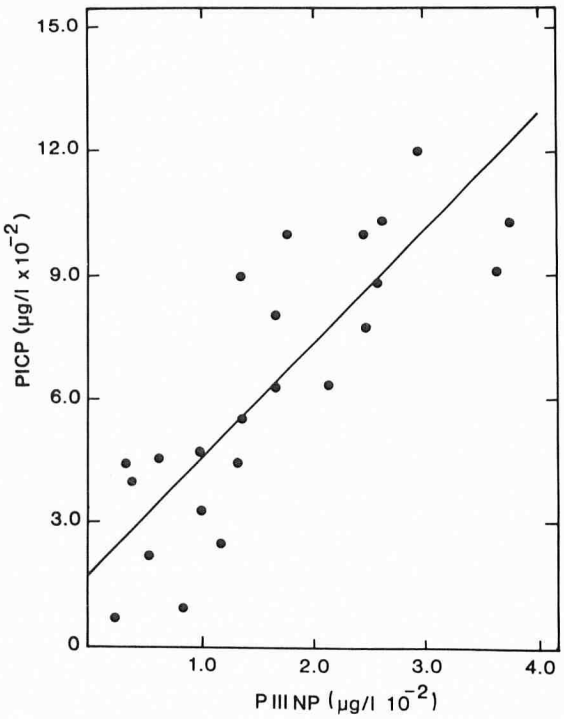


**Figure 2.** Correlation between the concentrations of the carboxyterminal propeptide of type I procollagen (PICP) and the aminoterminal propeptide of type III procollagen (PIIINP) in suction blisters. Blisters were induced on the abdominal skin of volunteers. The correlation efficient is 0.84 ($p < 0.001$).

after blister induction this was 36.5% and at 72 h it was 37.3% of the total collagen production.

**Effect of Topical Glucocorticoid Treatment on PICP and PIIINP in Suction Blisters** Clobetasol-17-propionate decreased the concentrations of PICP and PIIINP in suction blisters by about 90% (Figs 3–6). During aging of the blisters partial recovery of PICP took place in 72 h; in two subjects PICP recovered almost to the level of the control blisters (Fig 3). Already a treatment with clobetasol-17-propionate lasting for 1 d decreased the PICP concentration by 75 ± 19% (mean ± SD of three subjects), the maximum inhibition (92 ± 3%) being noted after a 2-d treatment (Fig 5).

When the skin was treated with different glucocorticoids, the largest decrease in PICP was noted with clobetasol-17-propionate (72 ± 20%) (Fig 6*A,B,C*). The corresponding decreases with hydrocortisone and with hydrocortisone-17-butyrate were 46 ± 20 and 67 ± 14%, respectively. The concentration of PIIINP was decreased by clobetasol-17-propionate treatment by 71 ± 13%, the corresponding figures for hydrocortisone and hydrocortisone-17-butyrate being 17 ± 6 and 52 ± 33%, respectively (Fig 6*D,E,F*).

SBF were analyzed with gel filtration during glucocorticoid treatment. As shown in Fig 7, the elution times of PIIINP and PICP were similar in control skin and in skin treated with hydrocortisone, hydrocortisone-17-butyrate, or clobetasol-17-propionate. However, the heights of the peaks, which are directly related to the amounts of PIIINP and PICP, were markedly reduced in hydrocortisone, hydrocortisone-17-butyrate, and clobetasol-17-propionate–treated skin, the smallest peaks being found in blisters induced in clobetasol-17-propionate–treated skin (Fig 7).

