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Activation of TLR2 Enhances Tight Junction Barrier in Epidermal Keratinocytes

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The epidermis has developed physical and immunological barriers that prevent infiltration of deleterious chemicals and pathogens. As a first step to understanding the relationship between these barriers, we investigated whether TLR2 activation functionally alters tight junctions (TJs) in cultured human keratinocytes. Stimulation with peptidoglycan, a ligand for TLR2, elevated the TJ-associated barrier in the space of 3 h. The increase in TJ-associated barrier function due to peptidoglycan stimulation was suppressed by the knockdown of TLR adaptor MyD88 or the pretreatment with TLR2-neutralizing Ab, indicating that TLR2 activation enhanced TJ-associated barrier. One and 3 h after peptidoglycan stimulation, expression levels of the TJ proteins occludin, claudin-1, claudin-4, and ZO-1 were unchanged. However, immunoprecipitation studies demonstrated that the association of phosphoatypical protein kinase Cζ/ζ, crucial for TJ biogenesis, with occludin was increased. Significantly, inhibition of atypical protein kinase Cζ/ζ activity completely blocked the immediate elevation of the TJ-associated barrier. Finally, peptidoglycan was applied to the stratum corneum surface of a human skin equivalent, and the TJ barrier was evaluated. In the space of 3 h after the stimulation, the amount of intercellular tracer in the stratum corneum incubated from the dermal side was reduced, indicating that the TJ barrier is strengthened via TLR2 activation. Taken together, our findings indicated that infiltration of pathogens into the epidermis immediately enhanced TJ function via TLR2 signaling. Furthermore, the dynamically controlled TJs in skin are considered fundamental in preventing further invasion of pathogens and maintaining cutaneous barrier homeostasis. The Journal of Immunology, 2011, 187: 000–000.
was significantly suppressed (15). Furthermore, utilizing TLR2- or MyD88-deficient mice, the TLR2–MyD88 pathway was clearly demonstrated to play an essential role in preventing infections (17). These findings suggest that keratinocytes are capable of defending against microbial agents not just as a physical barrier, but also by triggering an innate immune system response. Although proper physical and immunological barriers are maintained in the epidermis, the interaction between these barriers remains unknown. As a first step toward addressing this issue, we investigated whether TLR activation functionally alters TJs in cultured keratinocytes. Our study indicated that activation of TLRs enhanced TJ functions in the space of 3 h in the cultured keratinocytes, which suggested that the immediate enhancement of the TJ functions prevents further invasion of pathogens and/or their secreted products and maintains cutaneous barrier homeostasis.

Materials and Methods

Cells and reagents

Normal human epidermal keratinocytes (Kurabo, Osaka, Japan) were propagated in MCDB 153 medium (Nihon Pharmaceutical, Tokyo, Japan) with 0.1 mM Ca2+ and the following additives: 5 mg/ml insulin, 180 mg/l hydrocortisone, 14.1 mg/l 0-phosphorylethanolamine, 6.1 mg/l 2-aminooctanoic acid, 100 mg/l epidermal growth factor, and 0.4% (v/v) bovine pituitary extract. The cells were trypsinsized until single-cell suspensions and plated onto Transwell of 0.4-μm pore size (Millipore, Bedford, MA). When the cells were confluent, they were transferred to 1.8 mM Ca2+ medium. Four days after transferring to 1.8 mM Ca2+ medium, TJ ligands were added and TJ-associated barrier was evaluated.

Human skin equivalents were purchased from TOYOBO (Osaka, Japan) and used according to the manufacturer’s recommendations. Eight days after aerial exposure, 10 μg/ml peptide glycocan solution (2 ml) was added onto the stratum corneum surface of human skin equivalents; 30 min later, the solution was removed and TJ-associated barrier was evaluated. Peptide glycan (PGN) from Staphylococcus aureus, a ligand for TLR2, was purchased from Sigma-Aldrich (St. Louis, MO). For blockade of TLR2, keratinocytes were pretreated with chicken polyclonal to TLR2 (Abcam, Cambridge, MA) for 1 h before PGN stimulation. Chicken IgY (Abcam) was used for isogenic control. The following are the other TLR ligands used in this study: Pam3Cys-Ser-(Lys)4 (Imgenex, San Diego, CA), poly(I:C) (Cambridge, MA) for 1 h before PGN stimulation, Chicken IgY (Abcam) was used for isogenic control. The following are the other TLR ligands used in this study: Pam3Cys-Ser-(Lys)4 (Imgenex, San Diego, CA), poly(I:C) (Invivogen, San Diego, CA), LPS (Calbiochem, Darmstadt, Germany), flagellin (Axxora, San Diego, CA), MALP-2 (Imgenex), and CpG oligonucleotide type A (Invivogen). Atypical protein kinase C (PKC)z/pseudo-substrate inhibitor was purchased from Calbiochem (Darmstadt, Germany).

