# Hailey-Hailey Disease Keratinocytes: Normal Assembly of Cell-Cell Junctions *In Vitro*

Jerry E. Cooley,¶† Robert A. Briggaman,† Donna J. Cronce,† Albert J. Banes,* and Edward J. O'Keefe†
†Departments of Dermatology and *Orthopaedic Surgery, University of North Carolina, Chapel Hill, North Carolina, U.S.A.

**The blisters in the inherited disorder, Hailey-Hailey disease, may be caused by defective epidermal junctional complexes. We evaluated these structural complexes *in vivo* and *in vitro*. We induced a vesicular lesion in the apparently normal skin of a patient with Hailey-Hailey disease and studied a biopsy of this lesion by transmission electron microscopy. To determine whether acantholysis was related to a defect in the number or assembly of intercellular junctions, we cultured Hailey-Hailey disease keratinocytes in medium containing 0.1 mM $Ca^{2+}$ and increased the $[Ca^{2+}]$ to 1.1 mM in order to induce assembly of cell-cell junctions. Keratinocytes were examined by double immunofluorescence with antibodies to the desmosome protein, desmoplakin, and the adherens junction protein, vinculin, at intervals after the increase in $[Ca^{2+}]$. Characteristic Hailey-Hailey disease histopathology was observed by electron microscopy of the patient's skin after trauma, but we found no splitting of desmosomes. Based on the location, intensity, and rate of change of immunofluorescent staining, Hailey-Hailey and normal keratinocytes did not differ in their ability to assemble desmosomes and adherens junctions. Furthermore, we observed no significant morphologic differences between normal and Hailey-Hailey keratinocytes cultured in low and high $[Ca^{2+}]$-containing media; Hailey-Hailey cells contained abundant normal-appearing desmosomes in 1.1 mM $[Ca^{2+}]$. Since Hailey-Hailey disease keratinocytes can assemble normal-appearing adherens junctions and desmosomes *in vitro*, the functional defect may not lie in assembly of cell-cell adhering junctions, or additional perturbation may be required to expose the defect.** ***Key words: cadherins/vinculin/desmoplakin/adherens junctions/desmosomes. J Invest Dermatol 107:877–881, 1996***

It has been proposed that defective junctional complexes of Hailey-Hailey disease (HHD) (Hailey and Hailey, 1939) keratinocytes are the cause of a structurally weak epidermis (Wilgram *et al*, 1962; Gottlieb *et al*, 1970). This hypothesis is based on two characteristic features of HHD: (i) skin lesions occur in areas subject to trauma, and (ii) affected epithelium shows acantholysis without rupture of keratinocytes. Immunohistochemical studies using antibodies to different proteins in this junctional complex, including desmoplakin I and II, desmoglein I and III (pemphigus vulgaris antigen), desmocollins (Buxton *et al*, 1993), as well as keratin and actin (Inohara *et al*, 1990; Burge and Garrod, 1991; Bergman *et al*, 1992; Burge and Schomberg, 1992), have failed to identify an abnormality in the unaffected epidermis of HHD patients. Abnormal staining with these antibodies has been demonstrated in involved skin, but the changes have been thought to be secondary to the acantholytic process. Ultrastructural studies of HHD lesions have shown loss of desmosomal contacts, widening of intercellular spaces, retraction of tonofilaments, and characteristic bizarre membrane microvilli (Wilgram *et al*, 1962; Gottlieb *et al*, 1970), suggesting a defect in cell-cell attachment, but abnormalities in nonlesional HHD skin have not been described.

The assembly of desmosomes can be studied in keratinocytes *in vitro* by raising the calcium concentration of the culture medium (Hennings and Holbrook, 1983; Watt *et al*, 1984; Jones and Goldman, 1985; Green *et al*, 1987; O'Keefe *et al*, 1987). Formation of cell-cell junctions by human keratinocytes is inhibited in medium containing low $[Ca^{2+}]$ (0.1 mM), and keratin filaments are found in bundles in the vicinity of the nucleus under these conditions. Incubation of the cells in medium containing physiologic $[Ca^{2+}]$ causes rapid assembly of desmosomes. Keratin filament bundles become attached to desmosomal plaques and are found distributed widely in the cytoplasm. Over the course of 24 h, cells aggregate, stratify, and eventually form a multilayered epithelium, and markers for terminal keratinocyte differentiation develop. This "$Ca^{2+}$ switch" also stimulates the assembly of adherens junctions, another adhering type of intercellular junction of epithelial cells, over a similar or shorter period of time (Green *et al*, 1987; O'Keefe *et al*, 1987). The *in vitro* assembly of junctions in HHD, a disease of cell-cell adhesion, has not been studied.

