Characterization of Tight Junctions and Their Disruption by UVB in Human Epidermis and Cultured Keratinocytes

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It has not been confirmed whether tight junctions (TJs) function as a paracellular permeability barrier in adult human skin. To clarify this issue, we performed a TJ permeability assay using human skin obtained from abdominal plastic surgery. Occludin, a marker protein of TJs, was expressed in the granular layer, in which a subcutaneously injected paracellular tracer, Sulfo-NHS-LC-Biotin (556.59 Da), was halted. Incubation with ochratoxin A decreased the expression of claudin-4, an integral membrane protein of TJs, and the diffusion of paracellular tracer was no longer prevented at the TJs. These results demonstrate that human epidermis possesses TJs that function as an intercellular permeability barrier at least against small molecules (~550 Da). UVB irradiation of human skin xenografts and human skin equivalents (HSEs) resulted in functional deterioration of TJs. Immunocytochemical staining of cultured keratinocytes showed that occludin was localized into dot-like shapes and formed a discontinuous network when exposed to UVB irradiation. Furthermore, UVB irradiation downregulated the active forms of Rac1 and atypical protein kinase C, suggesting that their inactivation caused functional deterioration of TJs. In conclusion, TJs function as a paracellular barrier against small molecules (~550 Da) in human epidermis and are functionally deteriorated by UVB irradiation.

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INTRODUCTION

Tight junctions (TJs) are cell–cell junctions that form paired strands sealing the space between neighboring cells and control the paracellular passage of small molecules. TJs are highly developed in simple epithelia and separate the inside from the outside. It has been reported that TJs consist of transmembrane proteins such as claudins, occludin, and junctional adhesion molecules, and plaque proteins such as ZO-1, ZO-2, ZO-3, MUPP-1, and symplekin (Tsukita and Furuse, 2002).

In contrast to simple epithelia, stratified epithelia such as epidermis had been considered to be devoid of TJs functioning as an intercellular permeability barrier in the 1990s. Freeze-fracture analysis of TJ structures using electron microscopy found no continuous TJ strands (i.e., zonulae occludentes) but could find discontinuous TJ strands (i.e., maculae occludentes) (Elias *et al.*, 1977). These observations suggest that the TJs in the epidermis would be too fragmentary to function as an effective barrier. However, in the mouse epidermis, TJs functioning as an effective barrier to small molecules were identified by the application of a TJ permeability assay in combination with the use of antibodies recognizing occludin specifically localized at TJs (Furuse *et al.*, 2002). Sulfo-NHS-LC-biotin (~550 Da) injected into mouse epidermis diffused through the paracellular space from the basal toward the granular layer. However, this diffusion was abruptly prevented at occludin expression sites, because of the presence of TJs.

It has also been reported, in humans, that TJ proteins are expressed in the stratum granulosum, a layer of skin in which typical TJ structures (kissing points) have been detected (Brandner *et al.*, 2002, 2006). In skin disease, several studies have shown that the localization of TJ proteins is altered in the psoriatic epidermis (Watson *et al.*, 2001; Yoshida *et al.*, 2001; Lemini-Lopez *et al.*, 2006).

For adult human epidermis, however, it remains to be seen whether TJs have a role in the intercellular permeability barrier or undergo a functional change during cutaneous barrier disruption. To examine the existence of TJs functioning as a paracellular barrier in human skin, we injected Sulfo-NHS-LC-biotin into human skin obtained from abdominal plastic surgery and visualized it together with the marker protein, occludin and/or ZO-1, localized in TJs (TJ permeability assay).

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Abbreviations: HSE, human skin equivalent; SCID, severe combined immunodeficiency; TJ, tight junction

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Our experiments demonstrate that TJs can function as an intercellular permeability barrier to small molecules (~550 Da) in human epidermis. Irradiation with UVB in human skin xenografts and human skin equivalent (HSE) models was found to cause functional deterioration of TJs. In addition, *in vitro* experiments using cultured keratinocytes showed that UVB irradiation downregulated the active forms of Rac1 and atypical protein kinase C, suggesting that their inactivation caused functional deterioration of TJs.

RESULTS

Existence of TJs functioning as a barrier to paracellular tracer in human epidermis

Occludin and ZO-1 are highly concentrated at TJs. That is, these proteins are the best general markers for TJs identified to date (Saitou *et al.*, 1997; Yoshida *et al.*, 2001; Furuse *et al.*, 2002; Morita *et al.*, 2002). In contrast, claudin-1 and claudin-4 are prerequisite to the paracellular barrier function of TJs (Sonoda *et al.*, 1999; Furuse *et al.*, 2002). Double immunofluorescence staining with occludin and claudin-1 revealed the presence of occludin in small spots and the expression of claudin-1 throughout whole layers of the epidermis (Figure 1a(A) and (B)). The two proteins were overlapped in the granular layer beneath the stratum corneum (Figure 1a(C)). Claudin-4 was also expressed in the epidermis and overlapped with occludin (Figure 1a(D)–(F)). These results were consistent with previous reports, suggesting that functional TJs form a paracellular barrier beneath the stratum corneum in human epidermis (Brandner *et al.*, 2002; Langbein *et al.*, 2002). To determine whether TJs in the epidermis function as an

