Individual susceptibility to chronic irritant contact dermatitis

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Chapter 3.1

Cytokines at different stratum corneum levels in normal and sodium lauryl sulphate-irritated skin

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Abstract

Background Cytokines play an important role in inflammatory and repair processes occurring in the skin.

Objectives The objectives of this study were to determine the amounts of cytokines and protein isolated by tape stripping in the different layers of the stratum corneum (SC), and to compare normal skin with skin exposed *in vivo* to the irritant sodium lauryl sulphate (SLS).

Methods In 8 volunteers, we determined the amount of total and soluble protein and also interleukin-1α (IL-1α) in pooled tape strips obtained from the upper, intermediate and lower parts of the SC. Three different types of tape were compared (Diamond®, D-squame® or Sentega® tape). In a separate study, 20 volunteers were repeatedly exposed to 0.1% SLS over a 3-week period. The amounts of IL-1α, IL-1RA and IL-8 in strips obtained from the three different SC levels of SLS-exposed skin were compared with an unexposed site.

Results For normal skin, the amounts of soluble protein and IL-1α were similar for the three tapes. Diamond® tape showed the highest yield of total protein. The total protein yield per strip decreased to lower SC levels, whereas soluble protein and IL-1α normalized by soluble protein did not change across the SC. After SLS induced skin irritation, IL-1α decreased and IL-1RA and IL-8 increased at increasing depth into the SC.

Conclusions Tape stripping is a suitable method to determine SC cytokine concentrations in human skin. With this technique, it is possible to study changes in cytokine concentrations at different SC layers after skin irritation.

Introduction

Cytokines play an important role in inflammatory and repair processes occurring in the skin.[1] Upon an external insult, the release of interleukin-1α, which is constitutively present in keratinocytes and the stratum corneum (SC),[2-4] is the first step in the inflammatory cascade.[1,5] This IL-1α leads to stimulation of keratinocytes and fibroblasts to produce more early pro-inflammatory cytokines and chemokines, e.g. IL-1β, IL-6, IL-8 and tumour necrosis factor-α (TNF-α).[1,5] IL-1 receptor antagonist (IL-1RA) is also constitutively present and is released from keratinocytes and the SC upon stimulation. IL-1RA functions as an anti-inflammatory cytokine by blocking the IL-1 receptor without triggering a biological response.[1,6,7]

The cytokine content of the skin can provide more insight into individual susceptibility to cutaneous inflammatory diseases. The available techniques used for cytokine sampling in humans *in vivo* include punch biopsies[8], suction blister fluids[9] or skin-derived lymph.[10] The main drawbacks of these techniques are their invasiveness, laboriousness and the discomfort for the volunteers. As an alternative, the relatively non-invasive technique of SC tape stripping has recently been used.[6,7,11] With this technique, layers of the SC are sequentially removed by repeated application of pieces of adhesive tape. After extraction of the SC from the tapes, the cytokine and protein content is determined. Using tape stripping, not only constitutively present IL-1α and IL-1RA have been recovered from the SC but also low amounts of inducible cytokines and chemokines (IL-8, IL-2, TNF-α and IFN-γ).[6,7,12-15] Not only is measurement of cytokines possible in SC tape strips but also determination of cytokine mRNA and mRNA of other inflammatory mediators.[16-18]

Up to now, different investigators have used not only different types of adhesive tape but also different extraction protocols, e.g. type of extraction buffer and duration of sonication.[6,7,12-15] The amount of SC harvested per tape was shown to be dependent on the type of adhesive tape and the experimental protocol, e.g. applied pressure during tape stripping.[19-21] Furthermore, a non-linear decrease in the harvested amount with increased SC depth is seen.[21] To correct for these differences, the cytokine content on each strip is often normalized for the amount of soluble protein. Soluble proteins originate mainly from the extracellular matrix in which the corneocytes are embedded. Normalization for the total amount of SC (soluble and insoluble proteins) harvested on each strip has not yet been investigated. The amount of SC removed by a tape can be determined by measurement of the total protein amount,[22,23] which is obtained after treatment of the strips with a sodium hydroxide (NaOH) solution.