Shortly after HHD was first described, it became apparent that lesions could be experimentally induced by several methods, including trauma (Frank and Rein, 1942; Chorzelski, 1962). De Dobbeleer and Achten (1979) found that HHD epidermis under an occlusive dressing underwent acantholysis and that acantholytic cells appeared to have half-, or "split" desmosomes, suggesting that there was a defect in the intercellular core of desmosomes. Several reports have also suggested that the HHD phenotype can be recreated *in vitro*. Outgrown HHD epidermal cells from explant

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Reprint requests to: Edward O'Keefe, M.D., 3100 Thurston Building, CB 7287, Department of Dermatology, University of North Carolina, Chapel Hill, NC 27599.

Abbreviations: HHD, Hailey-Hailey disease.

¶ Current address: Department of Dermatology, University of Maryland School of Medicine, 405 W. Redwood St., 6th floor, Baltimore, MD 21201.

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culture displayed spontaneous cell-cell dissociation (Ishibashi and Kukita, 1983; Burge *et al*, 1991). Investigators studying skin organ culture of nonlesional HHD skin observed intraepidermal acantholysis (Ikeda and Ogawa, 1991). Others have reported the reproduction of characteristic histopathology *in vitro* by growing lesional (De Dobbeleer *et al*, 1989) or nonlesional (Regnier *et al*, 1990) keratinocytes on dead de-epidermized dermis. Even nonlesional HHD keratinocytes cultured on plastic dishes have been reported to be vacuolated and dyskeratotic (Regnier *et al*, 1990).

We have studied the assembly of junctions and morphology of cultured HHD keratinocytes in an attempt to identify an abnormality that might be related to the HHD phenotype.

## MATERIALS AND METHODS

**Materials** Affinity-purified antibodies to desmoplakin have been characterized previously (O'Keefe *et al*, 1989; Hamilton *et al*, 1991, 1992). Monoclonal antibody to chicken vinculin was obtained from ICN (Irvine, CA). Fluorophore-labeled second antibodies were from Kirkegaard and Perry (Gaithersburg, MD). Materials used to prepare MCDB 153 medium were from Sigma Chemical Co. (St. Louis, MO). Chemicals were reagent grade or better.

**Patients** Keratinocytes were cultured from one HHD patient and two normal controls. The HHD patient was a 38-y-old female whose diagnosis was based on vesiculobullous lesions with erosions occurring in the axillae, trunk, and submammary region; family history consistent with autosomal dominant inheritance; and biopsy showing intraepidermal acantholysis. The control cells consisted of keratinocytes from 15- and 35-y-old normal males.

**Induction of Lesions** A normal-appearing area of the back of the HHD patient was chosen, and a pencil eraser was rotated on the skin for about 10 half-rotations with light pressure for 5 s. Punch biopsies of this skin and an adjacent area of apparently normal skin were taken under minimal anesthesia and fixed in half-strength Karnovsky's solution. After being rinsed in 0.1 M sodium cacodylate, pH 7.4, the tissue was processed for electron microscopy as described below.

**Keratinocyte Culture** Keratinocytes were obtained and initiated into culture by a modification (O'Keefe *et al*, 1982) of the method of Rheinwald and Green (1977) and then subcultured in the absence of serum in MCDB 153 medium according to Boyce and Ham (1983) with 0.1 mM $Ca^{2+}$ supplemented with hydrocortisone (0.4 $\mu$g per ml), insulin (5 $\mu$g per ml), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), epidermal growth factor (0.1 ng per ml), bovine pituitary extract (140 $\mu$g per ml), and supplemental essential amino acids (histidine, $2 \times 10^{-4}$M; isoleucine, $7.5 \times 10^{-4}$M; methionine, $9 \times 10^{-5}$M; phenylalanine, $9 \times 10^{-5}$M; tryptophan, $4.5 \times 10^{-5}$M; and tyrosine, $7.5 \times 10^{-5}$M). The high $Ca^{2+}$ medium consisted of Ham's F-12:Dulbecco's modified Eagle's medium (1:1) as described below under *Immunofluorescence*.

