

iRHOM2-dependent regulation of ADAM17 in cutaneous disease and epidermal barrier function

Matthew A. Brooke¹, Sarah L. Etheridge¹, Nihal Kaplan², Charlotte Simpson¹, Edel A. O’Toole¹, Akemi Ishida-Yamamoto³, Olivier Marches¹, Spiro Getsios² and David P. Kelsell¹,*

¹Blizard Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, London, UK, ²Department of Dermatology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA and ³Department of Dermatology, Asahikawa Medical University, Asahikawa, Japan

Received October 24, 2013; Revised and Accepted March 13, 2014

iRHOM2 is a highly conserved, catalytically inactive member of the Rhomboid family, which has recently been shown to regulate the maturation of the multi-substrate ectodomain sheddase enzyme ADAM17 (TACE) in macrophages. Dominant iRHOM2 mutations are the cause of the inherited cutaneous and oesophageal cancer-susceptibility syndrome tylosis with oesophageal cancer (TOC), suggesting a role for this protein in epithelial cells. Here, using tissues derived from TOC patients, we demonstrate that TOC-associated mutations in iRHOM2 cause an increase in the maturation and activity of ADAM17 in epidermal keratinocytes, resulting in significantly upregulated shedding of ADAM17 substrates, including EGF-family growth factors and pro-inflammatory cytokines. This activity is accompanied by increased EGFR activity, increased desmosome processing and the presence of immature epidermal desmosomes, upregulated epidermal transglutaminase activity and heightened resistance to Staphylococcal infection in TOC keratinocytes. Many of these features are consistent with the presence of a constitutive wound-healing-like phenotype in TOC epidermis, which may shed light on a novel pathway in skin repair, regeneration and inflammation.

INTRODUCTION

iRHOM2 is a highly conserved, catalytically inactive protein belonging to the Rhomboid family of intramembrane serine proteases (1,2). In addition to its role in the regulation of EGF processing (3), iRHOM2 was recently identified as a novel regulator of the multi-substrate ectodomain sheddase enzyme ADAM17 (TNFα-converting enzyme; TACE) (4,5). ADAM17 is an enzyme required for the proteolytic cleavage and release of a wide spectrum of substrates from the cell surface (6,7), including TNFα, multiple members of the EGF family of growth factors (8–10) and components of the desmosome (11,12). In some cell types, iRHOM2 controls ADAM17 maturation, by regulating the transit of Pro-ADAM17 from the endoplasmic reticulum (ER) to the Golgi apparatus (4), where ADAM17 is activated by removal of its inhibitory pro-domain by pro-protein convertase enzymes such as Furin (13). Although iRhom2²/² mice are viable and show no obvious defects, they fail to control the replication of the pathogenic bacterium Listeria monocytogenes, as a result of impaired shedding of the ADAM17 substrate TNFα (5). Similarly, iRhom2²/² mice were recently shown to be protected from inflammatory arthritis to the same extent as mice lacking either ADAM17 or TNFα (14). In contrast, Adam17−/− mice die perinatally (9), recapitulating the phenotypes of mice lacking various EGFR ligands shed under the control of ADAM17, such as severe failures of epithelial (9) and cardiac (15,16) morphogenesis and maturation, which result from knockouts of TGFn and HB-EGF, respectively. The apparent paradox of the requirement for iRHOM2 in ADAM17 maturation, the lethality of Adam17 knockout mice and the seeming lack of effect of iRhom2 knockout may in part be explained by recent evidence showing that the closely related iRHOM1 may support ADAM17 maturation in iRHOM2-deficient cells (14). Indeed, both iRHOM1 and 2 have been shown to support the trafficking and maturation of ADAM17 in numerous murine cell types (17), with a high degree of redundancy observed between the two iRHOMs in tissues where both are highly expressed (including the skin). Murine knockouts of iRhom1 result in lethality after between 9 days and 6 weeks (dependent on mouse genetic..
background) and a phenotype much more severe than knockout of iRhom2, the phenotype of which appears to be immune cell-specific in mice (17).

Recently, we described the first example of ADAM17-deficiency in humans; unlike in the mouse model, this was compatible with life with one affected individual surviving into adulthood (18). This recessive syndrome was characterized by a severe inflammatory skin phenotype, greatly increased susceptibility to cutaneous and paronychial infection, bowel inflammation and a moderate cardiac phenotype. Tissue-specific knockouts of Adam17 in mice have shed light on this phenotype, with keratinocyte-restricted knockouts producing a skin phenotype very similar to that seen in humans, resulting from a defective epidermal barrier and impaired transglutaminase activity (19). Additionally, hypomorphic Adam17 mice show significantly increased susceptibility to inflammatory colitis (20).

