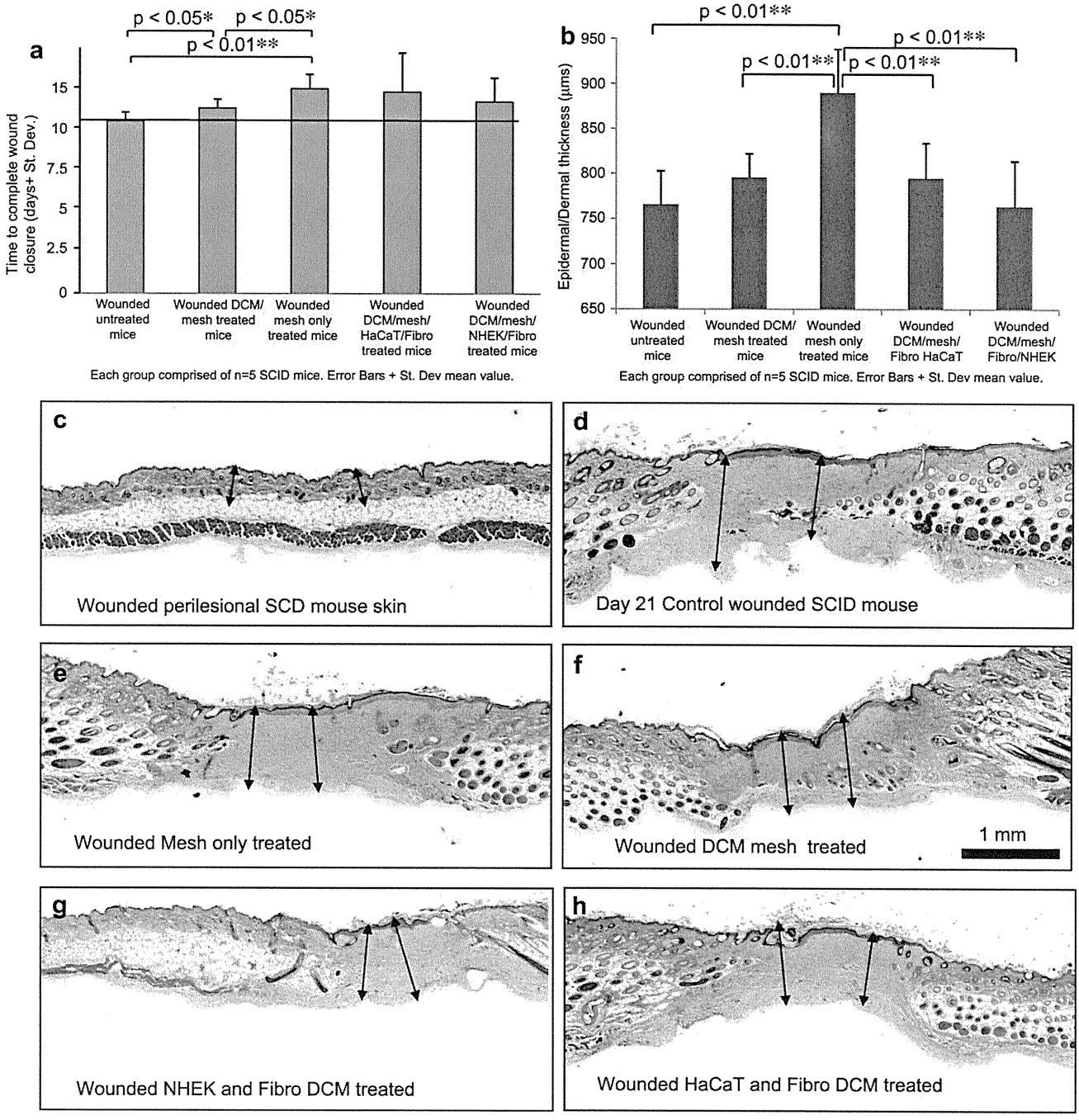

Fig. 6. Excisionally wounded immunodeficient SCID mice treated with DCM-mesh scaffold seeded with different skin cell combinations showed a significant increase in wound closure time compared to wound only, DCM and mesh treated and mesh treated wounds (a, $n = 5$ mice for each treatment group). There was no difference in the mean time to complete wound closure between the wound only, and the two DCM/mesh/skin cell-treated groups. Assessment of foreign body wound immunoreactivity/acute scarring response by using the mean combined epidermal/dermal tissue thickness (µm) 21 days after wounding demonstrated that the use of plastic mesh to support the graft at the air liquid interface during culture (and its subsequent incorporation into the DCM graft) increased acute wound thickness compared to all other treatments (b). This effect however, could be partially alleviated using DCM/mesh composite seeded with human skin cells (b). Histological analysis of paraffin embedded modified Masson's Trichrome stained wounded mouse skin demonstrated that compared to normal unwounded dermal thickness (c, mean depth 190 µm), the dermal wound thickness increased more than four fold in the untreated group (b and d), and by significantly more ($p < 0.01$) in the mesh treated group (graph in b and figures c and d versus e). All other wound treatments showed minimal affects on acute phase dermal thickening in the presence of DCM (with or without cells) with concomitant significant reductions in mesh-associated dermal scarring. Scale bar 1 mm or 1000 µm (c–h). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

4. Discussion

The material properties of electrospun denatured collagen microfiber (DCM) make this a promising candidate scaffold for skin-derived cell grafting. It comprises extracted bovine collagen and when combined with cells in a composite, avoids adverse foreign body immune responses after grafting more than many typical artificial polymers [9,21,34]. Bovine collagen was easy and cheap to prepare and quick to manufacture on an appropriate scale using a previously described process of acid extraction and