Our first objective was to study the protein and cytokine yield using three different types of tape. We performed two experiments in healthy volunteers. In the first experiment, we harvested the SC of normal skin using three different tapes. We determined the amount of IL-1α and total and soluble protein in pooled tape strips, obtained, respectively, from the upper, intermediate and lower parts of the SC. We selected three commercially available adhesive tapes: Diamond® tape, which we have used in several tape stripping studies in our laboratory; D-squame® tape, which is frequently used in dermatological research; and Sentega® tape, which was selected because this possesses a water-soluble adhesive that might be favourable for cytokine recovery from the tape.

Our second objective was to study the protein and cytokine yield across the SC (upper, intermediate and lower parts). In this experiment, cytokine amounts of IL-1α, IL-1RA and IL-8 within different levels of the SC were determined in normal skin and skin repeatedly exposed to the irritant sodium lauryl sulphate (SLS; 3-week irritation test, 0.1% SLS).

Methods

Subjects

Eight subjects participated in the first experiment and twenty subjects in the second experiment. The subjects were healthy volunteers, with no visible skin abnormalities and no history of skin disease. The study was approved by the Ethics Committee of the Academic Medical Center, Amsterdam, and all subjects gave their written, informed consent. The subjects were not allowed to (i) use soap or moisturizers on their forearms during the investigation and (ii) sunbathe or use a tanning bed in a period of 2 months prior to, and during, the investigation.

Experiment 1: normal skin

The SC on three sites on the dominant mid-volar forearm was removed using three different types of adhesive tape. One site was stripped using Diamond® adhesive tape (Diamond® Ultra Clear Tape, 19 mm x 25 mm; The Sellotape Company, Eindhoven, the Netherlands. D-squame® tape (Standard sampling discs, 22-mm diameter, Cuderm, Dallas, TX, USA) was used on a second site and Sentega® polypropylene tape with a water-soluble adhesive (PPCBT-K7, 20 mm x 25 mm, Sentega Etiketten BV, Utrecht, the Netherlands) on a third site. The sites were situated in a straight line with a distance of at least 2.5 cm in between ad-

joining sites. The tapes were assigned to the sites following a rotation scheme based on a 3 x 3 Latin square to avoid a possible confounding effect of the location of the tapes.

Templates of Scanpor® tape (Norgeplaster, Vennesla, Norway) were fixed to the skin around each site to limit the tape stripping area to a circle of 18-mm diameter. Pieces of tape were successively applied to the sites and homogeneously pressed onto the skin by moving a 1.0-kg stainless-steel roller[24] to and fro 10 times. The tapes were then removed at an angle of 170° with the skin. The sites were stripped in multiple directions until the SC was totally removed, as indicated by the shininess and redness of the surface and a transepidermal water loss (TEWL) above 100 g m^{-2} h^{-1}. TEWL was measured using an evaporimeter (VapoMeter SWL2g, Delfin Technologies, Kuopio, Finland). After application, each tape was cut into halves. One piece was added to a vial for determination of the total amount of protein and the other piece was added to a vial for determination of soluble protein and cytokines. The tapes were pooled with eight consecutive halves in one vial, except for the last vial for which usually less halves were available. The tape stripping of the three sites was completed simultaneously within 20 min. For the data analyses, the first eight tapes are referred to as the upper part and the second eight tapes as the intermediate part of the SC. The data of the remaining vials (1 up to 4 vials) were pooled representing the lower part of the SC.

The total amount of protein on the strips was analysed following a previously described procedure based on that of Dreher and colleagues.[22,25,26] Four millilitres methanol (J.T. Baker, Deventer, the Netherlands) was added to the vials, which were shaken for 1 h (TPM-2; Sarstedt, Numbrecht, Germany). Subsequently, the methanol was removed and 4 ml 1 M NaOH was added to the vial. The vials were then shaken for 2 h and left at room temperature overnight. After addition of 4 ml 1 M HCl, the total protein content was determined with the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), using the supplied bovine serum albumin (BSA) as a standard. Blank tapes were processed and assayed as a negative control.

