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Collagen XVII (BP180) Modulates Keratinocyte Expression of the Proinflammatory Chemokine, IL-8

Françoise Van den Bergh^{1,*}, Steven L. Eliason^{2,*}, Brian T. Burmeister^{2,5}, and George J. Giudice^{2,3,4}

¹Division of Rheumatology, Medical College of Wisconsin, Milwaukee, WI

²Department of Dermatology, University of Iowa Carver College of Medicine, Iowa City, IA

³Department of Biochemistry, University of Iowa Carver College of Medicine, Iowa City, IA

⁴Graduate Program in Immunology, University of Iowa Carver College of Medicine, Iowa City, IA

Abstract

Collagen XVII (COL17), a transmembrane protein expressed in epidermal keratinocytes (EK), is targeted by pathogenic autoantibodies in bullous pemphigoid. Treatment of EK with anti-COL17 autoantibodies triggers the production of proinflammatory cytokines. In this paper we test the hypothesis that COL17 is involved in the regulation of the EK proinflammatory response, using IL-8 expression as the primary readout. The absence of COL17 in EK derived from a junctional epidermolysis bullosa patient or shRNA-mediated knockdown of COL17 in normal EK resulted in a dysregulation of IL-8 responses under various conditions. The COL17-deficient cells showed an abnormally high IL-8 response after treatment with lipopolysaccharide (LPS), ultraviolet-B radiation or tumor necrosis factor, but exhibited a blunted IL-8 response to phorbol 12-myristate 13-acetate exposure. Induction of COL17 expression in COL17-negative EK led to a normalization of the LPS-induced proinflammatory response. Although $\alpha 6\beta 4$ integrin was found to be up-regulated in COL17-deficient EK, siRNA-mediated knockdown of the $\alpha 6$ and $\beta 4$ subunits revealed that COL17's effects on the LPS IL-8 response are not dependent on this integrin. In LPS-treated cells, inhibition of NF-kappa B activity in COL17-negative EK resulted in a normalization of their IL-8 response, and expression of an NF-kappa B-driven reporter was shown to be higher in COL17-deficient, compared with normal, EK. These findings support the hypothesis that COL17 plays an important regulatory role in the EK proinflammatory response, acting largely via NF-kappa B. Future investigations will focus on further defining the molecular basis of this novel control network.

Keywords

inflammation; bullous pemphigoid; hemidesmosome; junctional epidermolysis bullosa; dermal-epidermal junction

Correspondence: Dr. George Giudice, Department of Dermatology, University of Iowa, 500 Newton Rd., Iowa City, IA 52242, USA. george-giudice@uiowa.edu.

*These authors contributed equally to this work.

⁵Current affiliation: Department of Pharmacology, University of Illinois College of Medicine, Chicago, IL

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Conflicts of Interest The authors declare that there are no conflicts of interest.

Introduction

Type XVII collagen (COL17), also referred to as BP180 or BP antigen-2, is a transmembrane protein expressed by epidermal keratinocytes (EK) and localized to the epidermal anchoring complex, a structure involved in maintaining the integrity of the dermal-epidermal junction (1-3). COL17 was first identified as a primary target of IgG autoantibodies produced by patients with bullous pemphigoid (BP), an autoimmune disease characterized by cutaneous inflammation and subepidermal blistering (4, 5). Anti-COL17 autoantibodies of the IgE and IgA isotypes are also associated with BP (6, 7). Through the use of various *in vitro* and *in vivo* models of BP, our group and others have defined critical steps in the pathogenesis of BP, which include the binding of autoantibodies to COL17 at the dermal-epidermal junction, complement activation, recruitment of inflammatory cells to the upper dermis mediated by chemokines and complement components, and secretion of proteases by activated neutrophils (8).