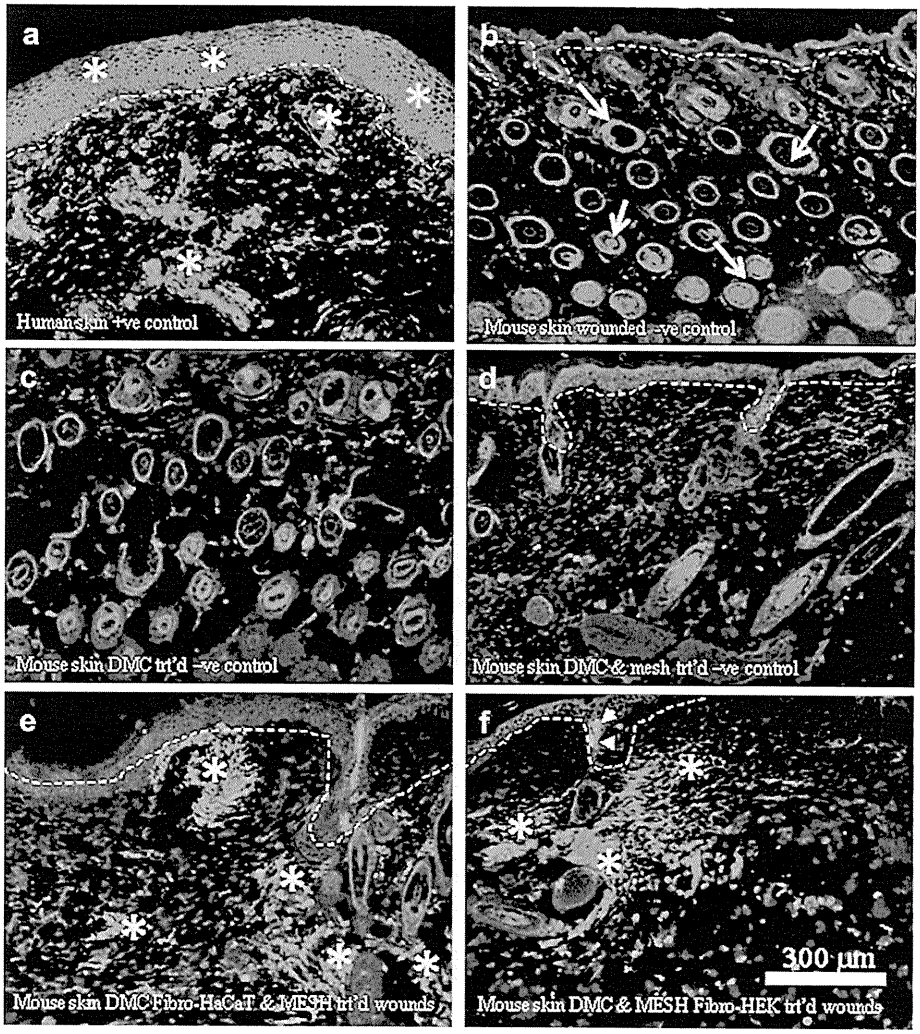

Fig. 7. Excisionally wounded SCID mice demonstrated human keratinocyte and fibroblast cell engraftment and survival in wounded mouse skin after 21 days using anti-human HLA specific antibodies after human cultured cell-DCM composite wound graft treatments. Control human skin stained with anti-human HLA antibody (a) showed bright fluorescence throughout the epidermis (asterisks above the dashed line) and bright patches within dermal tissue (asterisks). Conversely, untreated wounds (b), DCM (c), DCM/mesh only treated (d) mouse skin showed no focal areas of fluorescent staining for human keratinocyte or fibroblasts. However, non-specific reactivity with the antibody stained the hair follicle and shaft in many of these mouse tissues (see arrows in b). Finally, treatment of wounds with human HaCaT-fibroblasts (e) or primary keratinocyte-fibroblast DCM grafts (f) demonstrated significant staining of dermal cell foci (asterisks in e and f) and some epidermal staining (arrowheads in f) highlighting human cell survival and engraftment into the wound. Scale bar 0.3 mm or 300 µm (a–f). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

alcohol/UV sterilization to minimize potential biosafety issues but preserve, as much as possible, the natural collagen protein conformation [21]. Electrospinning was used to produce biomimetic-scaled scaffold fibers similar to dermal collagen fibers specific for this application. Such scaffold fibers mimic the close structural profile of the natural dermal extracellular matrix (ECM) to which the fibroblast and keratinocyte integrin receptors bind [21,35,37]. In addition, this scaffold was predicted and shown