Tylosis with oesophageal cancer (TOC; OMIM: 148500) is a dominantly inherited syndrome of palmoplantar keratoderma, oral and oesophageal leukoplakia, follicular keratosis and a striking susceptibility to oesophageal squamous cell carcinoma (OSCC). We recently reported the association of TOC in three large families (from the UK, USA and Germany) with heterozygous point mutations in RHBDF2, the gene encoding iRHOM2 (21). This finding was subsequently confirmed in a study on a separate Finnish family (22). Interestingly, although the mutations in each family arose independently, they display a remarkable clustering, with all four families’ mutations found within the same four residues (p.Ile186Thr in the UK and US, p.Asp188Ans in the Finnish and p.Pro189Leu in the German families) in a highly conserved N-terminal cytoplasmic tail domain unique to the iRHOMs. Intriguingly, these residues are also conserved in iRHOM1, which has been shown to share the ability of iRHOM2 to support ADAM17 maturation. This suggests that the affected domain may play a role in this process. Prior to the reports of a direct iRHOM2/ADAM17 interaction, we described abnormal, plasma-membranous iRHOM localization and dysregulated EGFR signalling in TOC keratinocytes (21), making the manner in which ADAM17 is affected by TOC-associated iRHOM2 mutations of particular interest. Here, we describe investigations of the iRHOM2/ADAM17 axis in the epidermis, keratinocytes and peripheral blood mononuclear cells (PBMCs) from TOC-affected individuals and unaffected controls. We demonstrate that ADAM17 maturation and activity is increased in TOC. These data support a key role for this axis in EGFR ligand shedding in the epithelium, keratinocyte barrier function, migration and bacterial infection.

RESULTS

iRHOM2 has a role in the regulation of ADAM17 in keratinocytes

To investigate the effect of TOC-associated iRHOM2 mutations on ADAM17, studies were carried out in immortalized keratinocyte cell lines derived from two TOC patients (TYLK1 and TYLK2—a male and a female) carrying the UK iRHOM2 mutation. These keratinocytes have been previously used in the study of the pathophysiology of TOC (21) and were immortalized by transfection with human papilloma virus (HPV)-16 open reading frames E6 and E7 (23-24). Throughout, these cell lines were compared with a pair of control keratinocyte cell lines immortalized in the same manner, both of which have previously been described [K17 (25) and Neb1 (26)]. To assess the role of iRHOM2 in ADAM17 maturation in keratinocytes, siRNA was used to separately knock down the expression of iRHOM2 and ADAM17 in the four cell lines studied (Fig. 1), after which western blotting of ADAM17 and iRHOM2 was performed. Western blotting of ADAM17, using an antibody whose epitope lies in the enzyme’s active site domain (and which therefore detects ADAM17 in both its pro-protein form, and the mature, active form which results from cleavage by pro-protein convertases in the Golgi), revealed the presence of a significantly increased level of the mature, active form of ADAM17 relative to pro-ADAM17 in untreated TOC cell lines compared with controls (P < 0.01; Fig. 1, *symbol). siRNA knockdown of iRHOM2 abolished this increased level of mature ADAM17 in TOC keratinocytes, leading to a significant reduction in mature ADAM17 in both TYLK1 and TYLK2 cell lines (P < 0.01; Fig. 1, § symbols) but not the two control cell lines. iRHOM2 knockdown would not be expected to completely block ADAM17 maturation, owing to the co-expression of iRHOM1 in keratinocytes. This suggests that iRHOM2 does play a role in ADAM17 maturation in keratinocytes. Knockdown of ADAM17 led to a significant reduction in

Figure 1. ADAM17 and iRHOM2 expression in control and TOC keratinocyte cell lines. Expression of iRHOM2 and ADAM17 is shown in two control (Neb1 and K17) and two TOC (TYLK1 and 2) keratinocyte cell lines in whole cell lysates collected 24 h after transfection with NTP siRNA, or siRNA against either ADAM17 (si-A17) or iRHOM2 (si-iR2). The graph shown represents results from three separate experiments. *signifies that expression of mature ADAM17 is individually significantly higher in both TOC cell lines than both control cell lines (P < 0.01, in all cases); #signifies that expression of both pro- and mature ADAM17 is significantly reduced in all keratinocytes treated with si-A17, compared with those treated with NTP (P < 0.01, in all cases); §symbols signify that expression of mature ADAM17 is significantly reduced in both TOC cell lines treated with si-iR2 compared with NTP, but not in either of the control cell lines (P < 0.05, in each case).
both pro- and mature ADAM17 in all four cell lines ($P < 0.05$, in all cases; Fig. 1, # symbol) but also interestingly caused a corresponding reduction in expression of iRHOM2 in all four cell lines, further illustrating the close relationship between the two proteins in keratinocytes. iRHOM2 expression in NTP-treated keratinocytes did appear slightly higher in TOC keratinocytes, but when this was quantified over three separate experiments, this difference was not found to be significant. iRHOM2 reduction was also observed in the epidermis from a human ADAM17 knockout individual (Supplementary Material, Fig. S1) and, in murine iRHOM1 and 2 knockouts, a reduction in the overall level of ADAM17 expression has also been observed (17).