**Immunofluorescence** Cells were plated on glass coverslips. One group of dishes from each cell strain (HHD patient and controls) was maintained in low $Ca^{2+}$ medium, while those from the other three groups were switched to a higher $Ca^{2+}$ medium at different times prior to fixation (15 min, 1 h, or 4 h). The high $Ca^{2+}$ medium consisted of Ham's F-12: Dulbecco's modified Eagle's medium (1:1) with 10% fetal bovine serum, hydrocortisone (0.4 $\mu$g per ml), insulin (5 $\mu$g per ml), transferrin (5 $\mu$g per ml), epidermal growth factor (10 ng per ml), and cholera toxin (10 ng per ml). All groups were fixed in 3.7% formaldehyde in phosphate-buffered saline, which for the high $Ca^{2+}$ groups contained 0.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$. Cells were extracted in phosphate-buffered saline containing 1% Triton X100 for 5 min at 24°C, washed, and incubated in 2% bovine serum albumin in phosphate buffered saline for 15 min to reduce nonspecific binding. Cells were then incubated overnight at 4°C with affinity-purified anti-desmoplakin antibody at 1:200 dilution as described (Hamilton *et al*, 1992) and then with mouse monoclonal anti-vinculin antibody at 1:50 dilution. Cells were washed in phosphate-buffered saline and incubated for 90 min at 24°C with fluorescein-conjugated goat anti-mouse and rhodamine-conjugated goat anti-rabbit antibodies. Coverslips were mounted on glass slides with polyvinyl alcohol and viewed with a Zeiss epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). Cells were photographed with Kodak Tmax film at an ASA of 400 and developed with Kodak Tmax developer (Kodak Inc., Rochester, NY).

**Application of Mechanical Load to Cells *In Vitro*** We seeded control cells or the patient's cells in Flex I collagen-bonded, rubber-bottomed culture plates (Banes *et al*, 1990) in high-calcium medium. Cells were allowed to attach to the surface and become confluent and stratify for 2 wk. Culture plates were seated on a gasketed baseplate in a $CO_2$ incubator. The volume beneath the culture plates was alternately evacuated and released by regulating solenoid valves with a microprocessor-driven instrument (Flexercell Strain Unit, Flexcell Intl. Corp., Mckeesport PA). Cells were then subjected to a load regimen at 1 Hz and from 10 to 30% elongation. Control and mechanically loaded cultures were then fixed and examined by electron microscopy.

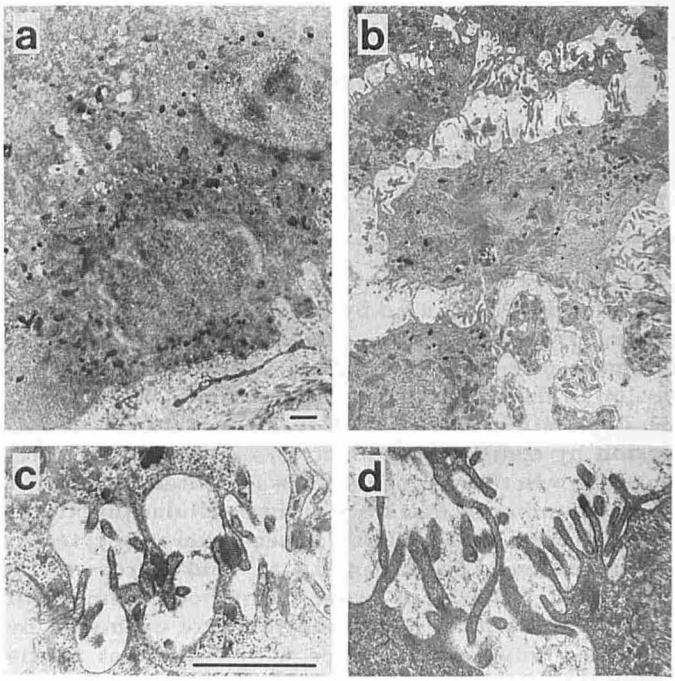


**Figure 1. Acantholysis in HHD after trauma.** A pencil eraser was used to induce acantholysis in clinically normal skin on the lower back of a patient with HHD, and biopsied normal and traumatized skin was examined by electron microscopy. *(a)* Untraumatized skin; *(b)* skin biopsied 2 min after induction of trauma with a pencil eraser; *(c,d)* higher power views of acantholytic epidermis in *(b)*. *Scale bar*, 2 $\mu$m *(a,b)* or 1 $\mu$m *(c,d)*.