to be able to undergo complete hydrolysis during the dermal reorganizational and maturation stages of wound healing to become safely degraded in its target tissue. This avoids the need for the composite to be removed after grafting allowing the wound to remain intact avoiding further damage and subsequent inflammatory processes.

Electrospun collagen provides a porous, dermal-like template for skin cell attachment that allows both keratinocytes and fibroblasts to survive and proliferate and for fibroblasts to penetrate through and migrate over this scaffold *in vitro*. The uncoated DCM scaffold EM data suggest DCM pores average 6–7 µm (ranging from 3 to 10 µm) that are significantly larger than the lower cut-off limit described for fibroblast penetration and migration (anything smaller than between 4.5 and 5 µm limits fibroblast penetration) [9,24]. Conversely, these large DCM pores do not appear to encourage single cell keratinocyte or cell sheet migration/penetration as seen in fibroblasts. However, it appears that two factors may be able to overcome this reduced keratinocyte migration on DCM; firstly a high initial keratinocyte seeding density and secondly pre-seeding the DCM with live human fibroblasts until confluent. This migration finding was unexpected as keratinocytes failed to assemble hemidesmosomes that encourage stable anchorage to the underlying matrix-scaffold (likely due to the lack of laminin 332) but assembled multiple focal contact associated lamellapodia important in migration. We hypothesize that either cell type releases matrix or utilizes exogenous soluble factors that encourage keratinocyte focal contact adhesion when maintained on DCM scaffolds.

