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Cell shedding from human plantar skin in vitro: evidence that two different types of protein structures are degraded by a chymotrypsin-like enzyme

A. Lundström and T. Egelrud

Department of Dermatology, University of Umeå, S-901 85 Umeå, Sweden

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Summary. A recently described endogenous proteolytic process in pieces of human plantar stratum corneum incubated in vitro has been further studied. This process leads to a decrease in cohesion between the cells that had been facing outwards in vivo. Using two methods, that differed with respect to efficiency, to detach surface cells with decreased cohesion, the process could be divided into two steps. The first step took place irrespective of the presence of ethylenediaminetetraacetate (EDTA) and led to a moderate decrease in cohesion between surface cells. The second step occurred only in the presence of EDTA and advanced to a point where the surface cells could be separated from the remaining cohesive tissue pieces by simple agitation. Both degradation steps could be inhibited by aprotinin and chymostatin but not by leupeptin. Zinc sulfate inhibited the first step. The results indicate that there are two different types of protein structures being degraded during the process of cell shedding in vitro. A chymotrypsin-like enzyme may be involved in the process.

Key words: Plantar stratum corneum – Cell shedding – Cohesive protein structures – Chymotrypsin-like enzyme

We have recently introduced an experimental system which we believe will be of value in efforts to elucidate the structures responsible for cell cohesion in plantar stratum corneum [13]. When a piece of human plantar stratum corneum is incubated in a simple buffer, a spontaneous proteolytic process can be demonstrated in the tissue. This process leads to a decrease in cohesion between cells at the surface that had been facing outwards in vivo, thus imitating desquamation. Regardless of the nonphysiological conditions under which these experiments are performed, significant information about some central aspects of plantar stratum corneum cell cohesion and consequently desquamation may be obtained from such studies.

In this report we have further characterized the process in which the cohesion between surface cells of plantar stratum corneum is decreased during in vitro incubation. Evidence was obtained that at least two types of protein structures are degraded in the process and that a chymotrypsin-like enzyme may be involved.

Offprint requests to: Anita Lundström (address see above)

Materials and methods

Aprotinin, chymostatin, and leupeptin were obtained from Boehringer Mannheim, Mannheim, FRG.

Incubation of plantar stratum corneum in vitro

These experiments were performed as described earlier [13], but included an additional procedure for detaching surface cells with decreased cohesivity. In brief, 0.2- to 0.5-mm thick slices of plantar stratum corneum were obtained with a skin transplantation knife from under the heels of volunteers with normal skin. After the tissue had been briefly soaked in phosphate buffered saline, loosely attached surface cells were scraped off under a dissection microscope. Tissue cylinders with a defined area, with regard to the surface that had been turned outwards in vivo, were prepared with a 2-mm biopsy punch. Cylinders were incubated at 37°C in 1.5 ml Eppendorf tubes (1–3 cylinders in each tube) with 1 ml 0.1 *M Tris*-HCl pH 8, 0.1% sodium azide, with additions as specified in the legends to the figures and tables. Chymostatin and leupeptin were added as solutions in dimethyl sulfoxide.

Detachment of surface cells with decreased cohesiveness

Vortex agitation. This method was effective only when the tissue had been incubated in the presence of ethylenediaminetetraacetate (EDTA). It was performed in a standardized manner as described earlier [13].

Brush method. Only very small amounts of cells could be released by vortex agitation of tissue that had been incubated in the absence of EDTA. When such tissue was manipulated under a dissection

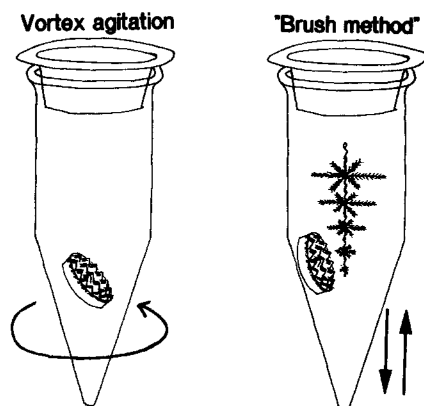

Fig. 1. Schematic outline of the two methods used to detach cells. (See 'Materials and methods' for details)