## RESULTS

**Blistered Skin Shows Acantholysis and Microvilli** Trauma produced by rubbing the epidermis of the lower back lightly with a pencil eraser for 5 s produced erythema within 1 min and vesiculation within several minutes. Adjacent nonlesional skin revealed normal ultrastructure **(Fig 1*a*)**. Characteristic HHD histopathology was present in the traumatized area after 2 min **(Fig 1*b*)** including acantholysis of suprabasal cells, apparent loss of desmosomes, retraction of keratin filaments from the cell periphery, and dramatic elongation of cell membrane microvilli **(Fig 1*b,c,d*)**. Although the light microscopic appearance of the affected epithelium suggests that acantholysis in HHD is suprabasal, the apical and lateral aspects of the basal layer of keratinocytes were clearly affected by the acantholytic process (not shown). Half-desmosomes, described in studies in which there was a delay between the time of induction of acantholysis and the time of biopsy (De Dobbeleer *et al*, 1989), were not found, either with or without attached tonofilaments.

**Assembly of Junctions Appears Normal in HHD** Normal **(Fig 2*a,c,e*)** and HHD **(Fig 2*b,d,f*)** keratinocytes were examined with antibodies to desmoplakin **(Fig 2)** during assembly of junctions. Cells in low $Ca^{2+}$ (0.1 mM) medium were arranged in loose colonies **(Fig 2*a,b*)**. Staining for desmoplakin was predominantly cytoplasmic, and only minimal intercellular punctate staining characteristic of junctions was noted. Within 15 min after addition of $Ca^{2+}$ (1 mM final concentration), a marked change in morphology was apparent in both types of cells **(Fig 2*c,d*)**. Borders of contigu-

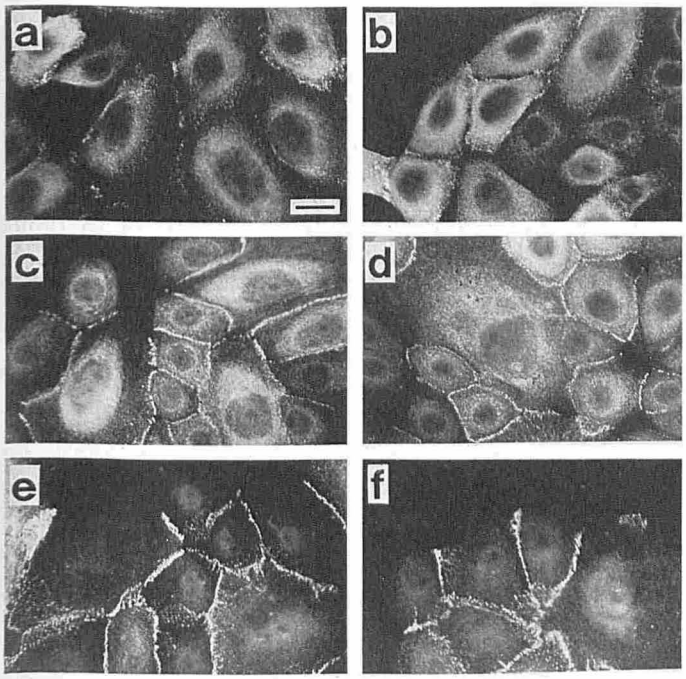


**Figure 2. Assembly of desmosomes is similar in normal and HHD keratinocytes.** Assembly of desmosomes was examined by immunofluorescence with antibody to desmoplakin I/II in normal *(a,c,e)* and HHD cells *(b,d,f)* by culturing cells in low [$Ca^{2+}$] (0.1 mM) and changing the medium to high [$Ca^{2+}$] (1.1 mM). *(a,b)* Low [$Ca^{2+}$]; *(c,d)* 15 min after changing the [$Ca^{2+}$] to 1.1 mM; *(e,f)* 4 h after changing the [$Ca^{2+}$] to 1.1 mM. *Scale bar*, 20 $\mu$m

ous cells appeared to fuse, and staining for desmoplakins decreased in the cytoplasm and increased along regions of cell-cell contact **(Fig 2*c,d*)**, indicating that desmosomes were being assembled from cytosolic components (O'Keefe *et al*, 1987). The decrease in cytoplasmic staining and increase in cell-border staining continued at 1 h (data not shown) and by 4 h, staining was limited to regions of cell–cell contact **(Fig 2*e,f*)**.