DCM scaffold appears efficient at maintaining cell adhesion and survival (compared to TCP substrates), however these precise adhesion rates are difficult to determine in our experiments since cell proliferation and survival might have affected the results

during the course of the Livedead® experiments. Previous studies have demonstrated that electrospun collagen fibers show reduced keratinocyte adhesion compared to unprocessed collagen or spun collagen fibers coated with ECM substrates [22,35]. In our experiments however, no deficit was noted in keratinocyte adhesion to fibroblast seeded DCM scaffold maintained in culture. These findings may reflect fibroblast cell deposition of new cell-matrix components (collagen I or fibronectin or vitronectin) onto the DCM scaffold before keratinocyte seeding as has been described for other scaffolds [9].

The porous nature of this scaffold and its ability to hold fluid between the fibers by capillary action means that graft survival can at least be temporarily supported by nutrients in wound tissue fluid, in the absence of a viable dermal blood supply. The ability of tissue fluid to support the graft would be made easier with a reduced cellular load in or on the scaffold, perhaps if grafted before complete confluence and/or epidermal maturation has taken place.

Conversely, the large 3-dimensional and variable pore sizes allow the reciprocal movement of both live fibroblast cells and cell-secreted cytokine growth factors into the wound bed to influence the important dermal wound healing processes like neovascularization. DCM biomaterial strength allows HSE graft transport and placement directly onto the wound from the *in vitro* cultures, avoiding problems with surgical handling. The random fiber orientation and DCM scaffold composition likely imparts stiffness that means wound contraction is less of an issue compared to other softer polymer materials where graft contraction can significantly limit the original surface area by as much as 20% [36].

Our data from experiment one, the excisional wounded animal model suggests that DCM scaffold alone is non-cytotoxic with dermal tissue and does not induce a foreign body immune response. This is in contrast to previous reports of electrospun fibers that only fail to induce acute foreign body immune responses when grafted with cells including: fibroblasts [34] and fibroblast/keratinocyte combinations in animal wounding models [22]. In the majority of these models the time frame for re-epithelialization to complete was within the critical two week period to reduce the chances of infection and subsequent hypertrophic scarring [22,24,34]. Wounded mouse models exhibit very different wounding time frame and responses from our previously described porcine model of burn wounding [37]. These differences relate to the scale of tissue, wounds and the differential structural composition of mouse skin resulting in healing largely by wound contraction, rather than re-epithelialization.

These factors are also likely to be important when assessing data from experiment two where DCM scaffold overcame the detrimental effects of grafting with the Nylon Mersilene™ mesh culture support with the DCM composite in inhibiting wound closure and promoting dermal thickening (it was included to support the DCM at the air liquid interface during culture to encourage keratinocyte differentiation). In future a better (less disruptive) alternative to mesh support will be identified for supporting scaffold composites, one that is hopefully easier to remove from the composites before grafting or a material that has less influence wound healing than the Nylon Mersilene™ mesh.

5. Conclusions

In conclusion, our data taken together show that DCM scaffold exhibits improved mechanical properties in terms of support and reducing graft shrinkage over unsupported cultured epithelial autografts (CEA) and avoids typical artificial polymer based scaffold immune responses. Furthermore, DCM cultured cell grafts do not require any donor site biopsies avoiding subsequent donor wounds as split thickness skin grafts procedures do. Further work would be beneficial to optimize keratinocyte adhesion, migration, proliferation and differentiation on DCM biomaterial to further improve its surface characteristics.

Acknowledgments

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Appendix. Supplementary data

The supplementary data associated with this article can be found in the on-line version at doi:10.1016/j.biomaterials.2011.03.023.