Immunofluorescence microscopy

The cells were fixed with ethanol for 30 min on ice and washed with a PBS solution containing 0.1% Triton X-100. The cells were then soaked in 1% BSA. Next, the cells were incubated overnight with the following primary Abs: anti-occludin mAb (Abcam), anti-calcitonin gene-related peptide (CGRP) mAb (MBL), according to the manufacturer’s instructions. Immune complexes precipitated with phosho-atypical PKCz were subjected to SDS-PAGE. After blotting to the polyvinylidene difluoride membrane, occludin was detected with the anti-occludin mAb (MOC37, a gift of M. Furuse, University of Kobe). For loading control, immunoprecipitants were also immunoblotted for phospho-atypical PKCz.

Immunoblotting and immunoprecipitation

For analyses of total protein levels of occludin, claudin-1, claudin-4, and ZO-1, atypical PKCz, atypical PKCp, phospho-atypical PKCz, and MYD88, keratinocytes were washed with ice-cold PBS and subsequently lysed in the buffer containing 200 mM Tris (pH 7.4), 2.0% SDS, protease inhibitor mixture (Roche, Manheim, Germany), and phosphatase inhibitor mixture (Roche). Equal amounts of total protein (20 μg) were subjected to SDS-PAGE. Samples separated by SDS-PAGE were transferred onto polyvinylidene difluoride membranes, and the membranes were soaked in 5% skimmed milk and incubated with the primary Abs and then HRP-conjugated secondary Abs. The signal was detected by ECL (Thermo Scientific, Rockford, IL) and exposed to x-ray film. Primary Abs against occludin, claudin-1, claudin-4, and ZO-1 were purchased from Zymed Laboratories (South San Francisco, CA), and Abs against atypical PKCz and MYD88 were purchased from Cell Signaling (Danvers, MA). The Ab for atypical PKCp was purchased from Abcam, and the Ab for phospho-atypical PKCz was purchased from MLB (Woburn, MA).

Transfection of keratinocytes with MYD88 small interfering RNA

Small interfering RNA (siRNA) was used to reduce MyD88 levels in normal human keratinocytes. The 21-mer siRNA sequences specific for MYD88 were purchased from Invitrogen (San Diego, CA). For the transfection into the keratinocytes, reverse transfection was performed with RNAiMAX (Invitrogen), according to the manufacturer’s instructions. Two days after the transfection, keratinocytes were washed with the medium containing 1.8 mM Ca2+. Four days after transferring to 1.8 mM Ca2+ medium, TJ ligands were added and TJ-associated barrier was evaluated.

In vitro system to evaluate TJ-associated barrier

Four days after transferring to 1.8 mM Ca2+ medium, TJ ligands were added into the differentiated keratinocytes in which TJs had already formed. Then transepithelial electric resistance (TER) was measured using a Millicell-ERS epithelial voltmeter (Millipore), as described in a previous study (6). TER by the keratinocyte sheet gives an indication of the TJ functions prevents further invasion of pathogens and/or their secreted products and maintains cutaneous barrier homeostasis. A significant increase in TER at 3 h after the stimulation. There was no effect of the ligand on TER during the first 3 h. In our previous study (6), elevation of TER values was accompanied by an enhancement of TJ functions, indicating that activation of TLRs enhanced the TJ barrier function in the space of 3 h.

Results

Addition of TLR ligands into keratinocyte cultures enhanced TER during the first 3 h

In the past several years, compelling studies have demonstrated that epidermal keratinocytes express at least seven kinds of TLRs (TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, and TLR9) (9–13). To clarify the relationships between TLRs and TJs, we stimulated differentiated keratinocytes, in which TJs had already formed, with the ligands for TLRs. The TJ-associated barrier was then evaluated by means of TER measurement (6). As shown in Fig. 1, stimulation of TLR1, 2, 3, 4, 5, 6, and 9 with ligands led to a significant increase in TER at 3 h after the stimulation. There was no effect of the ligand on TER at 3 h. In our previous study (6), elevation of TER values was accompanied by an enhancement of TJ functions, indicating that activation of TLRs enhanced the TJ barrier function in the space of 3 h.