Several lines of evidence underscore the importance of the proinflammatory cytokines, IL-6 and IL-8, in the pathogenesis of BP. High levels of these molecules are present in the blister fluid of BP lesions (9, 10) and circulating levels of these cytokines correlate with BP disease activity (11). Studies on BP models have revealed that neutrophil recruitment, due, in part, to the chemotactic activity of IL-8, is essential for blister formation (12-14). In the passive transfer BP model, mice depleted of IL-6 are not susceptible to the pathogenic effects of anti-COL17 IgG (14). The binding of BP autoantibodies to COL17 expressed on the surface of normal human epidermal keratinocytes (NHEK) was shown to trigger the production and secretion of IL-6 and IL-8 (15). Epitope mapping revealed that these bio-active BP autoantibodies recognize two distinct, but closely spaced, epitopes located within the non-collagenous linker domain, NC16A, on the extracellular portion of COL17 (15). Antibodies that recognize the portion of murine COL17 that is homologous with human NC16A are capable of inducing skin inflammation and subepidermal blistering in murine models of BP (16). Taken together, these findings suggest that anti-COL17 antibody-induced cytokine release in BP patients' skin contributes to the inflammatory cascade that ultimately leads to dermal-epidermal separation. These findings also provided a basis for our more general hypothesis that COL17 plays an important role in the regulation of the proinflammatory response in EK.

To test this hypothesis, we analyzed the IL-8 expression patterns of COL17-deficient and -sufficient EK that were induced by exposure to several well-characterized inflammatory stimuli, including lipopolysaccharide (LPS) from a Gram-negative bacterium, phorbol 12-myristate 13-acetate (PMA), ultraviolet-B (UVB) radiation, and tumor necrosis factor (TNF). Our findings point to COL17 as a newly identified modulator of the EK proinflammatory response.

Materials and Methods

Keratinocyte cell lines

NHEK and a COL17-deficient EK line (JEBEK) derived from a patient with non-Herlitz junctional epidermolysis bullosa (JEBnH) (17-19) were generously provided by Dr. Kim Yancey (U. Texas Southwestern Med. Sch.). COL17-positive JEBEK (JEBEK+) were generated by transduction of JEBEK with a retroviral-based COL17 expression vector (20). All EK cultures were grown in Keratinocyte-SFM (Invitrogen, Carlsbad, CA). As shown in Fig. S1, we confirmed by flow cytometric analyses that NHEK and NHEK-1° (primary culture of NHEK purchased from Cascade Biologicals, Carlsbad, CA) do express COL17 on their surface, while JEBEK do not. In the JEBEK+ line, 13.5% of the cells were positive for COL17 expression.

Inflammatory stimuli and inhibitors

UVB radiation (50 to 500 mJ/cm²) was delivered using TL100 W/01-311nm narrowband UVB lamps (National Biological Corporation, Beachwood, OH). Keratinocytes were also treated with the following inflammatory agents and signaling inhibitors (all from Sigma, St. Louis, MO): PMA (5-80 ng/ml in 0.1% DMSO), LPS (12.5-100 µg/ml), TNF (5 ng/ml), BAY-11-7082, an inhibitor of NF-κB (nuclear factor-kappa B) (250 nM to 10 µM), and SB 203580, a p38MAPK (mitogen-activated protein kinase) inhibitor (5 to 100 µM).

Immunological analyses

Antibodies to the following antigens were used for immunoblot and/or flow cytometric analyses (University of Iowa Flow Cytometry Facility) using protocols described previously (21): COL17 [R136 and R594 (21)]; α6 integrin (Abcam, Cambridge, MA); α3 and β4 integrin and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). Levels of α6 integrin in EK were quantified by flow cytometry, since an anti-α6 antibody that works well in Western blotting was not available. Conversely, the β4 integrin subunit was quantified by Western blotting, since this protein, which has a small extracellular domain, is difficult to detect by flow cytometry.

Transfections (siRNA and reporter constructs)

Experiments involving down-regulation of COL17 were carried out by co-transfecting a GFP-expressing plasmid and either the COL17-specific shRNA or control LacZ shRNA, as previously described (21). For knock down of α6β4 integrin, subunit-specific siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) were used as recommended by the manufacturer. AP-1 and NF-κB activities were analyzed by transfecting EK with the corresponding reporter constructs (Qiagen, Frederick, MD) and assaying the reporter activities using the Dual luciferase system (Promega, Madison, WI).

Analysis of IL-8 expression

IL-8 protein in culture supernatants was quantified using the Duo-set IL-8 ELISA system. (R&D, Systems, Minneapolis, MN). To measure IL-8 mRNA levels, quantitative PCR (qPCR) was performed on cDNAs prepared from EK mRNA (High Capacity cDNA kit; Applied Biosystems, Forest City, CA). For qPCR analysis the following Applied Biosystems primer sets were used: IL-8, Hs999999034_m1; IL-6, Hs00174131_m1; COL17A1, Hs00166711_m1; and GAPDH, Hs02758991_g1. Relative expression was determined using the 2^{-ΔΔC_t} method (22).