**ADAM17 maturation and localization is enhanced in TOC keratinocytes**

To further investigate the observation of increased ADAM17 maturation in TOC keratinocytes, immunocytochemical co-staining of ADAM17 alongside markers of the ER, Golgi apparatus and plasma membrane was performed in the four cell lines previously described. ADAM17 displayed clear staining at the plasma membrane and also within the Golgi apparatus (Fig. 2A). However, when comparing these cell lines, it was noticeable that the two TOC cell lines displayed greater levels of ADAM17 at the plasma membrane—its major site of activity (Fig. 2A). Western blotting using an antibody directed against the ADAM17 pro-domain revealed a greater presence of the 55-kDa band representing the pro-domain—produced by ADAM17 cleavage by pro-protein convertase enzymes—in TOC keratinocytes compared with controls (Fig. 2B). Next, organotypic three-dimensional cultures of these TOC cell lines and a control (K17) were performed. These 3D cultures yielded fully differentiated skin equivalent models for each and a control (Fig. 2E and F). These results in monolayer and organotypic culture suggested the possibility of increased levels of ADAM17 maturation and activity in TOC cells compared with controls and led us to investigate the effect of the apparently increased ADAM17 activity in both systems.

**TOC keratinocytes display increased shedding of ADAM17 substrates**

To investigate ADAM17 activity, we examined the shedding of a number of ADAM17 substrates from TOC keratinocytes and TOC PBMCs compared with controls. ADAM17’s best known substrate—TNFα—was shed at a significantly higher level by TOC keratinocytes than controls (in which levels of TNFα shed were below the limit of detection; Fig. 3A) when these cells were stimulated with the irreversible Protein Kinase C activator, Phorbol myristate acetate (PMA; 100 ng/ml). Importantly, this excess TNFα shedding was found to be dependent on ADAM17, as siRNA-mediated knockdown of ADAM17 (Figs 1 and 3F) was sufficient to inhibit the excess shedding observed. Similarly, secretion of TNFα from PBMCs isolated from three TOC patients was also significantly higher than matched controls following PMA stimulation (Fig. 3A). No significant difference in TNFα secretion was observed constitutively, or when these cells were stimulated with varying concentrations of lipopolysaccharide (LPS). These findings show that TOC keratinocytes harbouring mutant iRHOM2 protein are not only capable of shedding TNFα [in contrast to macrophages of iRHOM2-knockout mice, which cannot (4)] but do so in a more efficient manner than wild-type cells.

In a previous study, TOC keratinocytes proliferated and migrated significantly more than controls in a scratch assay and did so independently of exogenous EGF (21). Consequently, the shedding of ADAM17’s substrates in the EGF growth factor family, namely amphiregulin, TGFα and HB-EGF, was examined when cells were cultured in the absence of exogenous EGF. Shedding of all three growth factors was shown to be significantly higher in TOC keratinocytes than controls, with amphiregulin shedding around two orders of magnitude higher than that of TGFα and HB-EGF. In each of these cases, the elevated shedding observed could be significantly reduced by siRNA-mediated knockdown of ADAM17 in TOC keratinocytes (Fig. 3B), illustrating the ADAM17-dependent nature of the observed increase. Importantly, there was no change at the mRNA transcript level of each protein when ADAM17 siRNA was applied (Fig. 3F), illustrating the ADAM17-dependent nature of the shedding observed. mRNA transcript levels of EGFR ligands were in general marginally higher in TOC keratinocytes, although these differences would not appear to explain the large degree to which shedding levels were elevated in TOC cells.

Secretion of EGFR ligands from organotypic culture models of TOC was also examined. Amphiregulin secretion was again observed to be significantly higher in TOC cells than controls (Fig. 3C), whereas TGFα and HB-EGF shedding levels were below the limit of detection (not shown). As expected, the enhanced EGFR ligand shedding was associated with a corresponding increase in the phosphorylation of the EGFR at three separate tyrosine residues (Y845, Y1045 and Y1068) in TOC 3D cultures (Fig. 3E), suggesting that EGFR signalling is upregulated in these models.

We also examined the secretion of a number of pro-inflammatory cytokines that, although not directly shed by ADAM17, are important mediators of inflammation, epidermal wound healing and keratinocyte migration. These included interleukins 6 and 8, both of which were found to be secreted at significantly increased levels in TOC keratinocytes, with secretion of both sensitive to siRNA knockdown of ADAM17 (Fig. 3D). Owing to ADAM17’s inability to directly shed these cytokines, this is presumed to be an indirect effect of the increased shedding of, and signalling by, other ADAM17 substrates.
Figure 2. ADAM17 expression in TOC. (A) ADAM17 is highly expressed at the cell surface of TOC keratinocytes, and intracellularly, in compartments that co-localize with the Golgi marker GM130, higher levels of ADAM17 are observed at the cell surface of TOC cells compared with controls (left panel, and detail) (B) western blotting of the ADAM17 pro-domain (released upon ADAM17 maturation) also shows much higher levels of this free pro-domain in TOC. (C) 3D cultures of TOC and control keratinocytes yield fully differentiated skin equivalent models (see also Supplementary Material, Fig. S2). (D) Western blotting of lysates from 3D cultures reveals the same pattern of increased mature ADAM17 and free ADAM17 pro-domain as is seen in keratinocyte monolayers. These data represent results from a single experiment. (E) 3D skin equivalent cultures also display much higher levels of ADAM17 at the cell surface of TOC keratinocytes compared with controls (quantified in graph 2F). These data represent results from three separate experiments. Scale bars on all histology and immunofluorescence images (A, C and E) represent 100 μm.
Desmosomes show signs of immaturity and increased turnover in TOC