Table 1. The efficiency of vortex agitation and the "brush method" in releasing cells from plantar striatum corneum incubated in the absence and presence of EDTA

	Released cells	
	Vortex agitation	"Brush method"
+EDTA	18.1 ± 5.3	13.0 ± 5.9
−EDTA	0.8 ± 1.6	19.2 ± 9.9

Tissue cylinders (2 mm) were incubated in tubes containing media with and without 5 m*M* EDTA. After 12 h incubation the tubes were agitated in a vortex mixer for 10 s at maximum effect, the remaining tissue cylinders were then transferred to test tubes with fresh media and treated with "brush method" (see „Materials and methods"). Results are given as µg protein per square mm tissue. Each given value represents mean ± SD for nine tubes, one tissue cylinder in each tube

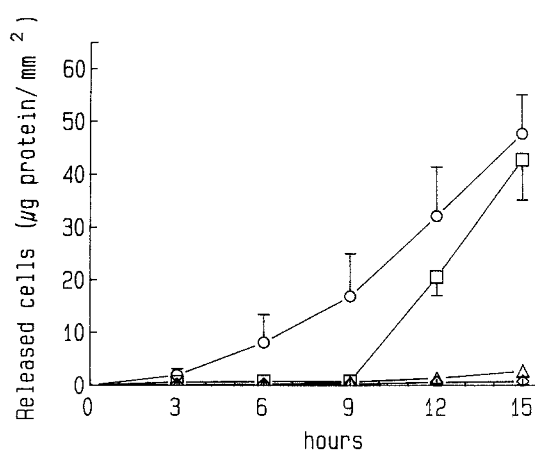

Fig. 2. The effect of EDTA on cell release from plantar stratum corneum in vitro. Three 2-mm tissue cylinders were incubated in each tube. The tubes were agitated in a vortex mixer every 3 h, released cells were collected, remaining tissue cylinders were transferred to tubes with fresh medium and the incubations continued. Results are presented as cumulative amounts of released cells, mean and SD for four tubes each containing three tissue cylinders. *Circles*, 5 m*M* EDTA present; *diamonds*, no EDTA present during the experiment; *squares*, 5 m*M* EDTA added to the incubation medium after 9 h incubation without EDTA; *triangles*, 5 m*M* EDTA + 15 µ*M* aprotinin added to the incubation medium after 9 h incubation without EDTA

microscope, however, it was noted that aggregates of cells could be removed from the surface that had been turned outwards in vivo by gentle scraping, but not from the other surfaces. Therefore, a standardized method to detach cells which was more efficient than vortex agitation was developed. After the incubation, a 15 × 6 × 2 mm conical interdental brush made of nylon with a 10-mm shaft of steel wire (Butler, Chicago, Ill., USA, product no. 614P) was placed in each tube containing tissue. The tubes were stoppered and packed in 50 ml stoppered centrifuge tubes. The centrifuge tubes, filled with 1.5 ml Eppendorf tubes, were then agitated using a Gallenkamp flask shaker (Gallenkamp, Loughborough, UK) at maximum effect for 15 min. Phase contrast microscopy revealed that this treatment released intact cells and aggregates of intact cells. When examined using light microscopy, tissue cylinders that had been treated with this method appeared to have lost material only from the surface that had faced outwards in vivo, i.e., all cut surfaces remained intact. The two methods to release cells are schematically presented in Fig. 1.

Quantification of released cells

After the remaining tissue cylinders had been removed from the incubation media, the released cells were collected by centrifugation, washed in phosphate buffered saline, and extracted with 1 *M* sodium hydroxide [13]. The amounts of alkali soluble protein were measured as described earlier [13] according to Lowry et al. [12]. The results are expressed as µg protein per square mm surface area.

Results

When a piece of plantar stratum corneum is incubated in 0.1 *M Tris*-HCl pH 8 there is a decrease in cohesion between cells close to the surface that had been facing outwards in vivo. Two methods were used to release cells with decreased cohesion from intact tissue (Fig. 1). Using the gentlest of these methods, vortex agitation, only very loosely attached cells could be released. As shown in Table 1 this method could release cells only from tissue that had been incubated in the presence of EDTA. With the "brush method" on the other hand, cells could be released also from tissue incubated without EDTA (Table 1).