The amounts of soluble protein and cytokines was determined based on a generally used approach.[6,7,12-15] In brief, to determine the amount of soluble protein and cytokines, 2 ml phosphate-buffered saline (Merck, Darmstadt, Germany) with 0.005% Tween-20 (Sigma-Aldrich, Zwijndrecht, the Netherlands) was added to each vial and the vials were left on ice for 30 min. Extraction was performed using an ultrasound sonifier equipped with a probe (Salm & Kipp, Breukelen, the Netherlands) for 15 min in ice water. The extract was centrifuged (1 min, 15,000 g) and supernatant aliquots of 225 µl were re-frozen at -80 °C until re-

quired for further analysis. The extracts were analysed for the cytokine IL-1α using specific enzyme-linked immunosorbent assay (ELISA) kits (Human CytoSets, Biosource International, Camarillo, CA, USA). Soluble protein was determined in the strips with the Micro BCA protein assay kit (Pierce, Rockford, IL, USA) using the supplied BSA as standard.

Experiment 2: SLS-exposed skin

In 20 volunteers, one site on the dominant mid-volar forearm was exposed to a 0.1% w/v SLS solution (200 µl, ≥ 99% purity, Fluka, Buchs, Switzerland) for 6 h a day, 4 days a week, for 3 weeks using patch test chambers (Finn chambers® of 18 mm diameter and Filter Paper Discs; Epitest, Tuusala, Finland) attached with adhesive tape (Scanpor® tape; Norgeplaster, Vennesla, Norway). Two subjects had to withdraw after the first week because of a strong erythema reaction to the SLS exposure. This experiment was performed before Experiment 1. The SC was removed from the exposed site and from an unexposed control site on the same volar forearm using pieces of Sentega® tape (see Experiment 1). The tape stripping was performed four days after the final exposure (day 21) and following the protocol described in Experiment 1. The tape stripping of the two sites was completed in 15 min. Strips were added to a 2 ml polypropylene tube (Fisher Emergo, Landsmeer, the Netherlands) and stored at -80 °C.

Extraction was performed using the protocol described in Experiment 1. The consecutive tapes from each site were divided into three tertiles. From the upper tertile, from the intermediate tertile and from the lowest tertile of the SC the two middle tapes were selected for the analyses. The extracts from these strips were analysed for the cytokines IL-1α, IL-1RA and IL-8 using specific ELISA kits (Human CytoSets, Biosource International, Camarillo, CA, USA). The amount of cytokines on each strip was normalized to the soluble protein content of the strips as determined with the Micro BCA protein assay kit (Pierce) using BSA as standard.

Statistics

Data are given as mean ± SD, unless otherwise indicated. Statistical analyses were performed using Pearson's correlation coefficient and one-way analysis of variance (ANOVA) for repeated measurements with a Bonferroni *post hoc* test. If appropriate, normal distributions were obtained by log transformation of the data. All tests were two-sided with $P < 0.05$ as the significance level, and performed using Prism 4 software (GraphPad, San Diego, CA, USA).

Results

Experiment 1: normal skin

The total number of strips needed to remove the SC from the unexposed skin sites was similar for all investigated tapes. With Diamond® tape, 24 [20-32] strips were used, with D-squame® 29 [22-32] and with Sentega® 24 [20-50] ($P > 0.1$; median [min-max]). Diamond® tape showed the highest cumulative yield of total protein (Table 1). On the other hand, the cumulative yield of soluble protein was similar for all three tapes ($P > 0.2$; Table 1).

For IL-1α, similar cumulative amounts were obtained by all three tapes ($P > 0.1$; Table 1). As the cytokines obtained by our study protocol originate predominantly from the extracellular matrix, normalization with soluble protein has been applied. Also after normalization, the three tapes did not show different results ($P > 0.1$; Table 1). We found considerable inter-individual differences in cumulative cytokine amounts, reflected in a very high coefficient of variation ranging from 57% to 78%. This variation remained high when the amount of IL-1α was normalized for soluble protein (Table 1).

Table 1. Cumulative amount of protein and interleukin-1α (IL-1α) obtained from stratum corneum tape strips using Diamond®, D-squame® and Sentega® tape.

