Evidence that Cell Shedding from Plantar Stratum Corneum In Vitro Involves Endogenous Proteolysis of the Desmosomal Protein Desmoglein I

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We have recently described a process leading to a unipolar cell shedding from pieces of plantar stratum corneum incubated in vitro, which seems to be dependent on the activity of a serine proteinase. This process has been studied further. Electron microscopy studies suggest that cell dissociation is preceded by a degradation of the intercellular parts of desmosomes. An antiserum was raised against the transmembrane protein desmoglein I (DG I) of bovine desmosomes. In extracts of layers of plantar stratum corneum with strong intercellular cohesion, this antiserum reacted with a protein of the same apparent molecular weight as bovine DG I. In dissociated cells this DG I-like protein could not be detected; instead components with molecular weights lower than DG I which reacted with the antiserum were found. During incubation of pieces of plantar stratum corneum, under conditions leading to unipolar cell shedding, there was a progressive decrease in the amounts of the DG I-like protein, and the appearance of the lower molecular weight components with DG I-like immunoactivity. This apparent degradation of the DG I-like protein was inhibited by aprotinin, chymostatin, and zinc ion, but not by leupeptin. The results suggest that proteolytic degradation of desmosomes may be an important part of the process leading to cell dissociation in plantar stratum corneum in vitro, and that desmosomes may play an important role in plantar stratum corneum cell cohesion. J Invest Dermatol 94:216–220, 1990

Epidermal steady state is dependent on a well-regulated desquamation that balances cell proliferation in the basal layer. The basal mechanisms of desquamation are largely unknown. We have recently reported that trypsin digestion of deeper layers of plantar stratum corneum leads to cell dissociation [1], which indicates that intercellular cohesion in this tissue is mediated by protein structures. In the electron microscope the trypsin-induced cell dissociation appears to be accompanied by a degradation of intercellular desmosomal plates [1]. We have also described an in vitro system in which a unipolar cell dissociation, imitating desquamation, occurs in pieces of plantar stratum corneum [2]. This process appears to be dependent on the activity of a serine proteinase. Even if the described in vitro system is highly artificial, studies on it may give important information. The protein structures that have to be degraded for cell dissociation to occur in vitro should be present also in palmo-plantar stratum corneum in vivo.

In the present study we have used immunochemical methods and electron microscopy to elucidate whether the in vitro cell shedding involves the degradation of intercellular parts of desmosomes. For the immunochemical experiments we have chosen to study the desmosomal glycoprotein desmoglein I (DG I). This is a transmembrane protein with its intracellular parts anchored in the desmosomal plaque. The extracellular parts are major components of the intercellular desmosomal plate and thus most likely of crucial importance for the cohesive function of desmosomes [3–6].

MATERIALS AND METHODS

Aprotinin (Trasylol®), chymostatin, and leupeptin were obtained from Boehringer Mannheim, W. Germany.

The monoclonal antibody DG 3.10, reacting with DG I [6,7], was a generous gift from Dr. M. Schmelz, Heidelberg. The monoclonal mouse antibody TS4 (cytokeratin antibody with broad specificity) was kindly provided by Dr. T. Stigbrand, Umeå.

Rabbit Antiserum Against DG I (Rabbit Anti-DG I) Whole desmosomes from bovine muzzles were prepared according to Gorbisky et al [8]. The desmosomal proteins were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) in 1.5-mm-thick 7.5% gels according to Laemmli [9]. DG I was identified as the third major Coomassie blue-stained band from the top of the separation gel with an apparent molecular weight of approximately 160 kDa. Gel slices containing approximately 50 μg DG I (approximately 50 × 2 × 1.5mm) were neutralized in phosphate buffered saline, homogenized in a glass homogenizer, suspended in an equal volume of Freund's complete adjuvant and injected subcutaneously at multiple sites of the back of a rabbit. The same amount of material in incomplete Freund's adjuvant was injected after 4 and 8 weeks. The rabbit was bled before the first immunization and after 10 weeks.