Statistical analyses

ANOVA was used to test for cell line effects under each treatment condition. In certain cases, a natural log transformation was applied to the data to satisfy the ANOVA assumption of homogeneity of variance. Pairwise comparisons of cell lines under various conditions were carried out using a test of mean contrast, and the resulting p-values were adjusted using Bonferroni's method to account for the number of tests performed.

Results

Cell detachment and replating induces a robust IL-8 response in COL17-deficient, but not COL17-sufficient, human keratinocytes

Analysis of cytokine production by the COL17-negative (JEBEK) and -positive (NHEK and JEBEK+) cell lines under standard culture conditions provided the first indication that COL17 expression can affect IL-8 expression. The cells were detached from their substrate by trypsinization and replated onto tissue culture plastic. A representative analysis of IL-8

protein and mRNA levels produced by these cells is presented in Fig. S2. The NHEK line produced only a small amount of IL-8 after replating, with a peak production of 31 pg/ml at 4h post-plating, while under the same conditions JEBEK produced a much higher level of IL-8 that peaked at 4 h (141 pg/ml IL-8, $p<0.02$). The peak production of IL-8 by JEBEK+ (81 pg/ml) was markedly lower than that of JEBEK ($p<0.03$), yet higher than that of NHEK ($p<0.05$). Analysis by qPCR revealed that after replating the IL-8 mRNA levels in JEBEK and JEBEK+ exhibited a rapid rise and fall, with peaks at the 2 h time point (2.8 and 1.2 relative units, respectively). No significant changes in the levels of IL-8 mRNA were detected in either of the COL17-sufficient EK lines (NHEK and NHEK-1°) during the post-plating period. These experiments were carried out by detaching the cells via a standard trypsinization protocol, but detaching the cells with EDTA yielded very similar results (data not shown), i.e., JEBEK produced dramatically higher levels of IL-8 at both the protein and mRNA levels compared with NHEK, while JEBEK+ showed an intermediate IL-8 response. These IL-8 expression patterns were also not affected by varying the substrates upon which the cells were plated, e.g., laminins 111 and 332, fibronectin, types I and IV collagen, HUVEC ECM or untreated tissue culture plastic (data not shown).

Using the replating assay, we compared the IL-8 responses of NHEK (HPV16 E6/E7-immortalized line) and NHEK-1°, a primary culture. These two EK preparations were shown to express comparable levels of COL17 on their surface (Fig. S1, a and b), and both expressed low levels of IL-8 in the replating assay (Fig. S2, a and b). The immortalized and primary cultured EK also exhibited very similar IL-8 responses when stimulated with the other inflammatory stimuli used in this study (data not shown). These data indicate that HPV16 E6/E7 immortalization does not have a large impact on the EK parameters analyzed in this study.

COL17-deficient keratinocytes exhibit an abnormally high IL-8 response to several inflammatory stimuli

We next investigated whether COL17 affected IL-8 expression in EK after exposure to inflammatory agents, including UVB, LPS, PMA and TNF. In order to avoid the above-described transient spike in IL-8 expression that occurs after cell passage, the COL17-positive and -negative cells were transferred to the assay plates and incubated for 24h prior to treatment with an inflammatory agent. Figure 1, **panels a and b**, shows the IL-8 expression patterns of COL17-positive and -negative cells at the 4 h time point following a dose of 500 mJ/cm² UVB. The level of IL-8 secreted by JEBEK (233 pg/ml) was much higher than those of NHEK and JEBEK+ (36 pg/ml, $p<0.01$; and 68 pg/ml, $p<0.02$, respectively), as determined by two-way ANOVA followed by a test of mean contrast with Bonferroni correction. Under these same experimental conditions, the IL-8 mRNA level in the JEBEK was also significantly higher than the levels in the other two lines (each pairwise comparison, $p<0.04$). Figure S3 shows the UVB dose response of IL-8 produced by NHEK and JEBEK.