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Rapid immunochromatographic test for serum granulysin is useful for the prediction of Stevens-Johnson syndrome and toxic epidermal necrolysis

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Background: Life-threatening adverse drug reactions such as Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) sometimes start with clinical features of ordinary drug-induced skin reactions (ODSRs) and it may be difficult to make a correct diagnosis before severe mucocutaneous erosions occur. We have reported that serum granulysin levels are elevated (cut off: 10 ng/mL) in patients with SJS/TEN before generalized blisters form.

Objective: We sought to develop a rapid detection system for elevated serum granulysin to predict the progression from ODSRs.

Methods: Serum samples from 5 patients with SJS/TEN at 2 to 4 days before mucocutaneous erosions formed were analyzed. Sera from 24 patients with ODSRs and 31 healthy volunteers were also investigated as control subjects. We developed a rapid immunochromatographic assay for the detection of high levels of serum granulysin using two different antigranulysin monoclonal antibodies.

Results: The immunochromatographic test showed positive results for 4 of 5 patients with SJS/TEN but only one patient of 24 with ODSRs. The results correlated closely with those of enzyme-linked immunosorbent assays.

Limitations: The validation of the long-time stability in this test strip has not been investigated.

Conclusion: This novel test enables the prediction of SJS/TEN occurrence in patients even when only features of ODSRs are noted clinically. (J Am Acad Dermatol 2011;65:65-8.)

Key words: adverse drug eruption; diagnostic test; granulysin; Stevens-Johnson syndrome; toxic epidermal necrolysis.

Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are life-threatening adverse drug reactions characterized by blister formation and widespread skin detachment.[1] In the early stage, SJS/TEN presents clinically as edematous papules or erythema multiforme–like target rashes, which are very similar to those of ordinary drug-induced skin reactions (ODSRs). Such a clinical course makes it difficult to reach a diagnosis of SJS/TEN in the early stage, and this results in high mortality. There is an urgent need for a method to distinguish between early-stage SJS/TEN and ODSRs.

The method should be as fast as possible, because SJS/TEN usually occurs within a few days. Furthermore, the technique should be as clinically

Abbreviations used:

ODSRs: ordinary drug-induced skin reactions
sFasL: soluble Fas ligand
SJS: Stevens-Johnson syndrome
TEN: toxic epidermal necrolysis

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simple as possible, such as using immunochromatographic test strips that are available for the detection of influenza infections. Among several candidates for diagnostic markers, we examined soluble Fas ligand (sFasL) and found that it is elevated in the sera of patients with SJS/TEN in the early stage, before mucocutaneous erosions appear.[2,3] It would be very useful to be able to predict the occurrence of SJS/TEN, but sFasL serum levels are too low (cut off: 100 pg/mL) for use in a rapid diagnostic device.

Chung et al[4] recently reported that granulysin is highly expressed in blisters of patients with SJS/TEN. We found that both serum granulysin and sFasL are higher in patients with early-stage SJS/TEN than in patients with ODSRs.[5] Serum levels of granulysin are 100 times higher (cut off: 10 ng/mL) than those of sFasL. Based on these observations, we developed a rapid immunochromatographic assay for the detection of high-level serum granulysin to diagnose and predict the early stage of SJS/TEN.

CAPSULE SUMMARY

- Drug reactions sometimes start with edematous papules, and it may be difficult to distinguish life-threatening drug reactions from ordinary drug reactions early in their course.
- We recently found that serum granulysin levels are increased in patients who later develop Stevens-Johnson syndrome or toxic epidermal necrolysis.
- We report a novel immunochromatographic assay to detect high levels of serum granulysin. With this test, we can predict whether patients with nonspecific edematous papules will develop severe drug eruptions.