Activation of TLR2 signaling enhances TJ-associated barrier in the first 3 h

Just beneath the stratum corneum, TLR2 is expressed in the granular cells as a pathogen sensor (9, 12), and TJs also exist between the granular cells to prevent paracellular diffusion of small molecules (4, 5). Again, if the stratum corneum barrier is damaged, TLR2 is...
believed to be the first to detect invading microorganisms such as *S. epidermidis*. Taking into account all of these factors, the relationship between the TLR2 and TJs was examined in detail. In the following studies, the PGN effect on the TJ permeability of keratinocytes was determined by measuring TER.

We stimulated TLR2 with PGN (10, 50 μg/ml); 3 h later, TER was measured to evaluate the TJ function. The values of TER were significantly elevated as early as 3 h compared with the nontreated control (Fig. 2A).

MyD88 is the common adaptor protein involved in TLR signaling (18). We knocked down MyD88 by MyD88 siRNA to investigate whether TLR/MyD88 signaling is required for the elevation of the TJ barrier. Six days after MyD88 siRNA transfection, MyD88 protein was completely suppressed compared
with that of the scramble siRNA transfection (Fig. 2B). In the keratinocytes transfected with scramble siRNA, PGN stimulation significantly increased the TER values after 3 h compared with the nonstimulated ones with scramble siRNA (Fig. 2B). This increase was suppressed when the keratinocytes with MyD88 siRNA were stimulated by PGN (Fig. 2B).

To examine the underlying mechanisms of the upregulation of the TJ-associated barrier by TLR2 activation, we blocked TLR2 with a TLR2-neutralizing Ab and evaluated the TJ-associated barrier. In the isogenic IgY (chicken IgY) control, TER values increased slightly in the space of 3 h (Fig. 2C). When the isogenic IgY control was stimulated by PGN, TER increased significantly in the space of 3 h compared with the nonstimulated isogenic IgY control (Fig. 2C). In contrast, the keratinocytes pretreated by a TLR2-neutralizing Ab showed a suppression of the immediate increase in TER with PGN stimulation (Fig. 2C).

These observations indicated that activation of TLR2/MyD88 signaling immediately enhanced the TJ-associated barrier that prevents the passage of small molecules through the spaces between cells.

**Association of phospho-atypical PKCζ/α with occludin is increased by TLR2 activation in the first 3 h**

Using the system described in Fig. 2C, the underlying mechanisms of the TJ-associated barrier enhancement were examined. First of all, we examined the expression level of TJ proteins after PGN stimulation by means of Western blot analyses. In human keratinocytes, the occludin Ab identified several bands as reported for occludin isofoms present in the human epidermis (19). There are no obvious changes in any of the bands of occludin. The other TJ components showed no change in expression level with PGN stimulation at 1 and 3 h either (Fig. 3).

We then examined another factor that can regulate TJ function. There are two types of atypical PKC, atypical PKCζ and atypical PKCα, which are strongly related but encoded by separate genes (20). All commercially available Abs for phospho-atypical PKC recognize both phospho-atypical PKC, ζ and α. Helfrich et al. (21) showed that atypical PKCζ/α and phospho-atypical PKCζ/α were expressed in the junctional area of plasma membrane, and, notably, strong membrane staining was seen when using an Ab that recognizes phosphorylated forms of atypical PKCζ/α, indicative of the active form. Previous studies have shown that, during TJ assembly, atypical PKCζ/α associated with anti-occludin immunoprecipitants strengthened TJ barrier function through phosphorylation of TJ proteins (22, 23). Therefore, we first examined the expression level of atypical PKCζ, atypical PKCα, and phospho-atypical PKCζ/α, indicative active forms, after PGN stimulation. Western blot analyses revealed that the expression levels of atypical PKCζ, atypical PKCα, and phospho-atypical PKCζ/α were unchanged (Fig. 4A). Next, we performed immunoprecipitation studies with a phospho-atypical PKCζ/α Ab. Immunoprecipitant with phospho-atypical PKCζ/α was subjected to the immunoblot for occludin, a marker protein specifically localized at TJs (4, 5). The occludin Ab identified three bands as well as Western blot analysis of occludin in Fig.
3. One and 3 h after PGN stimulation, all of the bands of occludin precipitated with phospho-atypical PKC\(\zeta/i\) showed an increase compared with the isogenic control (Fig. 4B). This increase was suppressed when keratinocytes were pretreated with a TLR2-neutralizing Ab, indicating that TLR2 activation promotes the association of phospho-atypical PKC\(\zeta/i\) with occludin and strengthens TJ-associated barrier in the space of 3 h.