The graphs in Fig. 1, c,d demonstrate that the general patterns of IL-8 expression induced by treatment of these three cell lines with LPS are similar to those induced by UVB. In response to 25 µg/ml LPS, JEBEK exhibited a much more robust IL-8 response at both the protein (152 pg/ml) and mRNA levels (12.3 relative units) compared with that of NHEK (54 pg/ml IL-8 protein, $p<0.02$; and 2.0 units of IL-8 mRNA, $p<0.01$) and JEBEK+ (97 pg/ml protein, $p<0.05$; and 2.9 units mRNA, $p<0.01$). Treatment of these cell lines with other doses of LPS (5, 10 and 50 µg/ml) for 2 or 4 h yielded the same negative relationship between COL17 expression and IL-8 response (data not shown). Likewise, when TNF (5 ng/ml for 4 h) was used as the stimulus, JEBEK expressed IL-8 protein and mRNA at levels

higher than those of NHEK (Fig. S4), following the same patterns as those induced by UVB and LPS.

These same three EK lines that vary in COL17 expression also exhibited differential IL-8 responses after exposure to PMA; however, the general pattern was quite different from those seen in UVB-, LPS-, and TNF-treated cells. Fig. 1e,f shows representative IL-8 expression data obtained after a 4 h treatment of 20 ng/ml PMA. The level of IL-8 protein expressed by JEBEK+ (239 pg/ml) was higher than that of JEBEK (182 pg/ml, $p < 0.05$), but lower than that of NHEK (351 pg/ml, $p < 0.02$). Using other PMA doses (10 and 40 ng/ml) and exposure times (1 and 2 h), we observed this same rank order of IL-8 expression among the 3 cell lines (data not shown). Thus, in stark contrast to the above data on IL-8 responses to UVB, LPS and TNF, we now find a positive relationship between COL17 expression and the IL-8 response to PMA. Interestingly, the relative levels of IL-8 mRNA measured in these 3 lines after PMA treatment showed no significant differences (Fig. 1f).

shRNA-mediated knockdown of COL17 expression in NHEK leads to an enhanced IL-8 response

The above data suggest that the level of COL17 expression in EK is inversely related to their IL-8 response after treatment with LPS. To facilitate further testing of this phenomenon, we transfected NHEK, JEBEK and JEBEK+ with COL17-specific and control shRNA constructs and compared the LPS-induced IL-8 responses of these cells (Fig. 2). COL17-knock-down NHEK responded to LPS with an IL-8 expression level that was significantly higher than that of the control NHEK ($p < 0.03$), but still lower than that of the COL17-deficient JEBEK ($p < 0.02$). JEBEK+(control shRNA) produced a level of IL-8 in response to LPS that was significantly lower than either JEBEK(control shRNA) ($p < 0.03$) or the COL17-knock-down JEBEK+ ($p < 0.05$). These data provide further support for the idea that COL17 expression levels are negatively correlated with the LPS-induced IL-8 response in EK.

Up-regulation of $\alpha 6\beta 4$ integrin in COL17-deficient keratinocytes does not account for the enhanced LPS-induced IL-8 response in these cells

COL17 and $\alpha 6\beta 4$ integrin are the two major transmembrane components of the hemidesmosomal anchoring complex (3). COL17 forms stable interactions with $\alpha 6\beta 4$ integrin (23, 24), and in conditional $\beta 4$ -integrin knockout mice, a marked reduction in the levels of COL17 and other hemidesmosomal components was observed in $\beta 4$ -deficient areas of the epidermis (25). These investigators also reported that the loss of $\alpha 6\beta 4$ frequently led to chronic skin inflammation (particularly in the ears of the $\beta 4$ knock-out mice); however, the molecular mechanisms underlying these phenomena are unknown. Because of these biochemical relationships between COL17 and $\alpha 6\beta 4$ integrin, we decided to test whether the observed COL17-related effects on the EK IL-8 response were linked to $\alpha 6\beta 4$ activity. For technical reasons (see Methods), $\alpha 6$ and $\beta 4$ levels were quantified by flow cytometry and Western blotting, respectively. As shown in Fig. 3, a and b, expression of both chains of $\alpha 6\beta 4$ integrin are up-regulated in JEBEK (COL17-negative) relative to levels in NHEK. The control siRNA-transfected NHEK and JEBEK labeled with an anti- $\alpha 6$ antibody exhibited flow cytometric MFI of 546 and 1,233, respectively. The intensities of the anti- $\beta 4$ integrin Western blot bands from control siRNA-transfected NHEK and JEBEK lysates were 63 and 102 relative units, respectively. These differences were reproducible in three experiments.