As shown below these two methods could be used to demonstrate that there are two separate proteolytic steps involved in the process of cell shedding from plantar stratum corneum in vitro. The first step, which leads to a moderate decrease in surface cell cohesion, takes place irrespective of whether EDTA is added to the incubation medium. The second step, which leads to a further decrease in cell cohesion, occurs only in the presence of EDTA.

Figure 2 shows cumulative amounts of cells released by vortex agitation after incubation of tissue pieces in the presence and absence of EDTA and when EDTA was added to the system after 9 h "preincubation". Significant amounts of cells were released only in the presence of EDTA. The rate of cell release increased drastically during the first few hours after the addition of EDTA to tissue that had been "preincubated" at 37°C in the absence of EDTA. The total amount of released cells at the end of the experiment (after 15 h incubation) was nearly the

Table 2. Effect of aprotinin, chymostatin, and zinc ion on the "first proteolytic step" in cell release from planta stratum corneum in vitro

	Released cells		
Aprotinin	0	0.8 μM	15 μM
Exp 1	39.0 ± 3.4	7.1 ± 3.9 (18%)	0.7 ± 0.7 (2%)
Exp 2	32.8 ± 9.5	18.9 ± 3.9 (58%)	0.9 ± 0.7 (3%)
Exp 3	44.5 ± 6.4	9.2 ± 7.2 (21%)	0.7 ± 0.9 (2%)
Chymostatin	0	32 μM	160 μM
Exp 1	15.7 ± 5.9	6.0 ± 5.3 (38%)	0.5 ± 0.9 (3%)
Exp 2	13.4 ± 5.1	3.1 ± 2.2 (23%)	0.2 ± 0.2 (2%)
Exp 3	11.5 ± 3.3	7.5 ± 2.7 (65%)	2.2 ± 2.0 (19%)
Zinc sulfate	0	0.1 mM	1 mM
Exp 1	31.3 ± 5.4	25.1 ± 4.3 (80%)	0.7 ± 0.9 (2%)
Exp 2	24.2 ± 5.0	16.8 ± 9.9 (69%)	1.1 ± 0.7 (5%)
Exp 3	42.4 ± 6.1	8.0 ± 5.9 (19%)	4.9 ± 2.8 (12%)

In each experiment 2-mm tissue cylinders from one healthy volunteer were incubated for 12 h in the absence or presence of inhibitors at concentrations as indicated. For each inhibitor experiments were carried out with tissue from three donors. The results are expressed as µg protein per square mm tissue, in parenthesis as percent of controls (i.e., the amount of cells released in the absence of inhibitor). Each value represents mean ± SD; $n = 3$, two cylinders in each tube (aprotinin) or $n = 5$, one cylinder in each tube (zinc and chymostatin). Cells were released by the brush method. In the experiments with chymostatin all incubation media contained 5% (v/v) DMSO

Table 3. The effect of chymostatin on "the second proteolytic step" in cell release from plantar stratum corneum in vitro

Incubation medium	Released cells after 12 h (µg protein/mm^2)
Buffer without EDTA	0.4 ± 0.2
Buffer without EDTA for 8 h, then buffer with EDTA	34.2 ± 3.3
Buffer without EDTA for 8 h, then buffer with EDTA and chymostatin	0.8 ± 0.5
Buffer with EDTA	31.8 ± 3.2

In this experiment 2-mm tissue cylinders were incubated in media as indicated, concentration of chymostatin 160 μM and of EDTA 5 mM. All incubation media contained 1% dimethyl sulfoxide. All media were changed after 8 h. Cells were released after 12 h by vortex agitation. Each given value represents mean ± SD for four tubes (one tissue cylinder in each tube)

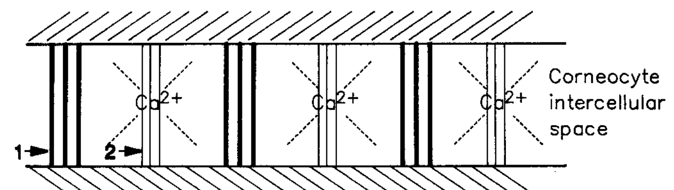

Fig. 3. Proposed model of structures responsible for intercellular cohesion in plantar stratum corneum. *1* Cohesive structure involving proteins that are susceptible to proteolysis irrespective of the presence of calcium. *2* Cohesive structures involving proteins stabilized against proteolysis by calcium

same when EDTA was added after 9 h "preincubation" as when it was present from the start of the experiment. When EDTA was added to "preincubated" tissue together with aprotinin – an inhibitor of serine proteinases [11] – the increase in cell release was minimal (Fig. 2).