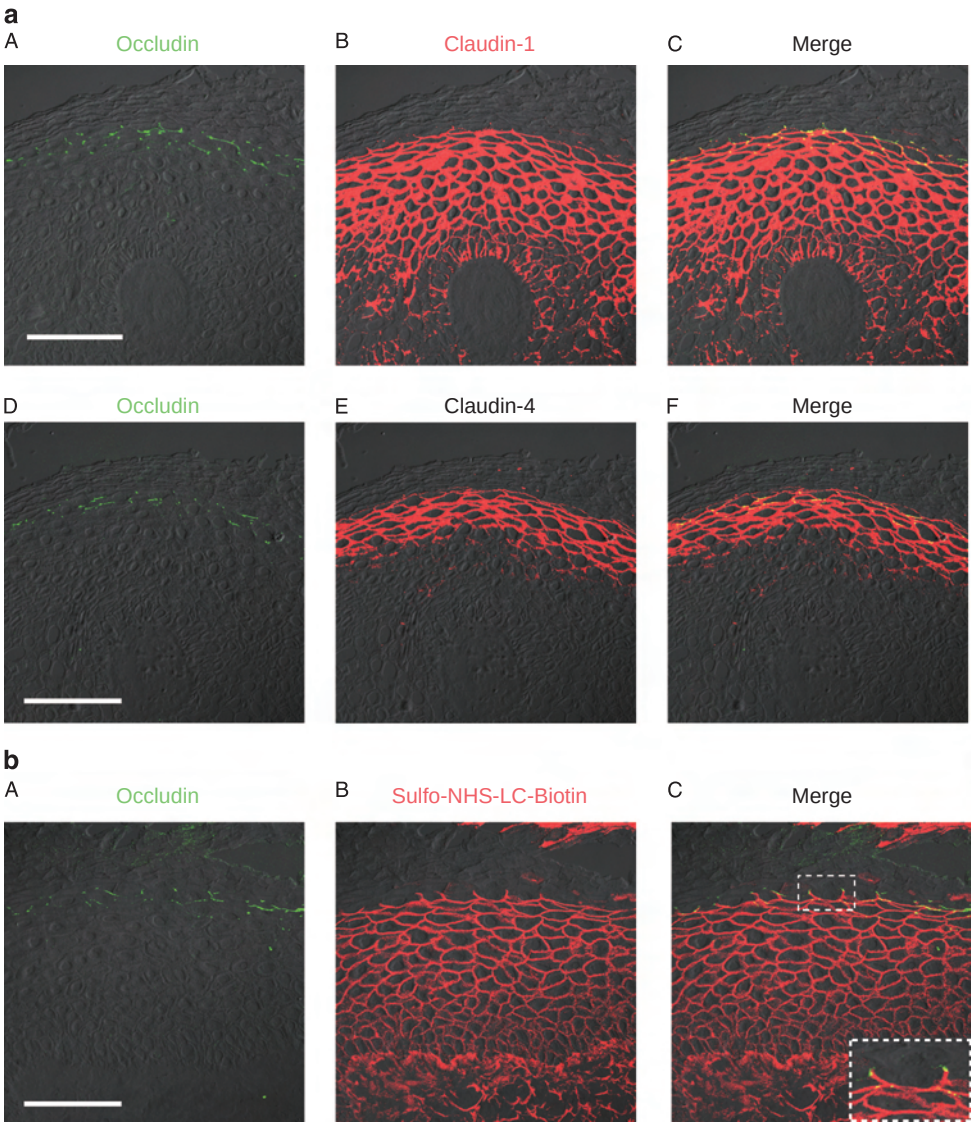

Figure 1. Immunofluorescence staining for tight junction (TJ) components and a TJ permeability assay using an intact human epidermis. (**a**) (A) Occludin (FITC) was expressed in the granular layer as dots. (B, C) Claudin-1 (RRX) was expressed at the plasma membrane in all layers of the epidermis and overlapped at the occludin (FITC) in the granular layer. (D, E, F) Claudin-4 (Cy3) was expressed in the granular layer of the epidermis and co-localized with occludin (FITC). (**b**) Sulfo-NHS-LC-Biotin was injected into human skin. Sulfo-NHS-LC-Biotin (B) and occludin (A) were made visible with anti-occludin mAb (FITC) and streptavidin–Alexa546. Sulfo-NHS-LC-Biotin was stopped at the occludin expression sites (C). Bar = 80 μm.

intercellular permeability barrier, we injected a membrane-impermeable paracellular tracer (Sulfo-NHS-LC-Biotin, molecular weight 556.59 Da) into the dermis and visualized its diffusion (TJ permeability assay). The injected Sulfo-NHS-LC-Biotin diffused through the intercellular space from the stratum basal to the stratum granulosum and was abruptly halted at occludin-positive sites (Figure 1b(C)).

To determine the contribution of TJ proteins to the barrier function of TJs, we performed a pharmacological blockade of TJ functions using ochratoxin A with human skin. Ochratoxin A is a mycotoxin known mainly to affect TJ barrier properties (Maresca *et al.*, 2001). Recent studies have demonstrated that ochratoxin A decreases the intercellular permeability barrier of TJs by removing claudin-4 from the TJ network without affecting cell viability (McLaughlin *et al.*, 2004; Lambert *et al.*, 2007; Yuki *et al.*, 2007). Claudin-4 decreased in a dose dependent manner in human skin samples incubated with ochratoxin A, compared with the control (Figure 2a). The TJ permeability assay revealed that skin incubated with 10 μM ochratoxin A failed to prevent the diffusion of Sulfo-NHS-LC-Biotin at occludin expression sites (Figure 2b). These findings indicated that TJs in human epidermis functioned as a primary barrier to the paracellular passage of Sulfo-NHS-LC-Biotin.