If the abnormally high levels of $\alpha 6\beta 4$ integrin in JEBEK account for their altered IL-8 responses, then a reduction in this integrin expression should tend to normalize these responses. We tested this idea by decreasing the expression levels of the $\alpha 6$ and $\beta 4$ integrin subunits in JEBEK and NHEK using siRNAs (Fig. 3, a and b). Note that for each integrin

subunit, the expression level in JEBEK treated with the integrin-specific siRNA has been reduced to an extent that approximates that in the control NHEK. This down-regulation of $\alpha 6$ or $\beta 4$ integrin subunit in JEBEK does not lead to a significant change in the LPS-induced IL-8 response of this line (Fig. 3c). It is clear from these data that the abnormally high expression levels of $\alpha 6$ and $\beta 4$ integrin subunits in JEBEK do not account for this cell line's enhanced LPS-induced IL-8 response.

NF- κ B plays a role in mediating COL17's effects on the EK IL-8 response

NF- κ B and p38MAPK are known to play key roles in cell signaling that leads to the regulation of IL-8 expression in epithelial cells (26-29). To begin our analysis of the mechanisms underlying the enhanced LPS-induced IL-8 response in COL17-deficient EK, we treated JEBEK and NHEK with various concentrations of Bay-11-7082, an NF- κ B inhibitor (250nM, 500nM, 1 μ M, 2.5 μ M, 5 μ M, 10 μ M) or SB203580, a p38MAPK inhibitor (5, 10, 25, 50, 100 μ M) for 12 h prior to analysis of their LPS-induced IL-8 response (25 μ g/ml LPS for 4 h). As shown in Fig. 4a, in the absence of either inhibitor JEBEK expressed IL-8 at a much higher level than did NHEK, as previously demonstrated in this report. If this enhanced IL-8 response in JEBEK is mediated via NF- κ B, then inhibition of this pathway would tend to normalize this IL-8 response in JEBEK. Indeed, our data are consistent with this idea. Addition of increasing concentrations of Bay-11-7082 to the cells resulted in decreasing differences in the LPS-induced IL-8 responses of JEBEK and NHEK. In contrast, increasing concentrations of SB203580 did not tend to decrease the differences in NHEK and JEBEK IL-8 responses to LPS. Fig. 4b shows the results of a qPCR analysis of IL-8 mRNA levels in NHEK and JEBEK treated with LPS in the presence or absence of the NF- κ B inhibitor, BAY-11-7082. In the absence of inhibitor, NHEK exhibited a modest increase in IL-8 mRNA level after LPS treatment; however, a comparison of the IL-8 mRNA levels expressed by LPS-treated NHEK with and without this inhibitor showed no significant difference. Interestingly, the LPS-induced IL-8 mRNA level of JEBEK, which was much higher than that of LPS-treated NHEK, was reduced to a level very close to that of LPS-treated NHEK in the presence of BAY-11-7082. These data support the idea that NF- κ B plays a role in the enhanced LPS-induced IL-8 response seen in COL17-negative EK.

To further probe this issue, we transfected NHEK and JEBEK with luciferase reporter constructs under the control of either NF- κ B or AP-1 (a key IL-8 transcription regulator that is activated by the p38MAPK signaling pathway). The transfected cells were treated with LPS for 4 h and then assayed for luciferase activity. As shown in Fig. 4c, in cells not treated with LPS, the expression level of the NF- κ B-driven reporter was significantly higher in JEBEK than in NHEK ($p < 0.02$), and this expression differential was extended after LPS exposure ($p < 0.01$). Expression of the AP-1-driven reporter was also higher in JEBEK than in NHEK ($p < 0.05$); however, LPS treatment of JEBEK did not result in a significant increase in the AP-1 reporter expression of these cells. Taken together, the data from these inhibitor and reporter gene analyses are consistent with the idea that NF- κ B plays a prominent role in mediating COL17's effects on the LPS-induced IL-8 response in EK.