Considering the established role of both ADAM17 and EGFR signalling on desmosomal turnover [the two have been shown to cooperate to regulate desmoglein 2 at the cell surface (11,12)], we next investigated the status of desmosomes in the spinous layer of TOC-affected individuals’ skin. When the epidermis of three British TOC patients was analysed by transmission electron microscopy, it was observed that the TOC desmosomes lacked the electron-dense midline found in mature desmosomes of control skin (Fig. 4A). This absence of a midline is indicative of the desmosomes being in a calcium-dependent, immature state (as opposed to the hyperadhesive, calcium-independent state of midline-containing desmosomes) associated with wound healing, keratinocyte motility and mitosis (27).

Next, we examined the expression of the desmosomal cadherin desmoglein 2 (DSG2) by western blotting. Cleavage of
DSG2 is dependent on ADAM17 and EGFR signalling; therefore, when keratinocytes expressing ADAM17 are grown in the presence of EGF, DSG2 shedding can be expected to occur constitutively and result in the presence of a relatively low level of DSG2 (Fig. 5B, left panel). However, removal of EGF can be expected to lead to a reduction in EGFR signalling, and therefore DSG2 cleavage, in control cell lines and thereby increase levels of DSG2 in cell lysates. Meanwhile, TOC keratinocytes (owing to their increased level of constitutive EGFR ligand shedding) will retain a similar level of DSG2 shedding, and hence, show no increase in the presence of DSG2 by western blot. As expected, this pattern of DSG2 expression was observed (Fig. 5B, right panel). When grown in the presence of EGF, levels of DSG2 expression did not differ significantly between any of the four cell lines tested, whereas in the absence of EGF, DSG2 expression in both TYLK1 and 2 cells was significantly lower than either control cell line (P < 0.05, in all cases).

To further investigate ADAM17-mediated DSG2 cleavage, expression of its specific cleavage product (28) was measured in all cell lines, in the presence and absence of EGF. DSG2 is cleaved by ADAM17 in the juxtamembrane region (11,29) to give an approximately 100-KDa fragment, comprising the entire Dsg2 cytoplasmic tail, transmembrane domain and an extracellular juxtamembrane domain (12). This fragment has been shown to undergo endocytosis following cleavage, and the intensity of it is reduced in the presence of siRNA targeting ADAM17 (12). The antibody used here to detect DSG2 is raised against an epitope in the extracellular domain of DSG2 immediately abutting the membrane (30). This epitope is therefore likely to be close to the ADAM17 cleavage site, allowing the antibody to detect the end of the cleaved fragment in whole cell lysates following endocytosis of this fragment. Corresponding with expression of full-length DSG2, expression of this cleavage product did not differ between all cell lines in the presence of EGF, but was noticeably reduced in control cells in the absence of EGF, whilst remaining present in TYLK1 and 2 cells.

**Figure 4.** Desmosomes in TOC. (A) Analysis of desmosomes in TOC skin compared with controls showed the presence in TOC skin of desmosomes lacking the electron-dense midline (white arrows) found in mature, calcium-independent desmosomes of control skin. (B) levels of the ADAM17 substrate desmoglein 2 (DSG2) were reduced in TOC keratinocytes. This effect is EGFR-activation dependent and was most noticeable when keratinocytes were cultured in the absence of exogenous EGFR ligands. DSG2 expression levels were not significantly different between any of the four cell lines in the presence of EGF (analysed in three separate experiments, using two-way ANOVA followed by Bonferroni’s post-test) but were significantly higher in both control cell lines than both TOC cell lines in the absence of EGF (P < 0.05, in all cases). (C) Expression of a DSG2 cleavage product is also shown when keratinocytes are cultured in the presence and absence of EGF. Expression of this cleavage product is comparable when cells are in the presence of EGF, but notably reduced in control cells in the absence of EGF, whereas expression in TOC keratinocytes in the absence of EGF remains robust.

Epidermal barrier function and resistance to infection are increased in TOC

ADAM17-dependent signalling through the EGFR has recently been shown to regulate the function of transglutaminase 1 (TGM1) in the epidermal barrier (19). In epidermally restricted ADAM17 knockout mice, loss of ADAM17 shedding significantly reduced TGM1 activity in the upper layers of the epidermis (19). When TGM1 activity in the epidermis of TOC patients was quantified using an assay based on the incorporation of the biotinylated amine donor monodansylcadaverine (Biot-MDC), increased staining was observed in the granular layer of TOC skin compared with controls (Fig. 5A and C), indicative of increased activity. A significant increase in TGM1 activity was also observed in organotypic models of TOC keratinocytes,
where the enzyme activity was more broadly distributed throughout the suprabasal epidermal layers (Fig. 5B and D). These data suggest an increased level of EGFR-dependent TGM1 function in TOC skin and imply that TOC epidermis may demonstrate improved barrier function.