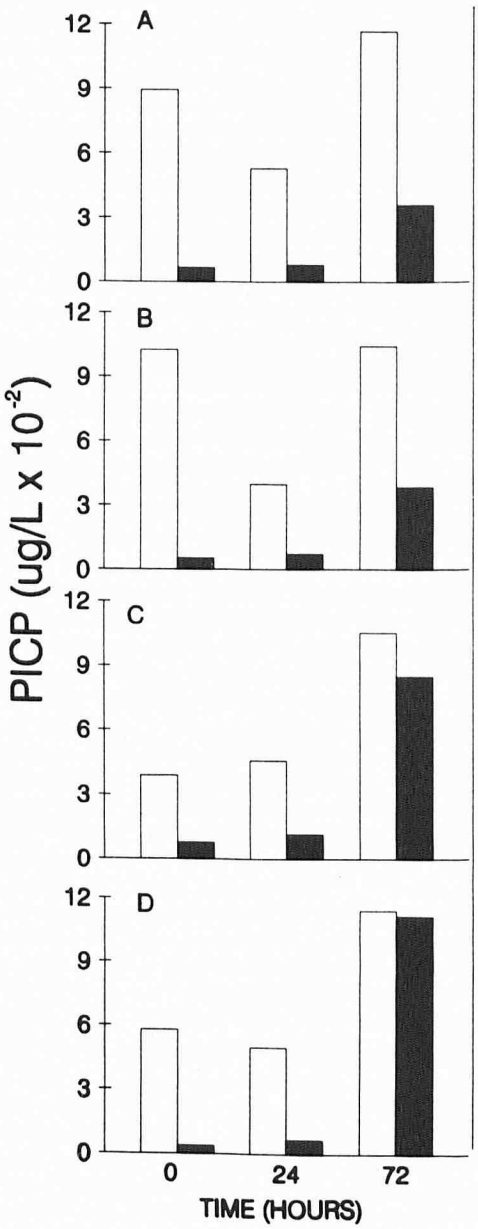


**Figure 3.** Effect of topical clobetasol-17-propionate treatment on the concentration of PICP in blister fluid. The abdominal skin of two subjects was treated three times a day for 3 d *(A,B)* and that of two other subjects three times a day for 4 d *(C,D)* before blister induction. The samples were taken immediately after blister induction (0 h), and 24 and 72 h after blister induction. *Black columns,* treated; *open columns,* controls.

**Table I.** Concentrations of PICP and PIIINP in Blister Fluid and Serum in Three Healthy Volunteers and the Relative Amounts of Locally Produced PICP and PIIINP

| Samples (n = 3) | PICP (µg/l) | Percent | PIIINP (µg/l) | Percent |
|---|---|---|---|---|
| Blister fluid | 895 ± 99 | 100 | 200 ± 49 | 100 |
| Locally produced[a] | 831 ± 105 | 92.8 | 199 ± 49 | 99.5 |
| Serum-derived[a] | 64 ± 31 | 7.2 | 1.2 ± 0.2 | 0.6 |
| Serum | 221 ± 107 | 24.7 | 4.1 ± 0.7 | 2.1 |

[a] Locally produced PICP and PIIINP have been calculated using the known molecular weights of PICP and PIIINP and by assuming that PIIINP behaves like a globular protein having a molecular weight higher than 42,000. The diffusion of serum proteins into blister fluid has been taken as described earlier [14–16].

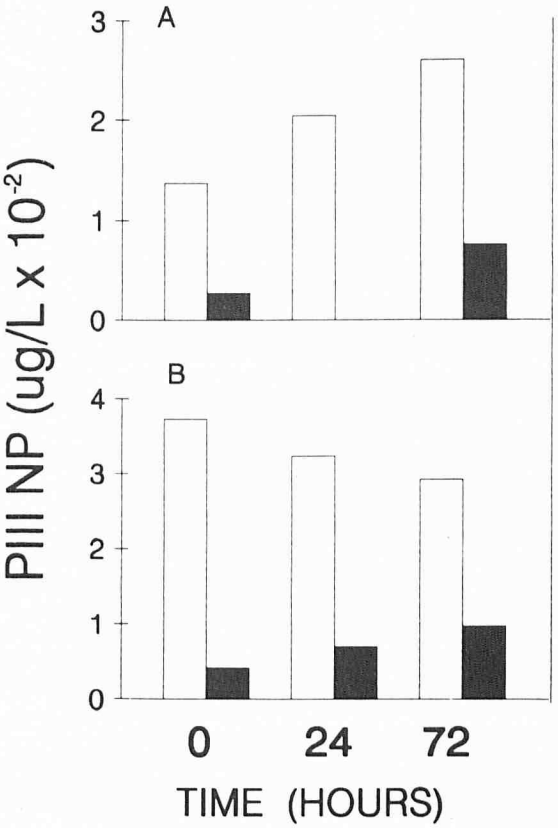


**Figure 4.** Effect of topical clobetasol-17-propionate treatment on the concentration of PIIINP in blister fluid. The abdominal skin of two subjects was treated three times a day for 3 d before blister induction. Symbols as in Fig 3.