In Vitro Incubations of Plantar Stratum Corneum The principle of these experiments has recently been described [2]. Slices of plantar stratum corneum, 0.2–0.5 mm thick, from under the heels of volunteers with normal skin, were obtained using a skin trans-
planted knife. Loose surface cells were scraped off with a scalpel. The tissue was then appropriately divided into smaller pieces and incubated at 37°C in media as specified in the figure texts. All media contained 0.1% sodium azide.

Electron Microscopy After incubation as above, 0.5 × 0.5 mm tissue pieces were prepared for electron microscopy as previously described [1]. Thin sections, contrasted with lead citrate and uranyl acetate, were viewed in a Jem 1200 EX transmission electron microscope at 80 kV.

Electrophoresis and Immunoblotting Tissues were extracted in a buffer containing 0.1 M Tris-HCl pH 9, 9 M urea, 2% SDS, and 1% (v/v) mercaptoethanol, 1 ml buffer per 20 mg tissue (weighed before hydration), for 15 h at 37°C. The extracts, still containing unsolubilized cell remnants, were prepared for one-dimensional SDS-PAGE by mixing them with 2/3 volume of a modified sample buffer (Laemmli’s sample buffer [9] with 2.5 times higher concentration of solutes), heated on a boiling water bath for 15 min, and centrifuged at 6,000 g for 5 min. This extraction procedure was chosen in order to obtain optimal extraction of stratum corneum proteins. The initial extraction resulted in a disintegration of the stratum corneum to single cell remnants, which could not be achieved with an extraction buffer containing only SDS and mercaptoethanol.

Analytical SDS-PAGE in 7.5% gels according to Laemmli [9], was performed with the BioRad Minigel equipment (BioRad, Richmond, CA) at 200 V for 45–60 min. Identical gels were used in all experiments for Coomassie Blue-staining and immunoblotting. Two-dimensional electrophoresis of bovine desmosomal proteins with nonequilibrium pH-gradient gel electrophoresis (NEPHGE) in the first dimension was performed according to O’Farrell et al [10]. For this purpose purified bovine desmosomes were extracted with the urea-SDS buffer as above. Before electrophoresis the nonionic detergent NP 40 was added to the extracts to final concentration 5% (v/v).

Electrophoretic transfer of proteins from electrophoresis gels to nitrocellulose membranes [11] was carried out in a Semi-Dry Electrophotter A (Ancos, Olsytkke, Denmark), following the instruction supplied by the manufacturer. Immunostaining was performed with Tween 20 as blocking agent [12]. Rabbit anti-DG I was used in dilution 1/1000. Alkaline phosphatase- (AP-) conjugated goat anti-rabbit or goat anti-mouse immunoglobulins (BioRad) in dilution 1/3000 were used as second antibodies. AP-conjugates were detected with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as substrates [13] (both substrates from BioRad).

Densitometric scanning of immunoblots was performed in a Shimadzu dual wave-length flying-spot scanner CS-9000 (Shimadzu, Kyoto, Japan) with reflected light at 560 nm in the zigzag mode.

RESULTS

Characterization of Anti-DG I The rabbit antiserum detected a component in viable plantar epidermis as well as in plantar stratum corneum with molecular weight approximately 160 kDa (Fig 1). On two-dimensional immunoblots of extracts of bovine desmosomes the antiserum produced the same staining pattern as a well characterized mouse monoclonal DG I-antibody [6,7] (Fig 2). On one-dimensional immunoblots of bovine desmosomal proteins anti-DG I stained only the 160 kDa periodic acid Schiff-positive component used for immunization. Desmoplakins, desmoglein II, or cytokeratins were not stained (not shown).