To confirm the involvement of atypical PKC\(\zeta/i\) in the enhancement of TJ-associated barrier, we inhibited atypical PKC\(\zeta/i\) using a pseudosubstrate inhibitor, and the changes of TJ-associated barrier were evaluated at 3 h after the PGN stimulation (Fig. 5A). Notably, the accelerated TER increase due to PGN stimulation was not observed following pretreatment with atypical PKC\(\zeta/i\) pseudosubstrate inhibitor, indicating that, in the first 3 h, the activity of atypical PKC\(\zeta/i\) is involved in the elevation of the TJ-associated barrier by PGN stimulation.

Taken together, our data demonstrated that TLR2 activation rapidly increased TJ function, which was not observed following pretreatment with atypical PKC\(\zeta/i\) pseudosubstrate inhibitor. Furthermore, the underlying mechanisms of accelerated TER increase in the first 3 h suggest an association of phospho-atypical PKC\(\zeta/i\) with TJ proteins.

FIGURE 4. Association of phospho-atypical PKC\(\zeta/i\) with occludin was increased by TLR2 stimulation at 3 h after PGN treatment. As shown in Fig. 2C, keratinocytes pretreated with chicken IgY or TLR2-neutralizing Ab were stimulated with PGN, and then harvested before PGN treatment and at 1 and 3 h after PGN treatment. Western blot analyses were performed to examine the effect of TLR2 activation on the expression levels of TJ proteins. The occludin Ab identified several bands, which are the same as \(M_0\) reported for occludin isoforms present in the human epidermis (19). The experiment was repeated three times with essentially identical results. Densitometric scanning of these bands was performed, and the data are shown in Supplemental Fig. 1.

FIGURE 3. TLR2 activation did not change the expression levels of TJ components at 1 and 3 h after PGN treatment. As shown in Fig. 2C, keratinocytes pretreated with chicken IgY or TLR2-neutralizing Ab were stimulated with PGN, and then harvested before PGN treatment and at 1 and 3 h after PGN treatment. Western blot analyses were performed to examine the effect of TLR2 activation on the expression levels of TJ proteins. The occludin Ab identified several bands, which are the same as \(M_0\) reported for occludin isoforms present in the human epidermis (19). The experiment was repeated three times with essentially identical results. Densitometric scanning of these bands was performed, and the data are shown in Supplemental Fig. 1.

PGN application to the stratum corneum surface of a human skin equivalent enhances TJ function in the space of 3 h

To investigate whether the penetration of PGN into the epidermis triggers the elevation of TJ barrier function, PGN was applied to the
stratum corneum surface of a human skin equivalent. Upregulation of TNF-α and IL-6 mRNA was observed by real-time RT-PCR analyses, indicating that PGN application triggered TLR2 activation (data not shown). TJ permeability assay revealed that the intensity of dot signal was markedly decreased at 3 h after PGN stimulation (Fig. 6).

The data obtained in this study indicated that sulfo-NHS-LC-biotin penetration passing through TJs was decreased, due to the enhancement of the TJ barrier function. These findings indicate that infiltration of pathogens and/or their secretions into the epidermis strengthens the TJ barrier via TLR2 signaling.

**Discussion**

Utilizing an in vitro system to evaluate TJ functions, we found that activation of TLR2 signaling enhanced the TJ-associated barrier in the space of 3 h. Elevation of TJ function by PGN was accompanied by the association of phospho-atypical PKCζ/i with occludin, which suggested the underlying mechanisms of an enhancement of the TJ-associated barrier in the first 3 h. To our knowledge, our study indicated for the first time that infiltration of pathogens into the epidermis enhanced TJ function via TLR2 signaling. The dynamically controlled TJs in skin are considered fundamental in preventing further invasion of pathogens and maintaining cutaneous barrier homeostasis.