Table 2 shows the effects of inhibitors when the "brush method" was used to release cells from tissue that had been incubated without EDTA. Aprotinin was an efficient inhibitor also in these experiments as was chymostatin (an inhibitor of chymotrypsin-like proteinases [17]) and zinc ion.

Table 3 shows that chymostatin inhibited also the second proteolytic step. Leupeptin, an inhibitor of many trypsin-like proteinases [17], had no significant effect on any of the two proteolytic steps (results not shown).

Discussion

It is unlikely that there is any de novo production of cohesive protein structures during in vitro incubations of plantar stratum corneum. It can therefore be assumed that the protein structures that contribute to cell cohesion in the in vitro system should have the same function in vivo. Correspondingly, as proteolysis is essential for cell shedding in vitro, the same should be true in vivo.

In this work we have shown that the unipolar cell shedding from plantar stratum corneum in vitro can be divided into two separate steps, both dependent on the activity of a proteolytic enzyme. The first step takes place in the absence of EDTA and can be detected only if a rather harsh method is used to detach surface cells. If EDTA is present in the incubation system, the decrease in surface cell cohesion reaches a point where the cells can be dissociated very easily from the rest of the tissue pieces, i.e., by simple agitation. Using this method essentially no cells can be released from tissue incubated without EDTA. This means that the second proteolytic step appears to be dependent on the presence of EDTA.

One possible explanation for the results obtained may be that two separate enzymes are responsible for the two proteolytic steps and that the second step is mediated by an enzyme inhibited by metal ions present in the tissue. An alternative, possibly more likely explanation may be that the protein structures that are degraded in the second step are protected against proteolysis by calcium ions. Cell adhesion molecules with this property have been described [5, 15, 16]. Moreover, Bissett et al. [1] have recently presented evidence that calcium and proteins are involved in cell cohesion in nonpalmo-plantar stratum corneum [1]. A proposed model of the different types of cohesive structures is outlined in Fig. 3.

In two recent papers we have presented evidence that cell shedding from plantar stratum corneum in vivo [6] as well as in vitro [14] is accompanied by a degradation of

the desmosomal glycoprotein desmoglein I. These results were seen as evidence that desmosomes play an important role in plantar stratum corneum cell cohesion, and that desmosomal degradation may be an important step in desquamation. We have also observed that the degradation of desmoglein I during in vitro cell shedding is not dependent on the presence of EDTA [14]. Since apparently intact desmoglein I is present throughout plantar stratum corneum except in the outermost, partially desquamated cell layers [6], this protein appears to be a likely candidate for being a protein that is degraded in the first step, i.e., in the absence of EDTA. This hypothesis is supported by the effects of various inhibitors. Aprotinin, chymostatin, and zinc sulfate, but not leupeptin inhibited the apparent degradation of desmoglein I [14]. This inhibitor profile is the same as that found for the first proteolytic step described in this paper.

The other major desmosomal glycoprotein, desmoglein II, has been reported to be protected by calcium against proteolysis [7]. Desmoglein II could thus be the protein that is degraded in the second step in our experiments, i.e., in the presence of EDTA. Evidence has been obtained, however, that this protein is partially degraded at a site close to the transition between viable and cornified epidermal layers [9, 10]. It is not known whether the resulting fragments retain any function as regards cell adhesion. Nondesmosomal glycoproteins with possible cohesive functions in the stratum corneum [2–4] should also be considered in this context.

The experiments with the various proteinase inhibitors suggest that a chymotrypsin-like serine proteinase is responsible for the degradation of intercellular cohesive structures during cell shedding from plantar stratum corneum in vitro. This was true for both proteolytic steps involved. In this context it may be of importance that inhibitors of trypsin- but not of chymotrypsin-like enzymes have been reported to be present in the stratum corneum [8]. Further studies of the involved enzyme are in progress.

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