As patients with no functional ADAM17 are highly prone to bacterial infections, particularly by *Staphylococcus aureus* (18), we postulated that modulating the iRHOM2/ADAM17 pathway may affect bacterial infection dynamics in keratinocytes. The adhesion and invasion efficiencies of *S. aureus* were quantified in control and TOC keratinocytes in the presence or absence of siRNA-mediated ADAM17 knockdown. Knocking down ADAM17 in control keratinocytes resulted in a significant increase in *S. aureus* adhesion and invasion (Fig. 5E), a result in concordance with increased infection susceptibility in patients harbouring loss of function mutations in ADAM17 (18). Conversely, *S. aureus* infection of TOC keratinocytes was significantly reduced, with both adhesion and invasion efficiencies reduced compared with control keratinocytes. Moreover, treating TOC keratinocytes with ADAM17 siRNA led to significantly increased *S. aureus* adhesion and invasion efficiencies (Fig. 5E).

iRHOM2-mediated ADAM17 regulation of IL-6 and IL-8 production during *S. aureus* infection was investigated. A significant increase in IL-6 and IL-8 production was observed in *S. aureus*-infected TOC keratinocytes compared with control keratinocytes (Fig. 5F). Furthermore, siRNA-mediated ADAM17 knockdown led to a significant decrease in both IL-6 secretion by control and TOC keratinocytes and IL-8 release by control and TOC keratinocytes (Fig. 5F).

**DISCUSSION**

In this study, we have shown that dominantly inherited mutations in the inactive Rhomboid protein iRHOM2 associated with the inherited cutaneous and cancer-susceptibility syndrome TOC increase ADAM17 maturation, localization to its major site of...
activity at the plasma membrane and shedding of many of its substrates. During the genetic studies of TOC, the remarkable clustering of TOC-associated mutations was noted: in the four large, unrelated families with \( RHBD2 \) mutations so far identified, the three distinct mutations observed cluster within a motif of just four codons. All three mutated residues are very highly conserved in vertebrates and found within a long N-terminal loop domain that is unique to the iRHOM proteins iRHOM1 and iRHOM2. This mutation clustering therefore strongly implies that a particular site-specific function of iRHOM2 is affected in TOC. Interestingly, iRHOM1 has recently been shown to share the ability of iRHOM2 to regulate the maturation of ADAM17 (14), with high levels of functional redundancy between the two proteins observed in multiple cell types (17). The effect of these iRHOM2 mutations on ADAM17, and the confinement of the affected domain to proteins that regulate ADAM17 maturation, suggests that the mutated domain is intimately involved in this ADAM17-regulatory function, perhaps being involved in the direct association of the two proteins, or the transit of iRHOM2 between the ER and Golgi compartments. Recently, knockout of \( iRhom2 \) in mouse embryonic fibroblasts (mEFs) was shown to strongly reduce the ability of these cells to shed amphiregulin, HB-EGF and epiregulin (but not TGF\( \alpha \)), as well as the non-EGFR ligands (and ADAM17 substrates) EphB4, KitL2 and Tie2 (31). Furthermore, the abrogation of the shedding of KitL2 (used as a model substrate) in \( iRhom2^{−/−} \) mEFs could be rescued by overexpression of wild-type \( iRhom2 \), but not by an \( iRhom2 \) mutant lacking the N-terminal cytoplasmic domain, in which TOC-associated mutations are located (31).

ADAM17 acts as the ectodomain sheddase for five members of the EGF family of growth factors: TGF\( \alpha \), HB-EGF, amphiregulin, betacellulin and epigen (whilst the closely related ADAM10 is responsible for the shedding of EGF and epiregulin (8,9,32)). In culture, keratinocytes express amphiregulin, with much lower levels of TGF\( \alpha \) and HB-EGF (33). It was therefore especially interesting to note that shedding of all three of these growth factors was upregulated in TOC keratinocytes, even in unstimulated conditions. Amphiregulin—the growth factor known to provide by far the strongest degree of autocrine stimulation of keratinocyte growth (34)—was shed at a particularly high level. Upregulation of EGFR ligands in \textit{vivo} is characteristic of numerous benign and malignant hyperproliferative conditions of the skin. For example, hyperproliferative psoriatic lesions overexpress TGF\( \alpha \) (35), HB-EGF (36) and amphiregulin (37) as well as the EGF receptor (38). Amphiregulin is also known to be strongly upregulated in actinic keratoses, verrucae and squamous cell carcinomas (37). Meanwhile, transgenic overexpression of TGF\( \alpha \) in murine epidermis leads to cutaneous hyperplasia and hyperkeratosis, accompanied by the spontaneous formation of squamous papillomas at sites of wounding (39). EGFR ligand signalling also underlies the hyperproliferative effects of retinoids on keratinocytes, with retinoid-induced hyperplasia mediated by the marked induction of HB-EGF and amphiregulin (40,41). The significant increase in shedding of these factors by keratinocytes from TOC-affected areas could therefore reasonably be expected to play a role in the epidermal hyperproliferation that characterizes TOC. Signalling through the EGFR is also known to play an important role in cutaneous wound healing—particularly in the early phase response to wounding—by increasing keratinocyte proliferation and migration (42). The importance of this response is illustrated in EGFR knockout mice, whose skin lesions heal at a slower rate compared with controls (43). ADAM-mediated shedding of EGFR ligands is required for keratinocyte migration during wound healing and is immediately induced upon wounding (44), with factors upregulated in wounded skin including amphiregulin, TGF\( \alpha \) and HB-EGF (but not EGF or betacellulin) (42). In particular, TGF\( \alpha \) is responsible for \( \sim 80\% \) of keratinocyte migration activity during wound healing (45) and also promotes proliferation (46), whilst HB-EGF promotes re-epithelialization (47) and accelerates cutaneous wound healing when applied topically (48,49).