Plantar Stratum Corneum in Vitro A horizontal slice of plantar stratum corneum can be divided into two functionally different parts, one superficial part with cells that can be considered functionally desquamated, and one deeper part with strong intercellular cohesion. The different parts can be separated by means of moderate scraping with a scalpel which detaches loosely attached surface cells. The procedure is facilitated if the tissue pieces are briefly soaked in buffer before scraping. When a piece of plantar stratum corneum from which loose surface cells have been removed is incubated in a simple buffer (e.g. 0.1 M Tris-HCl pH 8), a continuous decrease in intercellular cohesion takes place at the surface that had faced outwards in vivo. This leads to cell dissociation at this surface. This process is dependent on the activity of a serine protease. If the incubation is performed in the presence of EDTA, the dissociated cells can be separated from the rest of the tissue piece by vigorous agitation [2].

Electron Microscopy Figure 3 shows an electron micrograph of the intercellular space between two partially dissociated cells in a piece of plantar stratum corneum that had been incubated in vitro under conditions where cell dissociation occurs. At locations where the two cells are still in close contact (1 in Fig 3) the intercellular desmosomal plates have the homogeneous, electron-dense appearance typical of stratum corneum desmosomes [14]. As a first sign of
cell dissociation there is a loss of electron density in the lateral parts of the desmosomal plates, and the intercellular distance appears to be slightly increased (2 in Fig 3). In the widening intercellular space the desmosomal plates become more electron-lucent and eventually disappear (3 in Fig 3). At other places (not shown) fragmented desmosomal plates, which had been detached from one or both cells could be found between dissociating cells.

**Immunohistochemical Analysis with Anti-DG I of Plantar Stratum Corneum Incubated in Vitro** After incubation of plantar stratum corneum as described above, extracts of dissociated cells and still coherent tissue were subjected to SDS-PAGE. Separated proteins were transferred to nitrocellulose membranes and reacted with anti-DG I. Bound antibodies were detected with AP-conjugated second antibodies. The results are shown in Fig 4A (Coomassie blue-stained gel) and Fig 4B (immunoblot). In extracts of coherent tissue, a component with a molecular weight around 160 kDa (the same molecular weight as bovine DG I and the component in viable human epidermis reacting with anti-DG I, see Fig 1) reacted with anti-DG I (Fig 4B, lanes 1–3). This component could not be detected in extracts of dissociated cells (Fig 4B, lanes 4 and 5). Instead, these extracts contained two components with apparent molecular weights of 95 and 80 kDa with DG I-like immunoreactivity. These components could not be found in extracts of coherent tissue (Fig 4B, lanes 1–3).

The same type of analysis was performed on extracts of whole stratum corneum pieces (coherent and dissociated cells were not separated after the incubations) that had been incubated for various periods of time at 37°C in the absence and presence of the serine proteinase inhibitor aprotinin. The results are shown in Fig 5A (Coomassie blue-stained gel), Fig 5B (immunoblot), and Fig 6 (denitometric scannings of the immunoblot shown in Fig 5B). In the absence of aprotinin (Fig 5B, lanes 1–4 and Fig 6A) there was a gradual decrease in the amounts of the 160 kDa component reacting with anti-DG I. After 24 h of incubation this component could no longer be detected. (After 24 h of incubation in the absence of aprotinin, essentially all tissue consisted of dissociated cells.) Anti-DG I reactive components with molecular weights 95 and 80 kDa, absent at 0 h, could be detected after 6 h and then throughout the experiment. In the presence of aprotinin (Fig 5B, lanes 5–7 and Fig 6B) significant amounts of the 160 kDa component could also be

![Figure 3. Electron micrograph of the intercellular space between two dissociating cells of plantar stratum corneum incubated in vitro. The tissue, prepared as described under Materials and Methods, was incubated for 15 h at 37°C in phosphate-buffered saline before it was prepared for electron microscopy. 1, 2, and 3 indicate areas shown in higher magnification in the lower part of the figure. 1 = apparently intact desmosomal plate, 2 = desmosomal plate with decreased electron density in its lateral parts, 3 = degraded desmosomal plate and increased intercellular distance. (The desmosome at 3 can still be identified due to the decreased electron density in the structure continuous with the cell envelope, cf 1 and 2 in the figure.) Bars: 100 nm.](image-url)