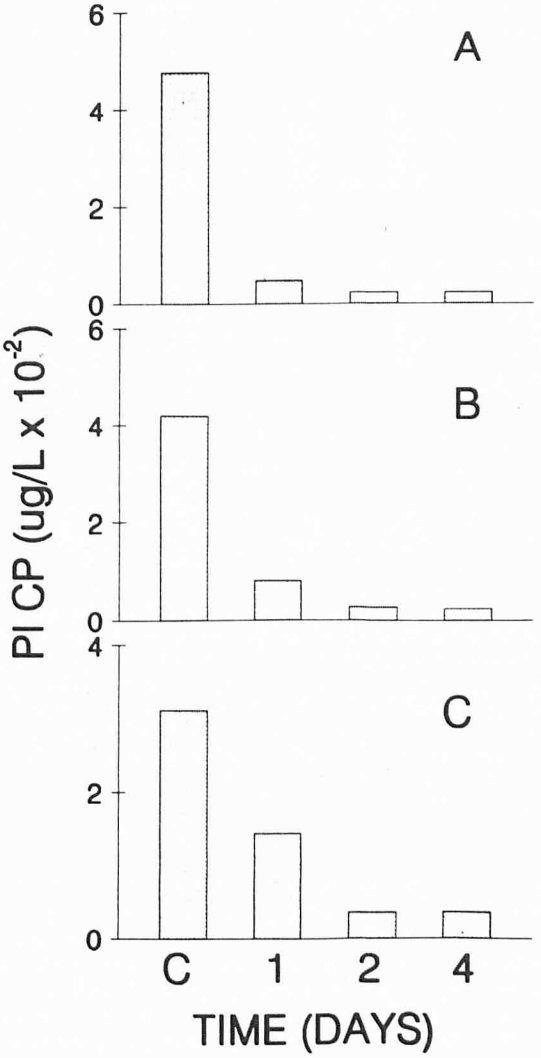


**Figure 5.** Time course for the clobetasol-17-propionate–induced decrease in PICP in suction blisters. The abdominal skin was treated for 1, 2, or 4 d with clobetasol-17-propionate, blisters induced simultaneously, and blister fluid samples were collected immediately after blister induction. Samples were also taken from control blisters (C) of the same subjects treated with vehicle only. The values are the means of duplicate assays.

## DISCUSSION

In the present study we measured type I and type III collagen synthesis in blister fluids of various blistering diseases and in suction blisters. This was carried out by assessing the concentrations of the carboxyterminal propeptide of type I procollagen (PICP) and the aminoterminal propeptide of type III procollagen (PIIINP). High concentrations of PICP were found in the blister fluid of certain blistering diseases, such as bullous pemphigoid, erythema multiforme, and erysipelas. These concentrations were clearly higher than those in serum. Active production of PICP into the blisters in these diseases may be due to the strong inflammatory state of the skin, because mediators of inflammatory cells are able to stimulate the collagen synthesis rate of the fibroblasts present in the dermis (for review see [18]). In addition it should be noted that spontaneous blisters in diseases were not fresh (as suction blisters are), and thus wound heading was already actively going on, which could increase the levels of propeptides, as previously demonstrated in surgical wounds [19,20].