The intercellular permeability barrier of TJs in human skin xenografted onto severe combined immunodeficiency (SCID) mice was deteriorated after UVB irradiation

UVB irradiation is an important inducer of biological changes in skin, including cutaneous barrier deterioration. Using human skin xenografted onto SCID mice (Hachiya *et al.*, 2009), we analyzed the TJ intercellular permeability barrier to Sulfo-NHS-LC-Biotin after UVB irradiation. Previously, it has been reported that the increase in transepidermal water loss peaked after 24 hours following UVB irradiation at three minimum erythema doses in healthy human skin (Frodin *et al.*, 1988). In human xenografted skin, transepidermal water loss increased significantly following a single 200 mJ cm^{-2} dose of UVB irradiation, peaking at 24, 48, and 72 hours after irradiation and returning to baseline levels by 144 hours (Supplementary Figure S1 online). In non-irradiated human skin, Sulfo-NHS-LC-biotin was diffusely distributed throughout the paracellular space but was completely halted in regions where ZO-1 was concentrated

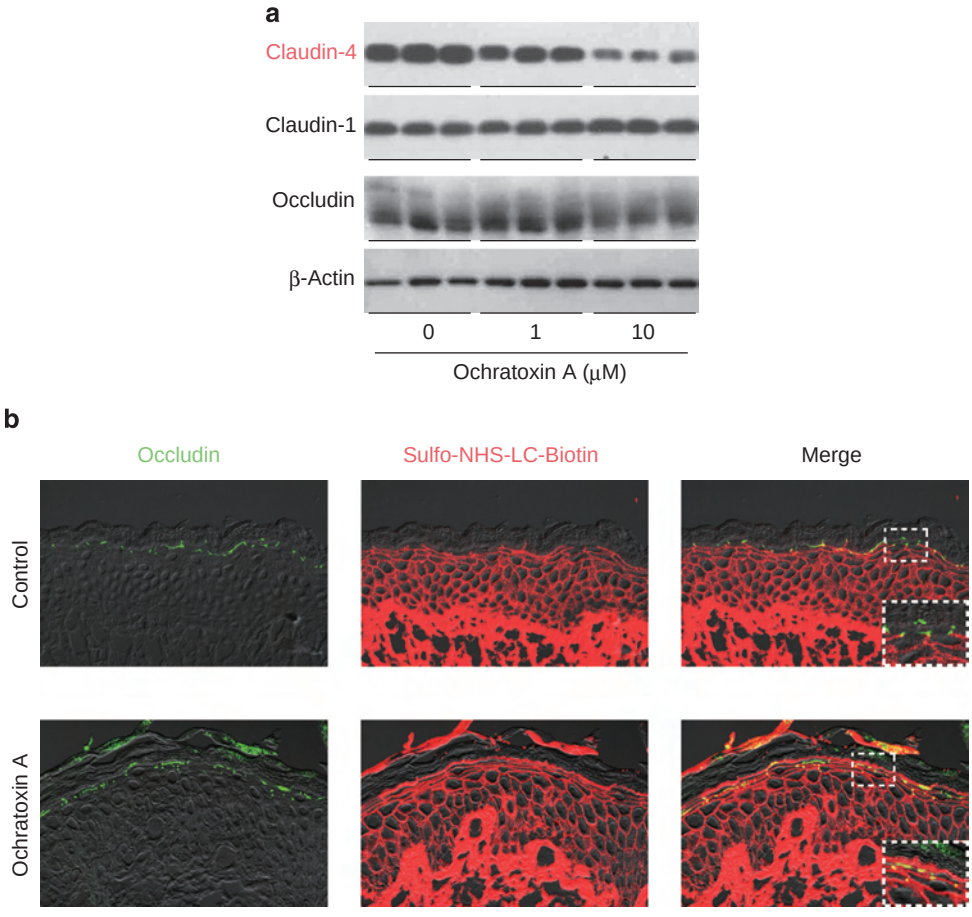

Figure 2. Western blot analysis for tight junction (TJ) components and a TJ permeability assay using organotypic culture of human skin with or without ochratoxin A. Human skin samples were incubated with or without ochratoxin A. After 24 hours, western blot analyses (**a**) and a paracellular tracer assay (**b**) were performed. (**a**) Expression levels of claudin-4 were decreased. (**b**) Human skin was injected with Sulfo-NHS-LC-Biotin. Sulfo-NHS-LC-Biotin and occludin were made visible using anti-occludin mAb (FITC) and streptavidin–TEXAS RED. Arrows indicate the portion in which diffusion of NHS-LC-Biotin was prevented. Diffusion of Sulfo-NHS-LC-Biotin was not prevented at the occludin expression sites in human skin with 10 μM ochratoxin A. Bar = 40 μm.