Excessive secretion of EGFR ligands may also offer clues as to the striking oesophageal cancer susceptibility observed in TOC patients [TOC remains the only known highly penetrant syndrome of genetic predisposition to OSCC (21,50)]. In sporadic OSCC, overexpression of the EGFR has been observed in 45.6–72.1\% of primary tumours (51,52), 88\% of OSCC lymph node metastases and 90\% of oesophageal squamous dysplasias (53)—suggesting EGFR signalling may play a role in tumour initiation. Direct EGFR gene amplification is seen in 12–28\% of OSCC (53–55) and is associated with significantly reduced cumulative survival (54), whilst EGFR overexpression has been shown to correlate with poor prognosis (56). Increased levels of EGFR ligand secretion have been associated with the development and progression of squamous cell carcinomas in both the oesophagus—where upregulation of TGF\( \alpha \) is associated with poor OSCC prognosis (57)—and numerous other tissues, including the epidermis (58,59), lung (60), mouth (61) and head and neck tissues (62). The observed presence of excessive EGFR ligand secretion may therefore play an important role in oesophageal cancer development. Independently of the EGFR, expression of ADAM17 itself has also been recently shown to correlate with progression of OSCC (63).

Furthermore, ADAM17-dependent signalling through the EGFR has recently been described to regulate the function of the epidermal barrier, via its effects on transglutaminase 1 (TGM1) in the upper epidermal layers. Epidermal-restricted knockout of murine \textit{Adam17} results in reduced TGM1 activity, reduced barrier function and an inflammatory dermatitis (19) (that could be rescued by the topical application of TGF\( \alpha \)) similar to that seen in human individuals with loss-of-function mutations in ADAM17 (18). Conversely, both epidermal sections from TOC patients and TOC keratinocytes in 3D culture showed increased levels of TGM1 activity. This is presumed to reflect the increased level of EGFR ligand shedding observed in TOC keratinocytes and suggests that TOC patients may demonstrate increased epidermal barrier function.

The phenotype observed in TOC tissues shows several hallmarks that may be indicative of the presence of a constitutive wound-healing-like state in the palmoplantar epidermis of TOC patients. In addition to the upregulation of EGFR ligand shedding and signalling, electron microscopy data demonstrate the absence of electron-dense midlines in the desmosomes of TOC skin—a state associated with a migratory, wound-healing state in keratinocytes (27)—and a reduction in presence of desmoglein 2 in TOC keratinocytes. Shedding of TNF\( \alpha \) and secretion of IL-6 are also upregulated in wound-healing epidermis (64), making constitutive expression of these cytokines also suggestive of a wound-healing state. IL-6 in particular is increased in wounding, persists in chronic wounds (42,65,66) and acts in a mitogenic (67) and
pro-proliferative (68) manner on keratinocytes. Meanwhile, IL-8 plays important roles in wound-healing epidermis (69,70) as well as its better-known roles in local inflammation. The sensitivity of IL-6 and IL-8 production to ADAM17 inhibition may appear somewhat surprising; however, secretion of both IL-6 and IL-8 has been shown to be stimulated by EGFR activation in a number of cell types (71,72), meaning this cytokine response may reflect reduced EGFR ligand signalling following ADAM17 knockdown. The tissue-restricted pattern of epidermal hyperproliferation observed in TOC may therefore be representative of an upregulated wound-healing state being present in epithelia that undergo particularly high levels of stress, such as the palmoplantar epidermis and the oral mucosa. These data demonstrate that the iRHOM2/ADAM17 axis plays a key role in skin barrier maintenance, inflammation and migration. Thus, activation of this pathway may be an attractive target for improved chronic and acute cutaneous wound repair.

**MATERIALS AND METHODS**

**Keratinocyte cell culture**

Control and TOC keratinocyte cell lines [as previously described (21)] were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% foetal calf serum, penicillin, streptomycin, 1-glutamine and the keratinocyte growth supplement RM+−/− (RM+: containing EGF, RM−: with all EGFR ligands absent), at 37°C and 5% CO2.