Parameter	**Diamond® tape** mean ± SD (CV%)	**D-squame® tape** mean ± SD (CV%)	**Sentega® tape** mean ± SD (CV%)
Total protein (µg/cm^2)	742 ± 186 (25%)*	487 ± 111 (23%)	568 ± 207 (36%)
Soluble protein (µg/cm^2)	64 ± 18 (28%)	74 ± 26 (36%)	63 ± 20 (31%)
IL-1α (pg/cm^2)	823 ± 470 (57%)	826 ± 645 (78%)	648 ± 432 (67%)
IL-1α (pg/µg soluble protein)	14.4 ± 9.5 (66%)	11.0 ± 7.0 (64%)	10.1 ± 6.4 (63%)

n = 8; CV%, coefficient of variation (in %). * $P < 0.05$ Diamond® tape vs. D-squame® and Sentega® tapes.

The amount of total protein per strip decreased to lower SC levels for all tapes (Figure 1a). The proteins harvested from the upper part of the SC (first 8 strips) amounted 46% ± 5%, 45% ± 9% and 51% ± 12% of the total SC protein amount for Diamond®, D-squame® and Sentega® tape, respectively. Contrary to the total protein, the amount of soluble protein obtained per strip did not change across the SC (Figure 1b).

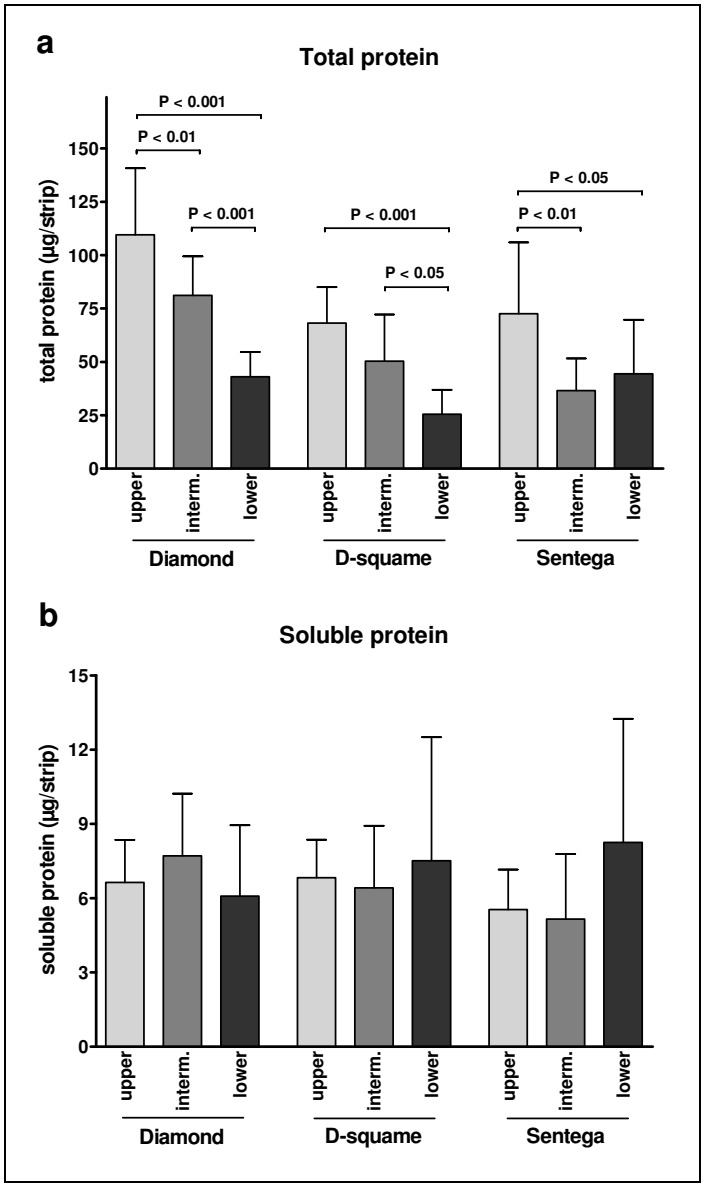

Figure 1. Amount of (a) total protein and (b) soluble protein recovered by tape stripping from the upper, intermediate and lower parts of the stratum corneum of normal skin sites using Diamond®, D-squame® and Sentega® tape. Data are expressed as mean ± SD (n = 8 subjects).