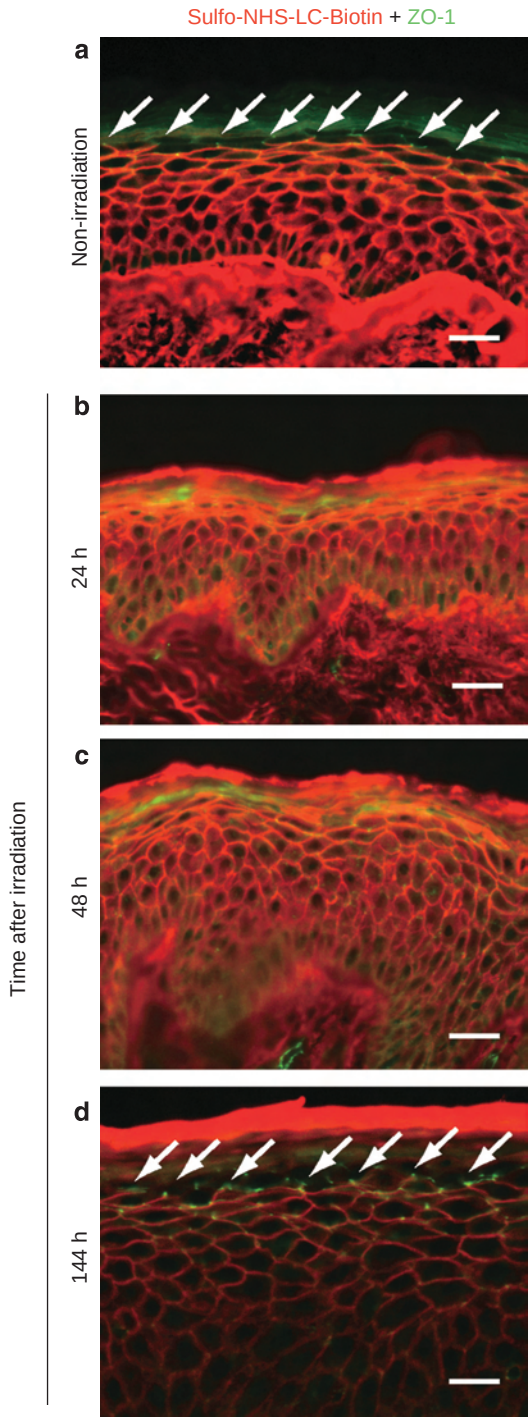

Figure 3. Tracer experiments illustrate disruption and recovery of intercellular permeability barrier of tight junction (TJ) after UVB irradiation in human skin xenograft. Normal human skin grafted onto severe combined immunodeficiency mice was irradiated with a single UVB exposure at a dose of 200 mJ cm^{-2} and harvested 24, 48, or 144 hours later. Non-irradiated grafted human skin was used as a control. Sulfo-NHS-LC-Biotin was intradermally injected and tissue sections were double stained with a mAb specific for ZO-1 (FITC) and streptavidin–TEXAS RED. Merged images of the two fluorescent stains are shown, as seen in non-irradiated skin (**a**), at 24 hours (**b**), 48 hours (**c**), and 144 hours (**d**) after UVB irradiation. Arrows indicate the portion in which diffusion of NHS-LC-Biotin was prevented. Bar = 40 μm.

(Figure 3a). At both 24 and 48 hours after UVB irradiation, injected Sulfo-NHS-LC-Biotin could pass through ZO-1 expression sites and reach the stratum corneum (Figure 3b and c). At 144 hours after UVB irradiation, diffusion of Sulfo-NHS-LC-Biotin was prevented at the region where ZO-1 was concentrated, similar to what was observed in non-irradiated skin (Figure 3d). These findings indicated that, in human epidermis grafted onto SCID mice, TJs could also prevent the diffusion of biotinylated tracer. However, this ability to prevent solute diffusion was found to be severely damaged by UVB exposure.

The intercellular permeability barrier of TJs in HSE models was disrupted after UVB irradiation

Seven days after lifting HSE cultures to the air–liquid interface, cultures were irradiated with a single UVB dose of 40 mJ cm^{-2} and the TJ permeability assay was performed 6, 24, and 48 hours later. An assay of mitochondrial enzyme activity indicated that UVB irradiation did not affect cell viability under these experimental conditions (data not shown). In non-irradiated control HSE, occludin expression was localized beneath the stratum corneum and diffusion of the Sulfo-NHS-LC-biotin was stopped at the occludin expression sites (Figure 4a). Six hours after UVB irradiation, TJs detected by occludin expression were found to retain the intercellular permeability barrier function against the diffused biotinylation tracer (Figure 4b). On the other hand, at 24 and 48 hours after UVB irradiation, the biotinylated tracer passed through the sites of occludin expression and reached the stratum corneum (Figure 4c and d). These findings demonstrate that TJs in HSE can also prevent the diffusion of a paracellular tracer, but the ability to prevent diffusion is severely aggravated by UVB exposure.

The arrangement of TJ proteins was severely disrupted in UVB-irradiated cultured normal human keratinocytes

After documenting the disruption of TJ function following UVB exposure in human skin xenografts and HSE, the mechanisms underlying the deterioration of TJs was investigated using cultured human keratinocytes. In our previous studies, immunofluorescent staining showed that keratinocytes had initiated the formation of TJs at 48 hours and formed continuous mature TJs at 96 hours after Ca^{2+}-induced differentiation (Yuki *et al.*, 2007). To investigate whether UVB irradiation impairs the ability to form mature TJs, keratinocytes were exposed to UVB irradiation at 48 hours after Ca^{2+}-induced differentiation at doses from 10 to 40 mJ cm^{-2}. Immunofluorescent staining was performed at 48 hours after UVB irradiation to evaluate the arrangement of TJ proteins. An assay of mitochondrial enzyme activity indicated that UVB irradiation did not affect cell viability under these experimental conditions (data not shown).

In non-irradiated keratinocytes, a continuous network of occludin was found along the plasma membrane (Figure 5a(A)). In contrast, keratinocytes irradiated with UVB exhibited dispersed, fragmented occludin expression in a dose-dependent manner. A discontinuous network of occludin enclosing cells suggested the sporadic formation of TJs

(Figure 5a(B)–(D)). The expression of claudin-1 was also detected as a distinct line at the cell–cell borders of non-irradiated keratinocytes, consistent with the expression of occludin (Figure 5b(A) and (B)). In contrast, keratinocytes irradiated with a UVB dose of 20 mJ cm^{-2} expressed fragmented claudin-1 in a discontinuous membranous pattern (Figure 5b(D)). Similar results were also found for claudin-4 expression between non-irradiated and UVB-irradiated keratinocytes (Figure 5b(E)–(H)). In concert with the observed expression patterns of TJ components, transepithelial electrical resistance of the keratinocyte sheet was decreased by UVB irradiation in a dose-dependent manner (Supplementary Figure S2 online). This indicated a weakening of the permeability barrier of the keratinocyte sheet to electrolytes. Taken together, the findings suggest that UVB-irradiated keratinocytes lose the ability to form mature TJs and are characterized by a discontinuous membranous pattern of TJ proteins.