Discussion

Transcription of the IL-8 gene occurs largely through the activities of NF- κ B and activating protein-1 (AP-1) (30, 31). The inflammatory stimuli, LPS, UVB, TNF and PMA, were chosen for use in this investigation because their effects on the activities of NF- κ B and AP-1 are known to be carried out through distinct mechanisms. LPS and TNF are sensed by EK primarily through the binding of these ligands to Toll-like receptor (TLR)4 and TNF receptor type 1 (TNF-R1), respectively (32, 33). The activated TNF-R1 stimulates the NF- κ B pathway through complexes that include TRAF (TNF receptor-associated factor), RIP (receptor-interacting protein), and TRADD (TNF-receptor-associated death domain) family

members (29, 34-37). LPS-triggered activation of NF- κ B and p38MAPK in EK typically involves a MyD88-dependent signaling pathway (29, 36, 38). UVB irradiation of HaCaT cells induces rapid release of nucleotides, which act as intercellular signaling molecules through activation of P2Y surface receptors that initiate downstream activation of AP-1 and NF- κ B (39). UVB also affects PKA, PKC and inflammasome activities that modulate IL-8 expression (30, 40-42). PMA's effects on IL-8 expression are known to involve PKC and epidermal growth factor receptor complexes (43-45).

The findings from this study provide the first evidence that IL-8 responses to all four of these inflammatory stimuli can be modulated by COL17. Utilizing EK lines that vary in COL17 expression, we observed a negative relationship between the cells' COL17 levels and their IL-8 responses to three of these stimuli -- LPS, UVB and TNF. The absence of COL17 in EK derived from a JEBnH patient and shRNA-mediated deficiency of COL17 in NHEK led to abnormally high IL-8 responses to these inflammation triggers. And a partial rescue of the COL17 deficiency in JEBEK by genetic manipulation led to a normalization of this line's IL-8 responses to LPS and UVB. A normalization of the JEBEK IL-8 response was also achieved by inhibition of NF- κ B, but not p38MAPK, activity. Consistent with this, an NF- κ B-driven reporter gene was found to be expressed at a higher level in LPS-treated JEBEK compared with LPS-treated NHEK. These findings point to NF- κ B as an important mediator of COL17's effects on the LPS-induced IL-8 response in EK. Interestingly, the fourth stimulus, PMA, was found to induce EK IL-8 responses that are positively correlated with COL17 expression. Thus, decreased expression levels of COL17 can have either an enhancing or suppressing effect on the IL-8 response, depending upon the external stimulus. These data imply that COL17 has distinct effects on multiple inflammation-related signaling elements. In the simplest scenario, COL17 can act to down-regulate a signal node that is present in the proinflammatory pathways used by UVB, LPS and TNF, but which is not used by PMA, and can also act to up-regulate another node that is important for PMA signaling, but not for the other three stimuli.

Our findings have implications in both acquired and inherited bullous dermatoses. COL17 is the primary antigenic target of pathogenic autoantibodies in BP (13, 46). Two of the essential and early phase steps in BP are the binding of anti-COL17 autoantibodies to the cutaneous basement membrane zone and initiation of skin inflammation (14, 47, 48). While this antibody-mediated inflammatory response has been shown to be dependent upon complement activation in BP mouse models, it is also clear that anti-COL17 BP autoantibodies can trigger a proinflammatory response in NHEK in a complement-independent manner (15, 49). In light of our present findings, we speculate that this second mode of promoting inflammation is caused by BP autoantibody-mediated alterations in COL17's effects on the EK pro-inflammatory response. One possible mechanism is that the autoantibodies disrupt COL17's normal interactions with other components of the hemidesmosomal complex, e.g., α 6 β 4 integrin, laminin 332, type IV collagen. Such intermolecular disruptions could play a role in the COL17-dependent changes in the proinflammatory responses documented in this report. In JEBnH, which is caused by mutations -- often null-type mutations -- in the gene encoding COL17, skin blistering is typically seen at sites of friction and trauma. The lesion-associated inflammation is generally interpreted as a consequence of tissue damage. However, in light of the findings presented here, we speculate that the skin of JEBnH patients is primed for dysregulated inflammatory responses that can contribute to lesion formation.