The possibility that the interstitial fluid accumulating in suction blisters could be used for quantifying collagen synthesis in the skin was evaluated in detail. The concentrations of PICP and PIIINP are of the same order of magnitude as those observed in the interstitial fluid of surgical wounds on the first days of healing [19,20]. This is also true for the relative proportion of type III collagen synthesis [20]. There is a significant correlation between the blister fluid concentrations of PICP and PIIINP, indicating a similar regulation of type I and type III collagen synthesis. The PIIINP and PICP antigens were characterized by gel filtration; in blister fluid both of these antigens were exclusively in the form of the free propeptides, indicating rapid and effective processing of the corresponding procollagens. Due to its peculiar molecular shape, PIIINP elutes earlier than PICP in gel filtration [17]. This is based on the fact that part of the PIIINP (trimeric form) is collagenous (= triplehelical) and as collagens in general, the molecule is an elongated rigid rod and thus has a larger "stokes radices" than a globular protein with a similar molecular weight.

The analytical method developed was applied for delineating the effects of topically used glucocorticoids, which affected the propeptides of both type I and type III procollagen. This is in agreement with previous studies in vitro in cell cultures and on animal models, in which corticosteroids decrease both the synthesis and the messenger RNA levels of type I and type III procollagen [4,5,21,22]. After a 1-d treatment the most potent drug, clobetasol-17-propionate, had decreased PICP production by more than 70%. The finding that glucocortcoids so quickly suppressed the levels of propeptides in the present study is in good agreement with previous studies, in which glucocorticoids in cell cultures and animal models have been shown to decrease markedly collagen synthesis in a relatively short time (12–24 h) [4,5,21,22]. Hydrocortisone and hydrocortisone-

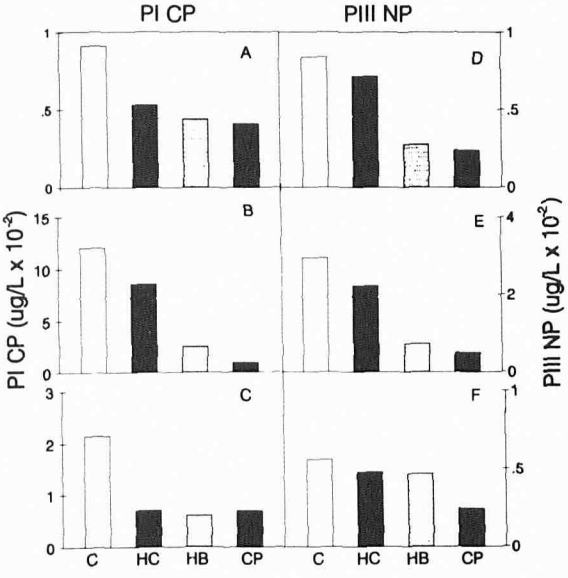


**Figure 6.** Effects of clobetasol-17-propionate (CP), hydrocortisone (HC), and hydrocortisone-17-butyrate (HB) on the concentrations of PICP and PIIINP in blister fluids. Clobetasol-17-propionate, hydrocortisone-17-butyrate, hydrocortisone, or vehicle were applied on the abdominal skin of three subjects three times a day for 2 d, and blister fluid samples were collected immediately after blister induction. The PIIINP levels in *D–F* were determined from the same blister fluid as PICP levels in *A-C*. The values are the means of duplicate assays.