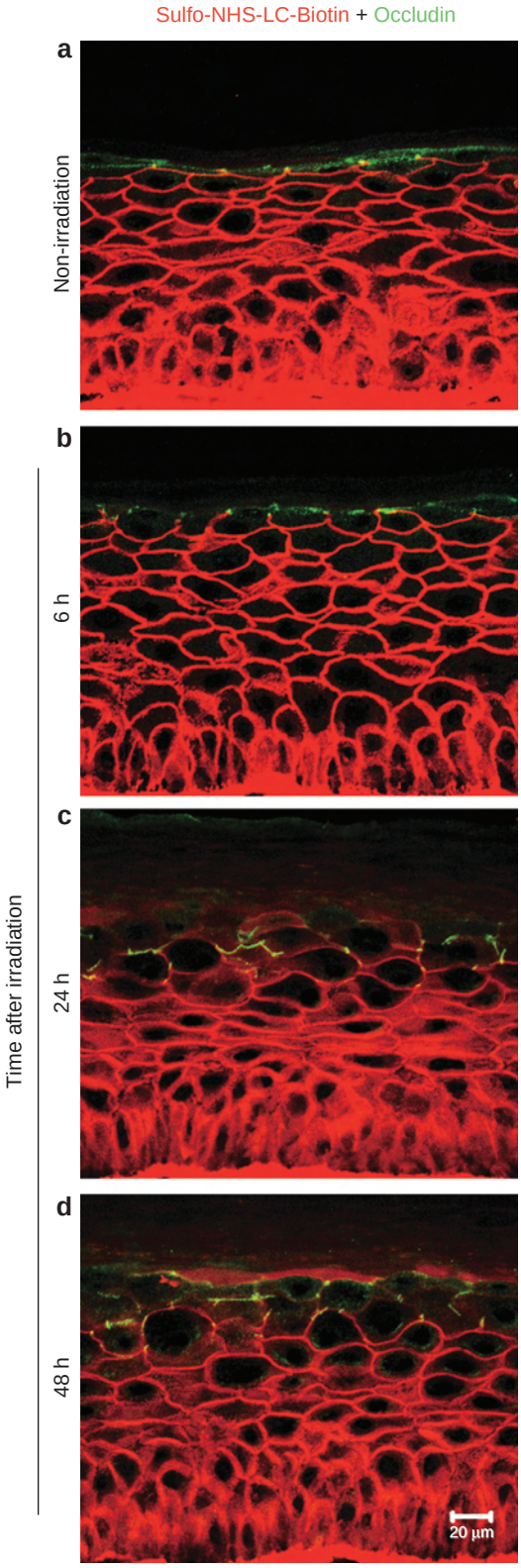

Figure 4. Tracer experiments used to determine tight junction (TJ) barrier integrity in human skin equivalents (HSEs). Mature HSEs were irradiated with a single UVB of 40 mJ cm^{-2} and harvested 6, 24, and 48 hours after exposure. Thirty minutes before each harvest, the culture medium was changed to phosphate-buffered saline containing Sulfo-NHS-LC-Biotin. In non-irradiated sections (**a**), Sulfo-NHS-LC-Biotin was blocked at the occludin expression sites. Sections from HSE harvested 6 hours after UVB treatment (**d**) retained the ability to block the tracer. At 6 (**b**) and 24 hours (**c**) after UVB treatment, the tracer was able to break through and reach the SC. Arrows indicate the portion in which diffusion of Sulfo-NHS-LC-Biotin was prevented. Bar = 20 μm.

UVB irradiation downregulated the active forms of Rac1 and atypical protein kinase C, crucial molecules for continuous TJ formation

It has been reported that continuous TJ formation is regulated by the polarity protein complex Par3/Par6/atypical protein kinase C and that this signaling complex is activated by Rac-induced protein phosphorylation (Hirose *et al.*, 2002; Macara, 2004; Chen and Macara, 2005). In addition, dominant-negative Rac1 and kinase-dead atypical protein kinase C have been reported to inhibit TJ maturation in mouse keratinocytes (Mertens *et al.*, 2005; Helfrich *et al.*, 2007). Therefore, we examined the activation state of Rac1 and atypical protein kinase C in UVB-irradiated keratinocytes. The Rac1 activation assay demonstrated that guanosine triphosphate–Rac1 (activated forms of Rac1) was markedly decreased by UVB exposure in a dose-dependent manner (Figure 6a(A)), whereas total Rac1 protein expression was not suppressed after UVB irradiation (Figure 6a(B)). In addition, the level of phosphorylated atypical protein kinase C was also reduced by UVB exposure in a dose-dependent manner (Figure 6a(C)), although total atypical protein kinase C was not changed by UVB irradiation. In contrast to guanosine triphosphate–Rac1 and phosphorylated atypical protein kinase C, protein expression levels of occludin, claudin-1, and claudin-4 were not altered after UVB irradiation (Figure 6b(A)–(C)). On this basis, we concluded that the downregulation of Rac1 and atypical protein kinase C activities is one possible explanation for the defects in TJ maturation.