Both normal **(Fig 3*a,c*)** and HHD **(Fig 3*b,d*)** keratinocytes were also examined with antibodies to vinculin **(Fig 3)** in order to observe the assembly of adherens junctions. Staining for vinculin in low $Ca^{2+}$ medium identified focal adhesions (cell-substrate attachment plaques) at the ventral aspect of the cell **(Fig 3*a,b*)**, but no

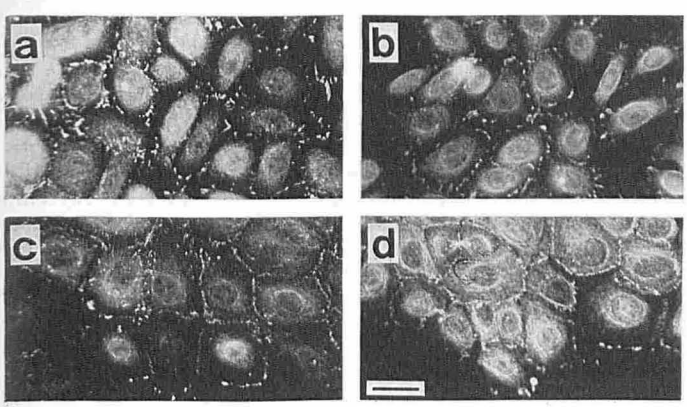


**Figure 3. Assembly of adherens junctions is comparable in normal and HHD keratinocytes.** The assembly of adherens junctions in normal *(a,c)* and HHD cells *(b,d)* was studied by immunofluorescence with antibodies to vinculin in medium containing 0.1 mM $Ca^{2+}$ *(a,b)* and 4 h after addition of medium containing 1.1 mM $Ca^{2+}$ *(c,d)*. *Scale bar*, 40 $\mu$m

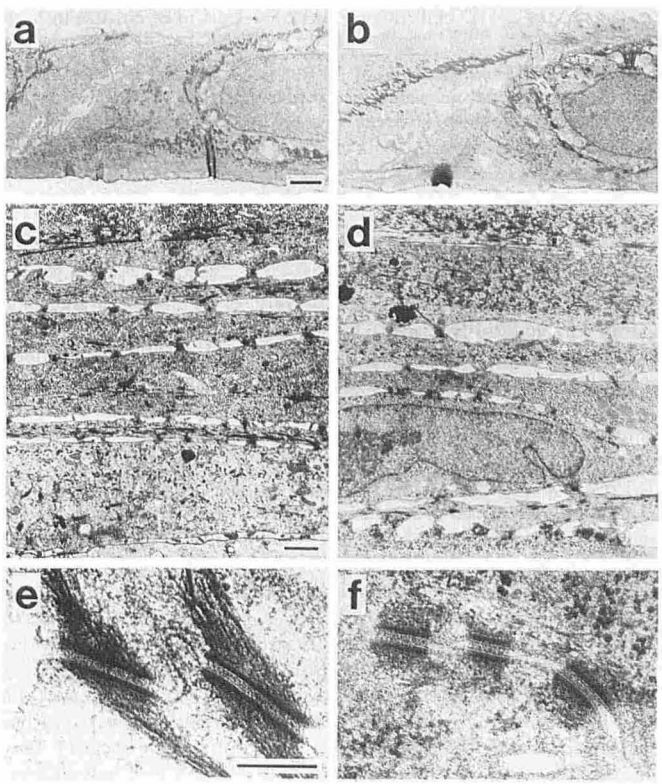


**Figure 4. Electron microscopy of normal and HHD keratinocytes *in vitro* reveals no differences.** *(a,c,e)* Normal cells; *(b,d,f)* HHD cells. *(a,b)* Keratinocytes at 0.1 mM $Ca^{2+}$, in which no junctions are formed; *(c,d)* cells cultured for 7 d in 1.0 mM $Ca^{2+}$ showing desmosomes; *(e,f)* higher power view of desmosomes in cells cultured in 1.0 mM $Ca^{2+}$. *Scale bar*, 2 $\mu$m *(a,b)*, 1 $\mu$m *(c,d)*, or 0.25 $\mu$m *(e,f)*