![Figure 4. Anti-DG I reactive components in coherent plantar stratum corneum and in dissociated cells. A: Coomassie blue-stained SDS-PAGE of extracts of plantar stratum corneum. B: Immunoblot with anti-DG I as first antibody. 1–3: Coherent plantar stratum corneum, undiluted (1), diluted 1/3 (2), diluted 1/9 (3). 4–5: Dissociated cells, undiluted (4), diluted 1/3 (5). A slice of plantar stratum corneum (0.5 × 5 × 5 mm) prepared as described under Materials and Methods was incubated for 15 h in 0.1 M Tris-HCl pH 8.5 mM EDTA. The cells that had dissociated during the incubation were released from the coherent tissue piece by vigorous shaking and collected by centrifugation. The amounts of extraction buffer (see Materials and Methods) added were adjusted to give approximately the same protein concentrations (as estimated by visual inspection of Coomassie blue-stained SDS-PAGE gels) in the two extracts. Several dilutions of the extracts were run to make possible a semi-quantitative comparison of the quantities of the different anti-DG I reactive components in coherent stratum corneum and dissociated cells.](image-url)
Figure 5. The effect of aprotinin on the degradation of a DG I-like component in plantar stratum corneum during in vitro incubation. A: Coomassie blue-stained SDS-PAGE. B: Immunoblot with anti-DG I as first antibody. Two-mm cylinders obtained with a biopsy punch from slices of plantar stratum corneum were divided into four equal-sized segments, which were randomly distributed to seven test tubes. The total amount of tissue in each tube was 3.0 mg. Incubations were performed in 1 ml of 0.1 M Tris-HCl pH 8, 5 mM EDTA in the absence and presence of 15 \( \mu \)M aprotinin. At the end of the incubation the media were removed and extraction buffer added (see Materials and Methods). 1–4, No aprotinin added; duration of incubations 0, 6, 12, and 24 h respectively. 5–7, Aprotinin present; duration of incubations 6, 12, and 24 h respectively.

Detected after 24 h (only a very small quantity of cells could be released by agitation of tissue incubated for 24 h in the presence of aprotinin). With aprotinin present there was also some production of the smaller components with DG I-like immunoreactivity, but there appeared to be an accumulation of the 95 kDa component as compared to the 80 kDa component.

In addition to aprotinin, zinc ion (Fig 7A) and chymostatin, but not leupeptin (Fig 7B) inhibited the degradation of the DG I-like 160 kDa component during in vitro incubation of plantar stratum corneum.

**DISCUSSION**

Desmosomes serve to create strong mechanical links between cells. They have one intracellular part, the desmosomal plaque, and one extracellular part, the desmosomal plate. At the molecular level the two structures are made up by specific proteins; the desmosomal plaque by desmoplakins, and the desmosomal plate by desmogleins. The latter proteins are transmembrane glycoproteins, anchored in the desmosomal plaque. Inside the cell the desmosomal plaque interacts with the keratin filaments. Thus the mechanical properties of individual cells are transferred to the tissue as a functioning unit via the desmosomes. The structure named the desmosomal plate results from the interaction between the extracellular, carbohydate-containing parts of desmogleins from two opposing cells (for a review of desmosomal structures see[3–5]). Thus a degradation of these parts of the desmogleins would lead to a loss of the cohesive capacity of desmosomes. At the ultrastructural level this would correspond to a deterioration of desmosomal plates.