DISCUSSION

Furuse *et al.* (2002) have reported that claudin-based TJs form a paracellular barrier in mouse epidermis. Many reports have

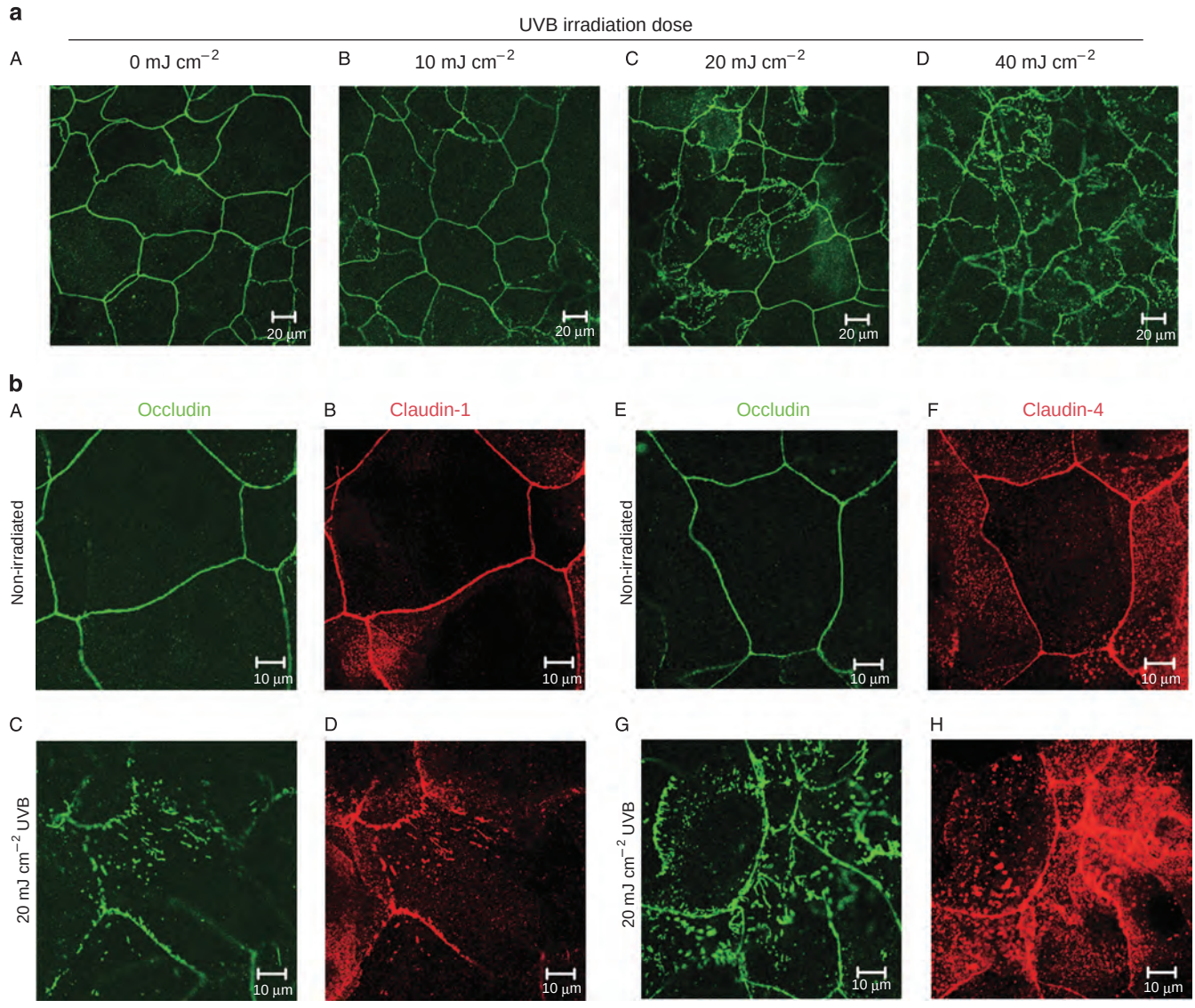

Figure 5. Alterations in the organization of tight junction (TJ) proteins in normal human keratinocytes following UVB irradiation. (**a**) Immunofluorescence analysis was performed to examine the localization of the TJ-specific marker, occludin (FITC), after UVB irradiation. Panels depict non-exposed cells (A) and cells irradiated with UVB doses of 10 (B), 20 (C), and 40 (D) mJ cm^{-2}. Bar = 20 μm. (**b**) Double immunostaining of occludin (FITC) with claudin-1 (RRX) or claudin-4 (Cy3). (A), (C), (E), and (G) show occludin staining. Claudin-1 and Claudin-4 staining is shown in (B), (D) and (F), (H), respectively. (A), (B), (E), (F) and (E), (D), (G), (H) indicate non-irradiated and UVB-irradiated cells, respectively. UVB irradiation dose = 20 mJ cm^{-2}. Bar = 10 μm.

suggested that functional TJs are present in human epidermis (Brandner *et al.*, 2002; Langbein *et al.*, 2002; Schluter *et al.*, 2004). However, there has been no direct evidence of TJ function as an intercellular permeability barrier in human epidermis. To clarify this issue, we performed immunohistochemical studies for TJ components and a TJ permeability assay using human epidermis. A subcutaneously injected paracellular tracer (Sulfo-NHS-LC-Biotin; ~550 Da) was halted because of the presence of TJs marked by occludin staining between the cells beneath the stratum corneum. Incubation of human skin with ochratoxin A in organotypic cultures decreased the expression level of claudin-4 and deteriorated the TJ intercellular permeability barrier to biotinylation tracer. It has been reported that ochratoxin A induces a reduction in transepithelial electrical resistance with a concomitant increase in the paracellular permeability of membrane impermeant tracers in human intestinal cells and epidermal keratinocytes (Lambert *et al.*, 2007; Yuki *et al.*, 2007). This loss of epithelial barrier function has been found to be due to removal of TJ proteins from the TJ network. In MDCK I cells incubated with C-CPE, claudin-4 is also selectively removed from TJs, concomitant with TJ barrier downregulation (Sonoda *et al.*, 1999). To our knowledge it is previously unreported that TJs function as a primary barrier to the paracellular diffusion of small molecules (~550 Da) in human epidermis.

The stratum corneum just above TJs clearly provides a strong barrier to the movement of water (Madison, 2003).