staining for vinculin was present at borders of contiguous cells, indicating that adherens junctions were absent. Fifteen minutes after the addition of $Ca^{2+}$, vinculin became increasingly concentrated in cell-cell junctions, and staining of focal adhesions was diminished. At 4 h, punctate staining similar to that produced by anti-desmoplakin was present along lines of cell-cell contact but not at free borders of cells, where vinculin-containing focal adhesions remained unchanged **(Fig 3*c,d*)**. When the intensity and location of desmoplakin and vinculin staining were compared, HHD keratinocytes appeared to assemble desmosomes and adherens junctions as well as normal cells, and staining intensity of junctions did not differ in control and HHD keratinocytes. (The increased cytoplasmic staining of HHD keratinocytes in **Fig 3*d*** is nonspecific, apparently related to increased confluency of the cultures, and probably unrelated to vinculin.)

**Cultured HHD Cells in Low and High $Ca^{2+}$ Are Ultrastructurally Normal** Keratinocytes from the patient with HHD were indistinguishable from cells from controls by electron microscopy. When grown in low [$Ca^{2+}$], both were unstratified, lacked desmosomes, had prominent microvilli, and contained perinuclear bundles of tonofilaments **(Fig 4*a,b*)**. Keratinocytes of both the HHD patient and a normal control grown in high [$Ca^{2+}$] for 1 wk were stratified into five to 10 cell layers **(Fig 4*c,d*)**. Both normal and HHD cells had abundant normal-appearing desmosomes along cell borders **(Fig 4*e,f*)**, and keratin filaments were spread about the cytoplasm and not aggregated (not shown). The apparent differences in the length of filaments inserting into the desmosomes in **Fig 4*e,f*** are caused by the different angles of sectioning; the association of the filaments with the desmosomal plaques is normal in both. Spontaneous acantholysis did not occur in the stratified

cultures of the HHD cells during the 2-wk period of culture in high $[Ca^{2+}]$.

In experiments subjecting the cultured cells to mechanical load in the Flexercell plates, some separation of the epithelial layers occurred with maximal forces (up to 30% elongation) from the instrument, but no acantholytic changes were noted, and significant differences between HHD and control cells were not found by electron microscopy (data not shown).

## DISCUSSION

Much of our understanding about the pathogenesis of HHD comes from ultrastructural studies of lesions in which characteristic changes in acantholytic cells have been identified, including retracted tonofilaments, elongated membrane microvilli, and reduced numbers of desmosomes. Some investigators have interpreted these changes to mean that retraction of tonofilaments precedes detachment of cells from one another, suggesting that tonofilaments or the tonofilament-desmosome complexes are defective (Wilgram *et al*, 1962; Pierard and Kint, 1969). Others have concluded that loss of cell-cell adhesion precedes retraction of the tonofilaments, suggesting that the intercellular connection, the attachment between the two halves of the desmosome, or something responsible for cell-cell adhesion is defective (Gottlieb and Lutzner, 1970; Thies *et al*, 1972; Burge *et al*, 1991).

De Dobbeleer and Achten (1979) attempted to settle this question by inducing lesions with friction under an occlusive dressing and studying biopsies at timed intervals. They concluded that the defect was in the intercellular component of the desmosome rather than in the tonofilament-desmosome connection because they observed desmosomes that appeared to have split into half-desmosomes. In our study of induced lesions we did not observe split desmosomes, although we would expect to see abundant half-(split) desmosomes at the edges of these cells or in the cytoplasm after the induction of acantholysis if this were the mechanism underlying acantholysis. The difference in results may be due to the method used to induce lesions. We induced acute lesions with friction and biopsied them promptly, whereas production of lesions of HHD by occlusive dressings creates a substantial delay between the time of acantholysis and the time of sampling, which may permit secondary changes to occur.

Some investigators have found changes corresponding to the HHD phenotype *in vitro*. Ishibashi and Kukita (1983) and Burge *et al* (1991) found that explant cultures of lesional HHD keratinocytes display spontaneous dissociation. Ikeda and Ogawa (1991) studied organ cultures of nonlesional HHD skin and also observed spontaneous *in vitro* acantholysis, a process that could be inhibited by betamethasone. To create an *in vivo*-like epidermis, De Dobbeleer *et al* (1991) seeded keratinocytes from HHD lesions on dead de-epidermized dermis from a normal donor. In their *in vitro* model, HHD epidermis exhibited spontaneous acantholysis with characteristic *in vivo* abnormal morphology. Regnier *et al* (1990) obtained similar results using nonlesional HHD keratinocytes. These investigators interpreted their results as evidence that the primary defect resides in keratinocytes alone, because normal dermis was present. It is of interest that HHD keratinocytes apparently displayed spontaneous acantholysis in these studies, without an identifiable precipitating factor. All these studies had in common the use of organ culture, which produces a fairly well differentiated epidermis. In our studies, which involved stratified keratinocytes that are less fully differentiated than those in skin organ culture, cells did not display spontaneous acantholysis.