The amount of IL-1α harvested per strip was similar in the three parts of the SC for D-squame® and Sentega® tapes (Figure 2a; $P > 0.2$). For Diamond® tape, the intermediate part contained a significantly higher amount of IL-1α than the lower part ($P < 0.05$). The amounts of IL-1α normalized by soluble protein did not differ in the three different parts of the SC ($P > 0.1$; Figure 2b).

We calculated the correlation coefficient between the concentration IL-1α normalized for soluble protein in the upper part of the SC and the parts below (average concentration of the intermediate and lower parts). For Diamond® tape we found $r = 0.97$ ($P < 0.001$), for D-squame® tape $r = 0.91$ ($P < 0.01$) and for Sentega® tape $r = 0.92$ ($P < 0.01$).

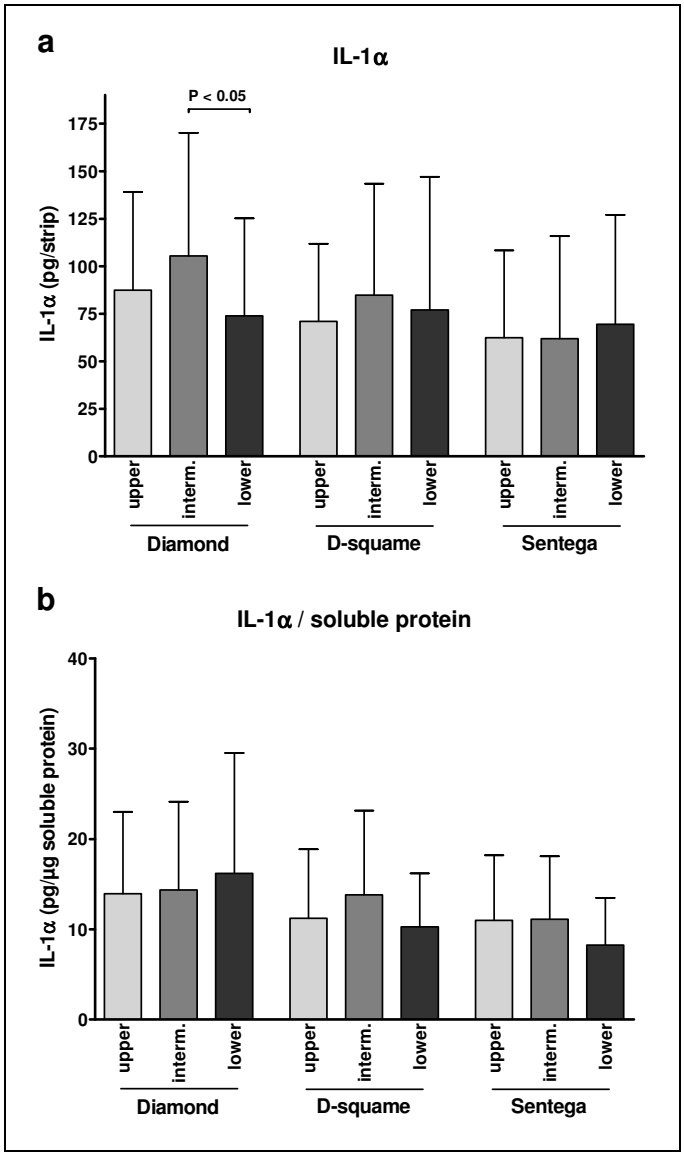

Figure 2. Amount of (a) interleukin-1α (IL-1α) per strip and (b) IL-1α normalized for soluble protein recovered from the upper, intermediate and lower parts of the stratum corneum of unexposed skin sites using Diamond®, D-squame® and Sentega® tape. Data are expressed as mean ± SD (n = 8 subjects).

Experiment 2: SLS-exposed skin

In this experiment, we used Sentega® tape for the harvesting of cytokines. A total of 30 [20-36] tapes were required for complete removal of SC from the site that was exposed to SLS, as compared to 26 [15-36] tapes at the control site (P = 0.06; median [min-max]). Lower cumulative amounts of soluble protein were recovered from the tapes of the SLS-exposed skin site (94 ± 43 µg soluble protein) compared to the unexposed site (142 ± 79 µg soluble protein; P = 0.02).