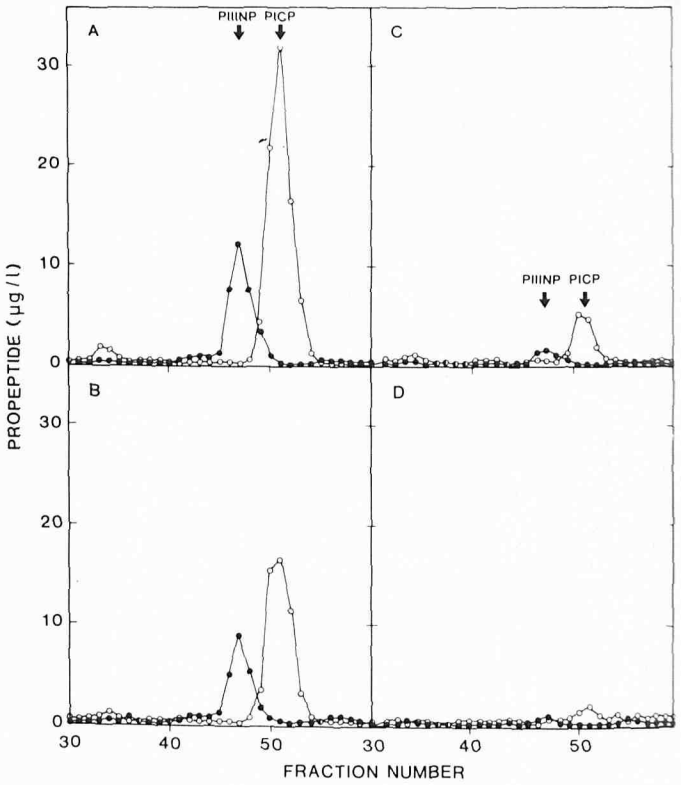


**Figure 7.** Gel filtration of PICP and PIIINP antigens in blister fluids. Blister fluid samples were collected from one subject from skin areas treated with vehicle *(A)*, hydrocortisone *(B)*, hydrocortisone-17-butyrate *(C)*, or clobetasol-17-propionate *(D)*. The samples were chromatographed as described, and 20-min fractions were collected and used for PICP and PIIINP assays. The elution positions of the PIIINP and PICP standards are indicated in the figure.

17-butyrate also induced marked decreases in the concentrations of PICP and PIIINP. This finding indicates that even topical hydrocortisone treatment may lead to skin atrophy. The idea is further supported by previous studies, in which topical hydrocortisone have been shown to decrease the activity of prolyl 4-hydroxylase, a key enzyme of collagen biosynthesis, in human skin [23]. It should be noted that in the present study the steroids were applied on healthy skin. It is possible that in diseased skin the effects are more pronounced, because the penetration of the steroids is increased when the barrier function of the stratum corneum is impaired. The inhibitory effect of the glucocorticoids seems to be reversible; in some subjects the collagen synthesis rate was already as high as in control skin by 72 h after stopping the treatment.

Marked changes observed in collagen propeptides during topical glucocorticoid treatment could not be due to changes on vascular permeability. This is supported by the fact that only a small portion of propeptides could be derived from serum and secondly potent glucocorticoid induces only minor changes in diffusion of serum proteins into blister compartment [24].

The results of the present study thus demonstrate that assays of type I and type III procollagen propeptides in suction blisters can be used for estimating collagen synthesis in vivo. These propeptides have previously been assayed when following wound healing in humans by collecting wound fluid through a thin silicone rubber tube [19,20]. In that model the concentrations of PICP and PIIINP increase markedly during healing, which further supports the idea that the propeptides reflect the activity of collagen synthesis in vivo. For measurements of PICP and PIIINP, relatively small fluid samples are satisfactory when the commercially available assays are made more sensitive, e.g., by using the technique of sequential saturation, as here. Because the PICP and PIIINP antigens are very stable proteins, no problems should arise during storage or handling of the samples. A large number of blister fluids can be assayed simultaneously.

The induction of suction blisters takes 1–2 h. This method is well standardized, practically non-invasive, and painless. The suction blisters heal without complications except for occasional and transient pigment changes. The method seems to be highly sensitive for detecting early changes of skin atrophy, even due to low-potential steroids such as hydrocortisone. The present experiments showed changes induced by this steroid on healthy skin; in skin diseases with an altered stratum corneum barrier function the effect may be even stronger.

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## ANNOUNCEMENT

The 9th International Symposium on Bioengineering and the Skin will be held in Sendai, Japan on October 19 and 20, 1992 after the Annual Meeting of the Japanese Society for Investigative Dermatology at the same site on October 16–18, 1992.

Honorary president: Y. Miki, MD. President: H. Tagami, MD. Secretary-treasurer: T. Ozawa, PhD.

For further information, please contact Tadashi Terui, MD, Secretary of the 9th ISBS meeting, Department of Dermatology, Tohoku University School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980, Japan. Tel: (81)-22-273-9275. Fax: (81)-22-276-2774.