In our studies, HHD keratinocytes appeared to aggregate into colonies normally and, based on the intensity of immunofluorescent staining, appeared to form normal junctions. We did not observe any consistent ultrastructural abnormalities (e.g., keratin aggregates, abnormal desmosomes) in HHD cells when compared to normal cells. In contrast to the findings of Regnier *et al* (1990) in a different *in vitro* system, keratinocytes cultured from our patient did not appear vacuolated or dyskeratotic.

A defect in keratin filaments can cause disruption of cells and formation of blisters; mutations in genes coding for basilar keratins K5 and K14 have been found in epidermolysis bullosa simplex and epidermolytic hyperkeratosis, respectively (Coulombe *et al*, 1991; Rothnagel *et al*, 1992). Although dyskeratosis is a feature of HHD, the available evidence does not suggest a primary defect in keratins. We found that immunoblots of extracts of normal and HHD-cultured keratinocytes probed with AE1 and AE3 antikeratin antibodies stained identically (data not shown), and Inohara *et al* (1990) and Bergman *et al* (1992) found normal staining in uninvolved skin with antibodies to cytokeratins. Welsh *et al* (1994) found no evidence of linkage to HHD to 17q or 12q, the chromosomal regions containing keratins of type I or type II. Furthermore, the pattern of acantholysis in HHD differs from that found in disorders associated with defects in keratins.

Alternatively, acantholysis may be related to defective cell-adhesion molecules such as cadherins (Takeichi, 1988), proteins that direct $Ca^{2+}$-dependent homophilic binding of adjacent cells and interact through their C-terminal domains, probably indirectly, with cytoskeletal filaments. Cadherin-related proteins in epidermis include E-cadherin, found in adherens junctions (Kemler *et al*, 1989), desmoglein I and desmocollins, found in desmosomes (Goodwin *et al*, 1990; Mechanic *et al*, 1991), and desmoglein III, present in desmosomes and possibly also elsewhere along the keratinocyte plasma membrane (Amagai *et al*, 1991). Because desmogleins I and III are autoantigens in two forms of pemphigus, they are likely to be important in maintaining intercellular adhesion in epidermis, and a mutation in a critical region of one of these proteins might produce acantholysis.

Burge and Schomberg (1992) found that staining for E-cadherin, desmogleins I and III, and plakoglobin appeared normal in HHD, indicating that these proteins are present, but such an immunohistochemical study does not exclude a functional defect. Although HHD has recently been mapped to a region of chromosome 3q (Richard *et al*, 1995; Peluso *et al*, 1995), the sequence and function of the gene involved have not been identified. Our study, indicating that it is possible for an HHD patient to assemble normal junctions, shows that normal assembly of junctions *in vitro* is compatible with the HHD phenotype. Because pathologic changes *in vitro* were prevented by betamethasone in a previous study (Ikeda and Ogawa, 1991), because some patients with HHD experience dramatic relief of signs and symptoms after systemic administration of corticosteroids, and because lesions occur in areas exposed to friction and higher body temperature (data not shown), the defect may reside in mechanisms such as inflammation or proteolysis.

A structural change might be "unmasked" by appropriate trauma. In studies of epidermolysis bullosa simplex *in vitro*, Morley *et al* (1995) showed that increased temperature may uncover a defect in structural proteins. Striking features of HHD are: (i) patients are unaffected for many years, (ii) most areas of skin remain normal until traumatized in some way, and (iii) relatively extensive removal of affected epidermis by dermabrasion followed by healing may resolve affected areas. These points suggest that junctions are "normal" until the epidermis is disturbed, at which time the defect is exposed, and that extensive healing somehow ameliorates the problem, and it is not surprising in light of these features that *in vitro* junction assembly appeared normal in our studies. It may be useful in this respect to try to "traumatize" the epidermis in this *in vitro* model. Although we were unsuccessful in efforts to induce acantholysis by mechanical means, it may be useful in the future to try to unmask the defect with a different type of trauma or by raising the temperature of the cultures.

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