Palmo-plantar stratum corneum is a tissue subjected to considerable mechanical stress. Its building blocks, the cornified cells with their tightly packed keratin filaments surrounded by a cross-linked protein envelope [14], are well adapted to their function. The entire nature of their surface serves to increase the intercelular contact area. In cohesive layers of palmo-plantar stratum corneum a considerable fraction of the intercellular space is occupied by desmosomal plates [15,16]. It seems reasonable to assume that these desmosomal junctions have an important function in maintaining structural integrity. This would imply a central role for mechanisms responsible for the degradation of desmosomes in desquamation.

In this report ultrastructural and immunohistochemical evidence was obtained that an in vitro process leading to a unipolar cell dissociation in plantar stratum corneum involves a degradation of intercellular parts of desmosomes. We have shown that there are ultrastructural changes in the desmosomal plates between dissociating cells similar to those observed when cell dissociation is induced in deeper layers of plantar stratum corneum by trypsin treatment [1]. We have also shown that during cell dissociation there is a progressive decrease of a protein with the properties of DG I in pieces of plantar stratum corneum, and the appearance of what may be degradation products of DG I. This apparent degradation of DG I was inhibited by aprotinin, an inhibitor of serine proteinases [17]. It was also inhibited by chymostatin, an inhibitor of chymotrypsin-like serine proteinases [18], and by zinc ions. In contrast, the degradation of DG I was not inhibited by leupeptin, an inhibitor of trypsin-like serine proteinases [18]. This inhibitor profile is the same as we have observed for the cell dissociation process in plantar stratum corneum.
in vitro, and for a protease associated with plantar stratum corneum cells (unpublished observations).

An obvious question concerns the relevance of the results presented herein for the in vivo situation. During the experiments the tissue becomes extensively hydrated, and protease inhibitors and other modulating factors may be extracted into the incubation medium. The ionic strength and composition as well as the pH of the incubation medium certainly deviate from physiological conditions. We find it reasonable to assume, however, that protein structures contributing to cell cohesion in the in vitro system should have a similar function in vivo. Thus, if cell dissociation in vitro requires a degradation of the intercellular parts of desmosomes, this suggests that desmosomes are important for cell cohesion in vivo, and that their degradation may be an important part of the desquamation process. We have recently presented evidence supporting these ideas [19]. Material reacting with anti-DG I (the antisem used in this study) was analyzed in serial horizontal sections of intact, non-hydrated plantar epidermis. A component with the properties of DG I was present in all epidermal layers, including the stratum corneum, except for the outermost, "functionally desquamated" cell layers. Instead a putative degradation product of DG I with molecular weight 80 kDa was found in these loosely attached surface layers [19].

It seems likely that there are protein structures in addition to DG I that may be involved in plantar stratum corneum cell cohesion. The degradation of DG I in vitro took place both in the absence and presence of EDTA in the incubation medium (cf Figs 4-6 and Fig 7). In the absence of EDTA there is a decrease in cohesion between surface cells, but this decrease is not sufficient to make possible cell release by simple agitation [2]. The effect of EDTA does not seem to be related to a simple breaking of divalent ion bridges, since cell dissociation after the addition of EDTA to tissue that has been preincubated without chelator is inhibited by protease inhibitors such as aprolin (unpublished observations). Thus the cell shedding from plantar stratum corneum in vitro seems to involve at least two proteolytic steps, one which occurs in the absence of EDTA and which may involve the degradation of DG I, and one step that may be inhibited in the presence of divalent ions. The effect of divalent ions may be to make proteins unsusceptible to proteolysis or to inhibit the involved proteolytic enzymes. The other major transmembrane glycoprotein, desmolgin II, has been reported to be protected against proteolysis by calcium ions [20]. However, evidence that this protein may be degraded at a site close to the transition between viable and cornified layers [21,22] seems to make an important role for it in stratum corneum cell cohesion uncertain. The possible cohesive function of non-desmosomal proteins [23] should also be considered.

We are indebted to Astrid Lundgren for skilful technical assistance.

REFERENCES