One and 3 h after TLR2 activation, association of phospho-atypical PKCζ/i with occludin was observed (Fig. 4B). It is noteworthy that atypical PKCζ/i is involved in the TLR2 signaling in the innate immunity of keratinocytes. However, questions remain
regarding by which mechanisms atypical PKCζ/ι is activated and TJ functions are enhanced after TLR2 engagement. Previous evidence indicates that TLR2-mediated NF-κB activation requires a Rac1-dependent pathway (24). Rac1-GTP associates with and activates atypical PKCζ/ι (25). Furthermore, Ebnet et al. (26) reported that atypical PKCζ/ι binds to PAR-3, and this complex recruited to TJ areas with junctional adhesion molecule. In addition, the integrity of TJs appeared to be regulated by the phosphorylation of occludin through protein kinases such as c-Src and atypical PKC (27–30). In conjunction with these reports, our findings raised the possibility that the dynamic association between activated atypical PKC and PAR-3/junctional adhesion molecule enables them to concentrate in TJ areas and enhances TJ functions through phosphorylation of TJ components at 1 and 3 h after PGN stimulation.

It is important to understand why the association of phospho-atypical PKCζ/ι with occludin resulted in the enhancement of TJ function. It is reported that atypical PKC localize at the TJs of polarized epithelial cells (31, 32). The TJs especially showed a significant enrichment in phospho-atypical PKCζ/ι, an indicative active form of atypical PKCζ/ι (21). Also, in vitro experiments using rGST-occudin showed that atypical PKC bind to the C-terminal tail of occludin (22). These findings suggest that atypical PKC can bind to TJ components, at least occludin. In addition, several reports show that the phosphorylation of occludin is associated with the enhancement of TJ function (22, 29, 30). Taken together, these findings prompted us to consider that the augmentation of occludin and atypical PKC might promote the phosphorylation of occludin, which is responsible for the enhancement of TJ function. However, further investigation is necessary for understanding the precise mechanism of regulation of the TJ function.

Activation of TLR2 involves recruitment of the cytoplasmic adaptor proteins Mal and MyD88 to the TLR2 complex, which results in activation of NF-κB and subsequent production of anti-inflammatory cytokines. The question we have to consider is the secondary effects of above cytokines on the TJ barrier. Continuing studies have highlighted the regulation of the TJ barrier by cytokines. Most proinflammatory cytokines, including IFN-γ, TNF-α, IL-12, and IL-1β, cause an increase in TJ permeability, whereas some anti-inflammatory cytokines such as IL-10 and TGF-β protect against the disruption of the TJ barrier and development of inflammation (33). At a dose of 10 μg/ml PGN stimulation, shown in our study, TNF-α and IL-6 mRNAs were temporarily increased at 3 h; however, they completely returned to the normal levels at 24 h after PGN stimulation (data not shown). Stimulation with PGN at high dose (or prolonged incubation) may induce excess NF-κB activation, thus leading to epidermal inflammation through exaggerated IFN-γ, IL-12, and IL-23 production, which in turn may suppress enhancement of TJ function. Taken together, one must acknowledge that the enhancement of TJ functions via TLR2 may be supplemented by the effect of cytokines, which need to be further defined in future studies.

Until now, the relationship between innate immune response and TJ functions was investigated using intestinal epithelial cells and cholangiocytes (34, 35). In intestinal epithelial cells, stimulation with TLR2 ligands led to enhanced TER via PKCζ and PKCζ/ι activation; however, involvement of atypical PKC had not been examined. On the contrary, LPS, a ligand for TLR4, disrupts tight junctions through dephosphorylation of tyrosine residues and phosphorylation of tyrosine residues of occludin. Considering these reports and our results, there is an implication that TLR activation stimulates different PKCs in different cell types, producing different effects.

Perturbation of stratum corneum integrity renders the skin permeable to infectious agents and/or their secreted products, which triggered activation of TLRs (14, 36). We demonstrated that, in cultured keratinocytes, activation of TLR2 signaling strengthened the TJ function in the first 3 h. Collectively, our study indicates that the immediate response of TJs prevents further invasion of pathogens and maintains cutaneous barrier homeostasis. Our research findings provide new insights for the fundamental understanding of the regulatory mechanisms underlying human cutaneous barrier function, and provide a basis for developing an efficient strategy for the treatment of xerotic skin disorders.

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Disclosures
The authors have no financial conflicts of interest.

References
EFFECT OF TLR2 ON THE EPIDERMAL TIGHT JUNCTION