The amounts of cytokines IL-1α, IL-1RA and IL-8 normalized for soluble protein in the upper, intermediate and lower SC parts obtained from the site repeatedly exposed to SLS and the control site are shown in Figure 3. Only very small amounts of IL-8 were measured at the

control site: 11 subjects had IL-8 values just above the limit of detection (LOD) of the assay (0.86 pg/strip). Samples with IL-8 values below the LOD were given a value of 0.43 pg/strip (= ½ LOD). For the whole SC, the average value of IL-1α normalized for soluble protein decreased by 30% after SLS exposure compared to the control site ($P < 0.05$), while IL-1RA increased 10-fold and IL-8 increased fourfold (both $P < 0.001$) (described in our previous paper[11]). In unexposed skin, similar cytokine concentrations were found in the three positions within the SC ($P > 0.10$; Figure 3), while for the SLS irritated skin, differences in cytokine concentration were seen (Figure 3). In the lower layer, IL-1α was decreased compared with the intermediate and upper layer ($P = 0.02$ and $P < 0.001$, respectively). IL-1RA tended to be decreased in the upper layer compared with the intermediate and lower layers ($P = 0.09$ and $P = 0.07$, respectively). IL-8 showed an increase in the intermediate layer compared to the upper layer ($P = 0.05$).

We also calculated the correlation coefficient between the amount of cytokines normalized for soluble protein in the upper part and in the parts below (average concentration of the intermediate and lower parts). In unexposed skin, we found $r = 0.94$ ($P < 0.001$) for IL-1α and $r = 0.82$ ($P < 0.001$) for IL-1RA. For IL-8, we found $r = 0.49$ ($P < 0.05$). In SLS irritated skin, we found $r = 0.83$ ($P < 0.001$) for IL-1α, $r = 0.48$ ($P < 0.1$) for IL-1RA and $r = 0.53$ ($P < 0.02$) for IL-8.