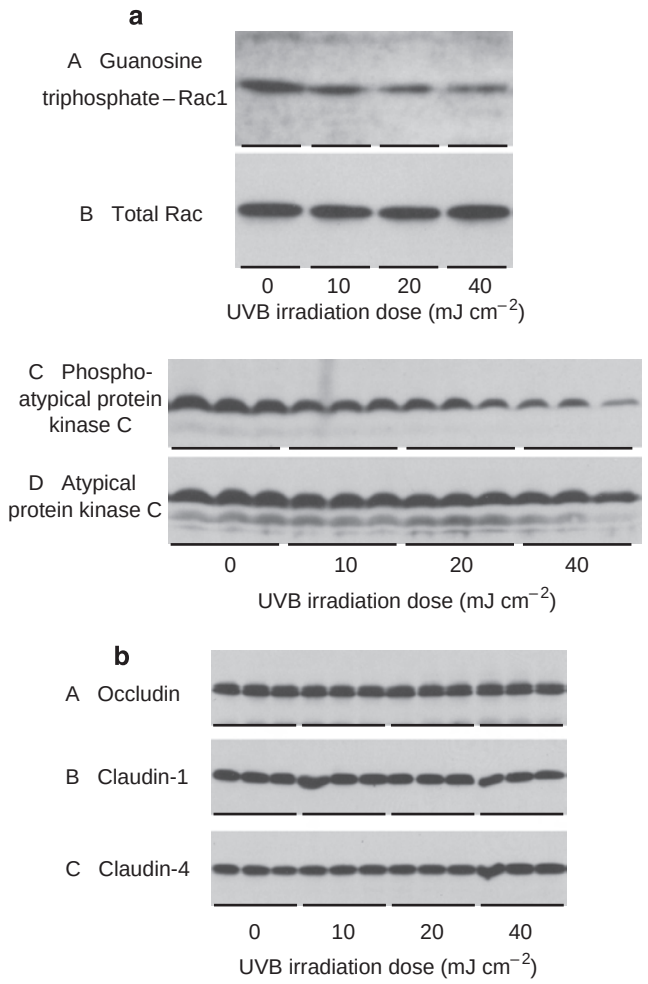

Figure 6. Alteration of the activation state of Rac1 and atypical protein kinase C following UVB irradiation. (**a**) The active form of Rac1, guanosine triphosphate–Rac, was quantified using a Rac activation assay (A), whereas the active form of atypical protein kinase C, phospho-atypical protein kinase C, was assessed by western blotting (C). UVB irradiation significantly decreased the expression of the active forms of Rac1 and atypical protein kinase C in a dose-dependent manner but had no effect on their overall protein levels (B, D). (**b**) Western blotting analysis with antibodies specific to occludin (A), claudin-1 (B), and claudin-4 (C) indicated that UVB irradiation at these doses did not alter the expression of these TJ proteins.

Our findings here demonstrated that epidermal TJs prevented the movement of Sulfo-NHS-LC-Biotin. No studies, however, have ever shown whether epidermal TJs can prevent the movement of water and/or ions. To better understand TJ function in the epidermis, it would be very useful to clarify the barrier properties (the cutoff size and ion selectivity for free diffusion across TJs) of TJs in the epidermis.

The intercellular permeability barrier of TJs was decreased at 24 and 48 hours after UVB irradiation. Similar results were obtained in UVB-irradiated mouse skin (Yamamoto *et al.*, 2008). It will be very important to consider the mechanism underlying the TJ dysfunction induced by UVB irradiation. Epithelial junction formation can be dissected into multiple steps in a sequential manner during the cell polarization process in a mouse epithelial cell line, MTD1-A cells (Yonemura *et al.*, 1995; Ando-Akatsuka *et al.*, 1999). Immunofluorescence analyses have demonstrated that at the initial phase of cell polarization, fibroblastic spot-like junctions containing E-cadherin and ZO-1 are formed as a nascent junctional complex (Vasioukhin *et al.*, 2000), followed by ZO-1 dissociation from E-cadherin that separately forms epithelium-specific belt-like junctions (Ando-Akatsuka *et al.*, 1999). In this process, atypical protein kinase C activity is required for the maturation of junctions from the spot-like formation of adherence junctions and TJs to the final belt-like structure seen in MTD1-A cells and HaCaT cells (Suzuki *et al.*, 2001, 2002; Aono and Hirai, 2008). In addition, Mertens *et al.* (2005) have demonstrated that lower endogenous Rac1 levels and less atypical protein kinase C activity, as determined by Rac1 activation and atypical protein kinase C autophosphorylation assays, could suppress maturation of belt-like adherence junctions and TJs. In our experiments, lower levels of guanosine triphosphate–Rac1 and phosphorylated atypical protein kinase C were observed and occludin was localized discontinuously in UVB-irradiated cells, suggesting that UVB irradiation disrupted Rac1 and atypical protein kinase C signaling, which then inhibited the maturation of TJs.

The downregulation of TJ proteins is another possible cause of TJ dysfunction. Occludin, claudin-1, and claudin-4 in cultured keratinocytes were all unchanged after UVB irradiation in our experiments. However, we cannot rule out the possibility that the TJ dysfunction results from decreases of other TJ components expressed in the epidermis (Brandner *et al.*, 2002; Amasheh *et al.*, 2005).

Lemini-Lopez *et al.* (2006) reported an intriguing phenomenon in psoriatic skin by using epidermal sheets to allow observation of a simple horizontal plane of the epidermis. In normal skin, occludin, a specific marker of TJs, is tethered to the cell membrane in a continuous linear pattern. However, in lesional skin, occludin shows a discontinuous pattern along the periphery of the cells, which is similar to the staining pattern that we observed in UVB-irradiated cells (Figure 5a and b). This result, in combination with our *in vitro* experiments, suggests that the intercellular permeability barrier of TJs might be decreased because of downregulation of endogenous Rac1 and atypical protein kinase C activity in psoriatic skin.