**3D culture**

The 3D organotypic raft models of human epidermis using control and TOC keratinocytes were established as previously described (73,74). Briefly, J2-3T3 fibroblasts maintained in DMEM containing 10% heat-inactivated foetal calf serum were trypsinized and resuspended (2.5 × 105/ml) in an ice-cold, pH-neutralized DMEM-rat tail collagen (4 mg/ml; BD Biosciences, San Jose, CA, USA) solution. The collagen–fibroblast slurry was polymerized in a 12-well plate (2 ml/well) at 37°C for 30 min and maintained in J2-3T3 culture medium for 24 h in a humidified tissue culture incubator at 37°C and 5% CO2. Control or TOC keratinocyte lines were trypsinized and resuspended in DMEM RM containing 5 ng/ml EGF; 5 × 105/ml keratinocytes were seeded onto a collagen–fibroblast plug established in duplicate for each experiment. Keratinocyte–collagen plugs were maintained as submerged cultures for 48 h to ensure an intact, confluent epidermal sheet of keratinocytes covered the entire surface of the dermal equivalent prior to air exposure. A sterilized metal spatula was used to transfer the keratinocyte–collagen plug to the top of a stainless steel meshed wire grid raised above the surface of a 60-mm Petri dish. FAD RM− was added to the bottom chamber of the wire mesh in contact with the base of the collagen plug, thereby exposing the keratinocytes to an air-liquid interface. The culture medium was replaced every other day from organotypic cultures for 48 h to ensure an intact, confluent epidermal sheet. Duplicate cultures were established from each control and TOC keratinocyte line, and the raft culture experiment was repeated on three separate occasions.

**Western blotting**

For western blotting studies, isolates of keratinocytes or PBMCs were lysed directly by the addition of lysis buffer containing 0.125 M Tris–HCl pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 0.001% (w/v) Bromophenol Blue and 1.44 M β-mercaptoethanol. Cell lysates were separated by SDS–PAGE, transferred onto nitrocellulose membranes and immunoblotted following standard procedures. Membranes were incubated overnight at 4°C with primary antibodies against full-length ADAM17 (Abcam Ab2051, Cambridge, UK), the ADAM17 Pro-Domain (Abcam Ab39161), desmoglein 2 (a gift from My Mahoney; Jefferson Institute, Philadelphia, PA, USA), three different phospho-EGFRs (Cell signalling Technology 3777, 2237 and 6963, Danvers, MA, USA) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam ab9485) and were developed using ECL plus chemoluminescence agent (GE Healthcare). Densitometric quantification of western blots was performed using ImageJ image analysis software [National Institutes of Health (NIH), Bethesda, MD, USA], with levels of each protein normalized to the loading control (GAPDH) for each individual sample.

**Immunocytochemistry**

For immunocytochemical studies, keratinocytes were cultured on 16-mm glass cover slips and fixed using a solution of 4% paraformaldehyde in PBS. Immunocytochemistry was then performed according to standard procedures with primary antibodies against ADAM17 (Abcam Ab2051) and the Golgi marker GM130 (BD 610822, Oxford, UK) followed by incubation with (green-fluorescently tagged) anti-rabbit and (red-fluorescently tagged) anti-mouse secondary antibodies. Mounting medium containing DAPI was used as a nuclear stain.

**Histology and immunohistochemistry**

For histology of 3D organotypic cultures, formalin-fixed, paraffin-embedded tissue sections were stained with haematoxylin and eosin. For IHC, tissue sections were deparaffinized and then heated for 45 min at 96°C in a water bath and immunostained as previously described (75) using ADAM17 antibody (Abcam Ab2051). IHC imaging of 3D organotypic cultures was performed using an epifluorescence Zeiss Axiovision Z1 microscope containing an Apotome slide module and a high-resolution AxioCam MRm digital camera. Image analysis was performed with the Zeiss Axiolab software (Thorwood, NY, USA). The ImageJ software (NIH) was used to calculate the pixel intensity of ADAM17 at cell-cell borders; these values were normalized for total area to calculate the mean fluorescence intensity.

**ELISA**

To assess the production of ADAM17 substrates (and other cytokines), keratinocytes were incubated for 24 h in medium containing no exogenous EGFR ligands, which was then collected. For assessment of TNFα production, this medium was supplemented by the addition of 100 ng/ml PMA. PBMCs were isolated from fresh blood specimens using Ficoll Paque Premium isolation medium (GE Healthcare Life sciences,
Buckinghamshire, UK) and were cultured for 24 h in the presence of PMA, at 37°C in RPMI medium. Collected medium specimens were analysed for the proteins of interest using relevant DuoSet ELISA kits (R&D Systems, Abingdon, UK). In all cases, the data are presented as mean ± SEM. Collection of supernatants was performed in triplicate for each cell line, and results from the two control and two TOC keratinocyte cell lines were pooled for presentation. Results were analysed by Student’s t-test using GraphPad Prism (Graphpad, San Diego, CA, USA).

**siRNA knockdown of ADAM17**

siRNA knockdown was performed on cells in culture using OnTarget SMARTPool siRNA specific to ADAM17 (Thermo Fisher Dharmacon, Lafayette, Colorado, USA). In brief, keratinocytes were incubated for 24 h with penicillin- and streptomycin-free medium, containing 100 nM ADAM17 or non-targeting pool (NTP) siRNA, alongside the transfection reagent DharmaFECT 1 (Thermo Fisher Dharmacon). ADAM17 expression was assessed by western blot or quantitative real-time PCR.