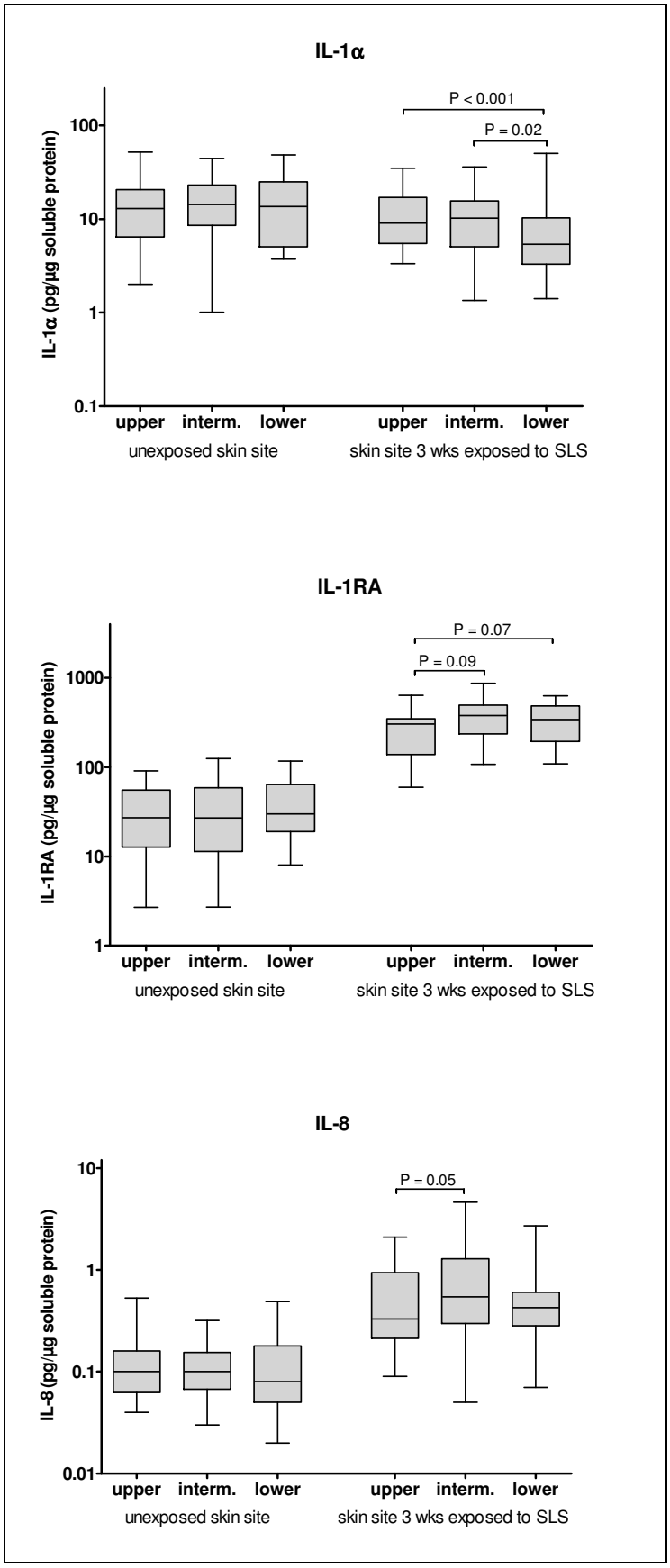

Figure 3. Cytokine concentrations [interleukin-1α (IL-1α), IL-1RA and IL-8] normalized by soluble protein content obtained from the upper, intermediate and lower parts of the stratum corneum from unexposed skin (n = 20 subjects) and sodium lauryl sulphate (SLS)-exposed skin (n = 18 subjects). Sentega® tape was used in this experiment.

Discussion

In this study, we have determined IL-1α and protein amounts in the SC harvested by three different adhesive tapes. Furthermore, we investigated cytokine amounts in the upper, intermediate and lower parts of the SC in normal skin and skin exposed to SLS.

The amount of SC harvested by adhesive tapes has been shown before to be highly variable and dependent on the type of tape, experimental conditions and position within the SC.[19-21] Therefore, in Experiment 1 we investigated the yield of soluble and total protein using three different tapes. Diamond® tape showed the highest total protein yield of all three tapes. Assuming that the SC was totally removed by all three adhesive tapes, this implies that the adhesive of this tape has the most favourable physico-chemical properties for the extraction of the SC proteins from the tape. Each type of tape harvested similar cumulative amounts of soluble protein. Interestingly, we observed visually that after extraction of the SC proteins, the typical imprint of the stripped SC pattern was still visible on most of the tapes. This was seen for all types of tape and after both extraction methods.

Our results on the amount of total protein harvested by successive tape stripping are in agreement with the earlier findings that the amount of SC removed by each strip decreases as the SC is progressively stripped.[19-21] The mass of SC removed is dependent on the adhesion properties of the adhesive and on the cohesiveness of the corneocytes.[21] In the superficial layers of the SC, the cohesion between the corneocytes is known to be reduced due to degradation of the corneodesmosomes.[27,28] Thus, the increasing SC cohesion with increasing depth of the layers may explain the decreasing amounts of SC per strip. With our tape stripping procedure, eight strips (approximately one-third of the total amount of strips) were needed to remove 45-50% of the total SC. This is comparable to the study of Jacobi et al.[19] were the investigators stripped 50% of the SC using about 30% of the total amount of strips.

In contrast to total protein, the amount of soluble protein did not change across the SC. Total protein originates more than 85% from insoluble corneocytes, whereas soluble protein as well as the determined cytokines mainly originate from the extracellular matrix. Hence, so we consider normalization of cytokine amounts with soluble protein instead of total protein to be justified. The question remains why the amount of soluble proteins remains constant across the SC. Perhaps, a relative increase in the extracellular component of the SC compensates for the decrease of total protein with increasing depth.

Our results show that all investigated tapes are suitable for determination of SC cytokines. In normal, untreated SC, we found similar cytokine concentrations in the upper layers and the rest of the SC. This indicates that for the measurement of the SC cytokine concentrations of groups of healthy individuals, it is not necessary to strip the whole SC. Harvesting only the upper layers of the SC saves time and yields a sufficient amount of cytokines for the analysis. However, when one considers to limit the sampling to the upper SC part, it has to be taken into account that although the cytokine level in the upper part of the SC correlated well with that in the part below, the predicted levels for the lower part have an appreciable error. For example, we performed linear regression of the group data obtained with Sentega® tape. When referring to the group mean of IL-1α in the upper part (11.7 pg/µg soluble protein), the estimated amount of IL-1α in parts below was 9.9 pg/µg soluble protein with a 95%-confidence interval from 3.7 to 16.1 pg/µg protein.