In conclusion, we demonstrated that TJs could function as an intercellular permeability barrier to small molecules (~550 Da) in human epidermis. Furthermore, irradiation with UVB into human skin xenografts and HSEs results in functional deterioration of TJs. These results led to our presumption that dysfunction of the cutaneous barrier is related to impaired TJ function. In the future, it will be very important to clarify the relationship between the epidermal TJ function and integrity of the stratum corneum. Our research findings provide new insights into the fundamental understanding of the regulatory mechanisms underlying human cutaneous barrier function. In addition, targeting Rac1 and atypical protein kinase C could be a promising treatment strategy for xerotic skin disorders.

MATERIALS AND METHODS

Human donors and organotypic culture

Human skin was obtained from Caucasian subjects who underwent abdominal plastic surgery at The Christ Hospital or University Hospital (Cincinnati, OH). Collection of discarded tissue was approved by the Institutional Review Board of Cincinnati Children's Hospital Medical Center and informed consent was obtained from the volunteers before surgery. The study was conducted according to the declaration of Helsinki Principles. Some experiments were performed immediately after surgery. For organotypic cultures, punch biopsies 6 mm in diameter were taken and placed dermis-side down in culture dishes and immersed in medium. Only the dermis was in contact with the medium, whereas the epidermis remained exposed to air. Complete Epilife with HKGS-V2 plus penicillin, streptomycin, and amphotericin (Cascade Biologics, Portland, OR) was used for the culture medium, with or without the claudin-4 inhibitor, ochratoxin A (Sigma-Aldrich, Dorset, UK). For grafting human skin onto SCID mice, skin from abdominoplasty was transplanted onto 5- to 7-week-old SCID mice as described elsewhere (Sriwiriyanont *et al.*, 2006). On confirmation of complete healing, UVB irradiation experiments were performed. Animals were handled according to the ethical principles of animal experimentation established by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center. For cell cultures, normal human epidermal keratinocytes were handled by the methods described previously (Yuki *et al.*, 2007). HSEs were purchased from TOYOBO (Osaka, Japan) and used according to the manufacturer's recommendations.

UVB irradiation

UVB irradiation was performed using fluorescent sun lamp tubes (Toshiba FL 20SE, Toshiba Electric, Tokyo, Japan) as the UVB source. Three different experimental models were used to test the functional alteration of TJs. The first model was the xenografted human skin model. After complete healing of xenografts, the human skin was irradiated with a single UVB exposure (200 mJ cm^{-2}). The second model was a commercially available HSE that was irradiated with a single UVB at a dose of 40 mJ cm^{-2}. The third model was an *in vitro* model using keratinocytes that were cultured as described above. Forty-eight hours after the induction of differentiation by the elevated Ca^{2+} concentration, cells were irradiated with UVB at doses that ranged from 10 to 40 mJ cm^{-2} to examine the effect of UVB irradiation on the formation of TJs. Cells were cultured for another 48 hours before the function and organization of TJs were examined.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed by the method described previously (Furuse *et al.*, 2002; Yuki *et al.*, 2007) using the following antibodies: anti-occludin mAb (MOC37 mAb) was kindly gifted from Professor Mikio Furuse (Department of Cell Biology, Graduate School of Medicine, Kobe University) (Saitou *et al.*, 1997); anti-ZO-1 mAb, anti-claudin-1 polyclonal antibody, and anti-claudin-4 mAb were purchased from Zymed Laboratories (San Francisco, CA).

SDS-PAGE and immunoblotting

Protein extraction and SDS-PAGE gel electrophoresis were performed by the methods described previously (Yuki *et al.*, 2007). For assessment of TJ proteins, anti-occludin polyclonal antibody, anti-claudin-1 polyclonal antibody, and anti-claudin-4 mAb (Zymed Laboratories) were used. For examination of atypical protein kinase C activity, anti-atypical protein kinase C polyclonal antibody (Cell Signaling, Danvers, MA) and anti-phospho-atypical protein kinase C (Cell Signaling) antibody were used.

Rac activation assay

Rac activity was determined using a Rac activation assay biochemical kit (Cytoskeleton, Denver, CO), according to the manufacturer's recommendations.

TJ permeability assay

The TJ permeability assay was performed to assess TJ function using a surface biotinylation technique according to the method developed by Furuse *et al.* (2002). Briefly, ~50 µl of 10 mg ml^{-1} EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) in phosphate-buffered saline containing 1 mM $CaCl_2$ was injected into the dermis of human skin, which had been grafted onto SCID mice. As for HSE, HSE was incubated with 2 mg ml^{-1} EZ-Link Sulfo-NHS-LC-Biotin from the dermal side. After a 30-minute incubation, the skin samples were removed and frozen in liquid nitrogen. Frozen sections, 5-µm thick, were fixed in 95% ethanol at 4 °C for 30 minutes and then placed in 100% acetone at room temperature for 1 minute. The sections were soaked in 1% BSA/phosphate-buffered saline for 15 minutes, incubated with either anti-occludin mAb or anti-ZO-1 mAb for 1 hour, washed three times with blocking solution, and incubated with a mixture of secondary antibody-conjugated FITC and streptavidin–Texas Red (Calbiochem, Darmstadt, Germany) for 1 hour.

Transepidermal water loss

Transepidermal water loss was determined over a 196-hour interval following UVB irradiation by measuring water evaporation from the surface of xenografted human skin using a DermaLab device (Cortex Technology, Hadsund, Denmark).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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