Integrin α 6 β 4, like COL17, is a component of the epidermal anchoring complex, and COL17 has been shown to associate with both the α 6 and β 4 subunits (23, 24, 50). Skin-conditional deletion of the α 6 gene in mice has been shown to be associated with spontaneous inflammation and alterations in keratinocyte differentiation (51, 52). Here, we

found that both the $\alpha 6$ and $\beta 4$ integrin subunits are up-regulated in the COL17-deficient JEBEK, which raised the question about whether COL17's effects on IL-8 expression are dependent upon $\alpha 6\beta 4$ activity. Since down-regulating the expression of these integrin subunits did not result in a reduction in the robust LPS-induced IL-8 response in JEBEK, we conclude that the observed COL17 effects on this IL-8 response are not dependent on $\alpha 6\beta 4$ activity.

In conclusion, the findings presented in this report implicate COL17 as a key player in the regulation of EK proinflammatory responses, a previously unrecognized function of this protein. In addition to the potential relevance of this activity in autoimmune and inherited blistering diseases described above, we further postulate that this newly identified COL17 activity contributes to the initiation of epithelial inflammation within a variety of contexts, e.g., allergy, cancers, metabolic diseases, physical trauma, and exposure to noxious agents. These new findings identify COL17 as a potential target for strategies designed to control skin inflammation under certain conditions. Future investigations will focus on unraveling the molecular networks that mediate COL17's influence on IL-8 expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AP-1	activator protein-1
BP	bullous pemphigoid
COL17	type XVII collagen
EK	epidermal keratinocyte
NHEK	normal human epidermal keratinocyte line
JEBEK	epidermal keratinocyte line from a junctional epidermolysis bullosa patient
LPS	lipopolysaccharide
NF-κB	nuclear factor-kappa B
NHEK-1°	primary cultured normal human epidermal keratinocytes
MAPK	mitogen-activated protein kinase
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
TNF	tumor necrosis factor
UVB	ultraviolet-B radiation

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Figure 1. Alterations in COL17 expression in EK are associated with a dysregulation of the pro-inflammatory response

EK with normal and abnormal COL17 expression levels were exposed to UVB radiation (500 mJ/cm² over a period of 1 minute; **panels a and b**), 25 µg/ml LPS (**c,d**), or 20 ng/ml PMA (**e,f**). The culture supernatants were harvested 4 h after the start of treatment and analyzed by ELISA for IL-8 protein (**a,c,e**). At this same time point, mRNA was isolated from the cells and analyzed by qPCR for IL-8 mRNA (**b,d,f**). Each bar in this figure represents the average of three assay results ± S.D. The data shown are representative of a minimum of three experiments. Compared with UVB-treated NHEK and JEBEK+, UVB-treated JEBEK produced higher levels of IL-8 protein (**panel a**; each p<0.02) and mRNA (**b**; each p<0.04). Likewise, LPS-treated JEBEK produced higher levels of IL-8 protein (**c**) and mRNA (**d**) compared with LPS-treated NHEK [p(protein)<0.02; p(mRNA)<0.01] and JEBEK+ [p(protein)<0.05; p(mRNA)<0.01]. In contrast, after PMA treatment (**e and f**) NHEK produced a higher level of IL-8 protein than either JEBEK+ (p<0.02) or JEBEK (p<0.02), but there were no significant differences in the IL-8 mRNA levels produced by these 3 cell lines. Black bars = JEBEK; Dark gray bars = JEBEK+; light gray bars = NHEK.

Figure 2. shRNA-mediated knockdown of COL17 expression in NHEK leads to an enhancement of the LPS-induced IL-8 response

(a) JEBEK and NHEK were transfected with a GFP expression construct alone (“mock”) or were co-transfected with GFP and either a control shRNA (lacZ) or COL17-specific shRNA, as indicated. The bar graph shows the COL17 labeling intensities of the GFP-positive cells, as determined by flow cytometry and represented as mean fluorescence intensity (MFI). The transfection efficiencies ranged between 32-36%. The data are representative of three experiments. (b) NHEK, JEBEK (labeled “JEB”) and JEBEK+ (“JEB+”) transfected with either the COL17-specific shRNA (labeled “C17”) or the lacZ shRNA construct were exposed to 25 μ g/ml LPS (black bars) or plain medium (gray bars) for 4 hours, after which the IL-8 levels in the culture supernatant fractions were measured by ELISA. After LPS treatment, the level of IL-8 produced by the COL17 knockdown NHEK was significantly higher than that of the control knockdown NHEK (indicated by an asterisk; $p < 0.03$). In response to LPS, COL17-positive JEBEK+(control shRNA) expressed significantly less IL-8 than either JEBEK(control shRNA; $p < 0.03$) or COL17-knock-down JEBEK+ ($p < 0.05$). As expected, the COL17 shRNA had no effect on the IL-8 response of JEBEK. The data shown are representative of three experiments. Each bar represents the average $\pm$ S.D. of triplicate assay results.