In contrast to untreated skin, after induced skin irritation, cytokine amounts were more variable among the different positions in the SC between subjects. In studying cytokines in exposed skin (e.g. to irritants or ultraviolet radiation) or in diseased skin, determination of cytokines in different layers or in the whole SC will be necessary.

The origin of cytokines measured in the tape strips is not fully clear yet. A part of the cytokines might have leaked from intracellular pools of damaged corneocytes due to the tape stripping and sonication procedure. We assume that this is only a minor source, as corneocytes are highly resistant to mechanical wear and chemical influences. Previous studies have shown that keratinocytes in the epidermis display membrane-bound IL-1α[3,29,30] and hence these cytokines may still be present after transformation of keratinocytes into corneocytes. Furthermore, cytokines secreted by the epidermal cells might diffuse from the viable epidermis into the extracellular matrix of the SC; however, experimental data on this process is lacking. In addition, cytokines are produced by eccrine sweat glands[31,32] and sebaceous glands,[33] but we assume that this secretion contributes to only a small part of the cytokines present inside the SC of the mid-volar forearm due to the low number of these glands.

After a 3-week SLS exposure, a decrease in IL-1α and an increase in IL-1RA with increasing depth into the SC have been observed. This might be explained by a downregulation of the inflammatory response over time. We hypothesize that the oldest, upper SC layers present the cytokine pattern of the earlier period of the irritation. The youngest layers at the bottom of the SC reflect more or less the cytokines present in the recently viable epidermis. The

longer the skin irritation lasts, the lower the IL-1α and the higher the IL-1RA amounts in the SC.

The amount of IL-1α of 10-14 pg/μg soluble protein in normal skin that we found in our study was almost identical to the amount found by Perkins et al.[12] in the sebum sampled on the forearm skin using a lipophilic polymeric film (Sebutape®). This suggests that, at least on the forearm skin, sebum contains similar cytokine concentrations as the extracellular matrix or that Sebutape® not only samples superficial sebum but also the extracellular matrix.

It appeared that after repeated skin irritation with SLS over a period of 3 weeks, approximately 30% less protein was recovered from the tape strips as compared with untreated skin. In another study, this effect was also observed after a single exposure to SLS and the authors speculated that this might be caused by a partial loss of the SC after SLS treatment, as SLS is known to change the cohesion between the corneocytes.[34] Hence, also in our volunteers, the upper layers of the SC might have scaled off in the course of the 3-week SLS treatment period.

In this study, we have shown that tape stripping is a suitable method for determination of cytokines in the SC of normal skin. The agreement between results of IL-1α found independently in two groups of volunteers who participated in our two studies implies that the method is reproducible. Further, the harvesting of cytokines using tapes enabled us to study changes in SC cytokines after skin irritation in different SC layers.

An advantage of the tape stripping technique is the minimal invasiveness and the short duration of the procedure. This suggests that the measured cytokine amounts are hardly influenced by the sampling process. Although it is known that the tape stripping procedure results in the activation of keratinocytes to produce cytokines, it takes several hours before the cytokine mRNA production in the keratinocytes is started.[35] When sampling epidermal cytokines by e.g. microdialysis or suction blisters, which takes several hours, the measured cytokine amounts might be influenced by the induced damage to the epidermal cells.

One of the limitations of SC sampling is that only a limited number of cytokines and chemokines can be determined. Recently, in skin-derived fluid, we were able to measure at least 17 different cytokines.[36] Furthermore, it has to be realized that the amount IL-1α and IL-1RA in the SC are highly influenced by sun exposure.[6,12] Therefore, tape stripping should preferentially performed in minimally or non sun-exposed skin.

SC tape stripping is a relatively fast and simple technique, which is suitable for large-scale studies in humans. A possible application may be studying the relations between some SC

cytokines and individual susceptibility to cutaneous inflammatory diseases. Also, an individuals' cytokine response to skin irritation may be monitored by using this tape stripping technique. Further studying the role of SC cytokines in the development or progression of skin disease might be a possible application. In short, SC tape stripping is a feasible technique for studying cytokine profiles in the skin with a wide variety of applications.

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