**Quantitative real-time PCR**

RNA was extracted from cultured cells using the Qiagen RNeasy mini kit (Qiagen, Crawley, UK) according to the manufacturer’s instructions. RNA was converted to cDNA using Invitrogen SuperScript II reverse transcriptase (Invitrogen, Grand Island, NY, USA) as the active enzyme. Quantitative real-time PCR using TaqMan chemistry on a Rotorgene Q thermocycler (Qiagen) was performed using primers against ADAM17, AREG, TGFA, HBEGF, TNFA, IL6, IL8 and GAPDH designed in-house. Primers were designed to bind across exon–exon boundaries, ensuring only cDNA was amplified. Samples were assayed simultaneously for GAPDH and the gene of interest via the use of probes labelled with different fluorophores. Expression of all genes was quantified relative to GAPDH using the ΔCt method (76).

**Electron microscopy**

For electron microscopy, TOC and control epidermis specimens were fixed in phosphate buffered 4% glutaraldehyde, post-fixed in 1% osmium tetroxide and dehydrated through a graded ethanol series. They were then cleared in propylene oxide and infiltrated with Araldite. The cells were embedded by inverting ‘BEEM’ capsules filled with partly cured Araldite over the monolayer and incubating them at 60°C for 48 h. After cutting away the silicone membrane, semi-thin sections (0.5 mm) for light microscopy were cut and stained with Toluidine Blue. Ultrathin sections (60–80 nm) were cut, mounted on copper grids and stained with uranyl acetate and lead citrate. They were examined in a J.E.O.L. JEM 1230 electron microscope and images collected with an Olympus ‘Morada’ 2 × 2 K digital camera.

**Transglutaminase activity**

For in situ detection of epidermal transglutaminase activity, we used the biotinylated amine donor substrate monodansylcadaverine (biot-MDC) assay, as previously described (77). In brief, frozen epidermal sections from normal controls and tylosis patients were preincubated with 1% BSA in 0.1 M Tris–HCl, at pH 7.4 for 30 min. The sections were then incubated for 2 h with 100-μM biot-MDC (or 10 mM EDTA as a negative control) and 5 mM CaCl2 in 0.1 M Tris–HCl at pH 7.4 (to assess transglutaminase 1 activity). The reaction was halted by the addition of 10 mM EDTA solution and washing with PBS, following which the sections were stained using Alexa Fluor 488 (Invitrogen) conjugated to Streptavidin and mounted in medium containing DAPI. To quantify TGM1 activity in the granular layer, the average fluorescence intensity of the epidermis of negative control skin was calculated using ImageJ image analysis software (as previous), then subtracted from Biot-MDC-positive specimen images. The fluorescence intensity in the granular layer of these normalized images was then measured.

**Staphylococcus aureus invasion and adhesion**

*Staphylococcus aureus* (NCTC 6571) was grown in lysogeny broth (LB) (Sigma Aldrich, Dorset, UK) in an orbital shaker in 5% CO2 atmosphere overnight. In duplicate, keratinocytes were cultured and treated with siRNA as previously described. After 48 h, cells were processed for *S. aureus* infection: the media on cells was replaced with 1 ml of serum and antibiotic-free media. *S. aureus* was added at a multiplicity of infection of 100 bacteria per cell. The plate was centrifuged at 1000 RPM for 10 min to initiate infection and incubated at 37°C/10% CO2 for 3 h. The media on the cells was collected as supernatants for ELISA experiments. One well that was used to determine the cell-associated bacteria was lysed immediately post-infection. Media containing 75μg/ml of gentamycin was added to the other well, which was used to determine the internalized bacteria. The plate was incubated at 37°C for 1 h and washed three times with 1 × PBS. Cells were trypsinized, collected in a tube and lysed with 0.5% Triton X-100 in PBS. Lysates were serially diluted in PBS and were plated onto LB agar. Bacteria were grown overnight at 37°C, and the number of colonies was counted. The number of adhering bacteria was calculated by subtracting the internalized bacteria from the cell-associated bacteria. The percentage adhesion and invasion efficiencies were calculated relative to the inoculum dose. For analysis adhesion and invasion, efficiencies for two control and two TOC keratinocyte cell lines were pooled and compared across three independent experiments to give an n value of 6. Statistical analysis was carried out using a two-tailed unpaired t-test.

**SUPPLEMENTARY MATERIAL**

Supplemental Material is available at HMG online.

**ACKNOWLEDGEMENTS**

We are grateful to Claus-Werner Franzke (University of Freiburg, Germany) for the gift of the Biotinylated MDC used in the transglutaminase activity assays, to My Mahoney (Jefferson Institute, Philadelphia, PA, USA) for the gift of the anti-DSG2 monoclonal antibody used for western blotting studies and to Graham McPhail (Department of Cellular Pathology, Barts and
The London NHS Trust, London) for the embedding of tissue for electron microscopy.

Conflict of Interest statement. None declared.

FUNDING

This study was supported by a British Skin Foundation PhD studentship (2008s) and a Medical Research Council/Queen Mary University of London PhD studentship (all awarded to D.P.K.). Additional funding came in part from a Barts and The London Charity project grant (458/1493; awarded to D.P.K.).

REFERENCES