Figure 3. The up-regulation of $\alpha 6\beta 4$ integrin expression in a COL17-deficient EK line is not essential for the enhanced IL-8 response of these cells

(a) JEBEK and NHEK were transfected with an $\alpha 6$ integrin-specific (si- $\alpha 6$) or control siRNA (si-ctrl) and then analyzed by flow cytometry for expression of $\alpha 6$ integrin. In the control-transfected cells (as well as in untransfected cells -- data not shown), JEBEK expressed a level of $\alpha 6$ integrin on their surface that was much higher than that of NHEK (MFI = 1,233 and 546 relative units, respectively). Transfection with the $\alpha 6$ -specific siRNA reduced $\alpha 6$ expression by 52% in JEBEK and by 40% in NHEK. **Panel b** shows the results of a representative Western blot analysis (n=3) of $\beta 4$ integrin expression in JEBEK and NHEK that were transfected with a $\beta 4$ integrin-specific (si- $\beta 4$) or a control siRNA (si-ctrl). In si-ctrl-transfected cells, JEBEK expressed 62% more $\beta 4$ integrin compared with that of NHEK. After transfection with the $\beta 4$ siRNA, $\beta 4$ integrin expression was reduced in JEBEK and NHEK by 46% and 36%, respectively. **Panel c** shows the analysis of LPS-induced IL-8 expression by JEBEK and NHEK transfected with the $\alpha 6$ / $\beta 4$ integrin-specific and control siRNAs described above. No significant differences were seen in the pairwise comparisons within each cell line. The data shown in each of the three panels are representative of three experiments. In panel c, each bar represents the average $\pm$ S.D. of triplicate assay results.

Figure 4. Inhibitor and reporter gene analyses point to NFκB as a mediator of COL17's effects on the EK IL-8 response

(a) NHEK (gray bars) and JEBEK (black bars) were treated for 12 h with Bay-11-7082 ("BAY", an NFκB inhibitor) or SB203580 ("SB", a p38MAPK inhibitor) prior to a 4 h incubation with 25 μg/ml LPS or vehicle control. Addition of increasing concentrations of Bay-11-7082 to the cells corresponded to decreasing differences in IL-8 levels of NHEK and JEBEK. This trend was observed throughout the range of Bay-11-7082 concentrations tested, only two of which are shown – 250nM, 500nM, 1μM, 2.5μM, 5μM, 10μM. Increasing concentrations of SB203580 (5, 10, 25, 50, 100μM; data for 10 and 50 μM are shown) did not show this same trend. (b) NHEK and JEBEK (labeled "JEB") were treated with TNF, LPS or control in the presence or absence of Bay-11-7082. Comparing with vs. without inhibitor, significant decreases (asterisks) in IL-8 levels were seen in TNF-treated NHEK ($p < 0.05$), as well as in TNF- and LPS-treated JEBEK (both p values < 0.05). (c) NHEK and JEBEK were transfected with either an NFκB-driven or AP-1-driven luciferase gene construct and then treated with LPS for 4h in the presence or absence of Bay-11-7082 or SB203580. Expression of the NFκB-driven reporter was much higher in LPS-treated JEBEK compared with either LPS-treated NHEK ($p < 0.01$) or JEBEK without LPS ($p < 0.01$). As far as the AP-1-driven reporter, expression in LPS-treated JEBEK was higher than in LPS-treated NHEK ($p < 0.05$), but was not significantly higher than in JEBEK without LPS. It is noteworthy that both reporter genes were expressed at higher levels in JEBEK vs. NHEK in the absence of LPS. The data shown in each of the three panels are representative of three experiments. Each bar represents the average $\pm$ S.D. of triplicate assay results.