METHODS

Patients

SJS refers to cases with mucosal erosions and epidermal detachment of less than 10% of the body surface area, and TEN refers to those with more than 30% involvement. Disease onset in patients with SJS/TEN was defined as the day when the mucocutaneous or ocular lesion first eroded or ulcerated (day 1).[3] From multiple Japanese institutions, we obtained serum samples from 35 patients with SJS/TEN.[3] Of these, we investigated 5 patients whose sera had been collected before the diagnosis of SJS/TEN (day −2 to −4). The patient information is listed in Table I. Serum samples from patients with ODSRs (n = 24) and healthy volunteers (n = 31) were also analyzed. Informed consent was obtained from all patients, and the procedures were approved by the Ethical Committee of the Hokkaido University Graduate School of Medicine, Sapporo, Japan.

Immunochromatographic assay

In the immunochromatographic test, a murine monoclonal antibody specific to human granulysin (RB1, MBL, Nagoya, Japan) was conjugated with microparticles and then placed on the glass membrane area of the test device in a dry state. Another granulysin monoclonal antibody (RC8, MBL) was immobilized on a nitrocellulose membrane to form a result line. Likewise, a control line was created by the immobilization of antimouse IgG. The granulysin in the serum sample specifically bound to the microparticles via RB1 and comigrated upward until the granulysin was sandwiched with the immobilized RC8, revealing a visible result line. The entire test procedure was completed within 15 minutes.

Enzyme-linked immunosorbent assay

The granulysin concentrations of the serum samples were measured with a sandwich-enzyme-linked immunosorbent assay as previously described.[6,7] In brief, 96-well flat-bottomed plates were coated with 5 mg/mL of RB1 antibody and stored overnight at 4°C. The plates were then washed and blocked with phosphate-buffered saline containing 0.1% Tween-20 (washing buffer) and blocked with 10% fetal bovine serum in washing buffer at room temperature for 2 hours. The samples and standards (recombinant granulysin, R&D Systems, Minneapolis, MN) were incubated for 2 hours at room temperature. Then they were reacted with 0.1 mg/mL of biotinylated RC8 antibody for 1 hour. The plates were then treated with 0.2 mg/mL of horseradish-peroxidase-conjugated streptavidin (Roche Diagnostics, Basel, Switzerland) for 30 minutes at room temperature. The plates were incubated with tetramethylbenzidine substrate (Sigma, St Louis, MO) for 30 minutes at room temperature, and then 1 mol/L sulfuric acid was added. The optical density was measured at 450 nm using a microplate reader (Mithras LB940, Berthold Technologies, Thoiry, France).

RESULTS

We first applied diluted recombinant human granulysin protein to the immunochromatographic test strips, to confirm the threshold and reliability of the assay. Approximately 10 ng/mL of sample yielded a result line, and 3 repeated investigations brought the same results (Fig 1, *A*).

Table I. Patient information

Patient No.	Age, y	Sex	Diagnosis	Affected skin area	Causative drug	Serum granulysin (d)
1	17	M	SJS	20%	Carbamazepine	52.1 (−3)
2	66	F	TEN	70%	Imatinib	14.2 (−3)
3	27	F	SJS	<10%	Unknown	42.2 (−4)
4	80	M	SJS	5%	Phenytoin	12.9 (−2)
5	25	F	SJS	Only mucosal lesions	Unknown	2.7 (−2)

F, Female; *M*, male; *SJS*, Stevens-Johnson syndrome; *TEN*, toxic epidermal necrolysis.

Based on this observation, we then applied serum samples to detect the elevated granulysin levels. Four of 5 SJS/TEN samples showed positive results (Fig 1, *B*). All the positive samples had elevated granulysin as detected by enzyme-linked immunosorbent assay analysis (30.35 ± 9.91 ng/mL, average ± SEM). The only sample with a negative result had granulysin at the normal level of 2.7 ng/mL. Conversely, one in 24 ODSRs samples and none of 31 healthy volunteers showed positive bands in this immunochromatographic assay. The test showed a sensitivity of 80% and a specificity of 95.8% for SJS/TEN versus ODSRs. The results of the immunochromatographic test correlated closely with early diagnosis for SJS/TEN ($P = 1.02 \times 10^{-3}$, analyzed by Fisher exact probability test).

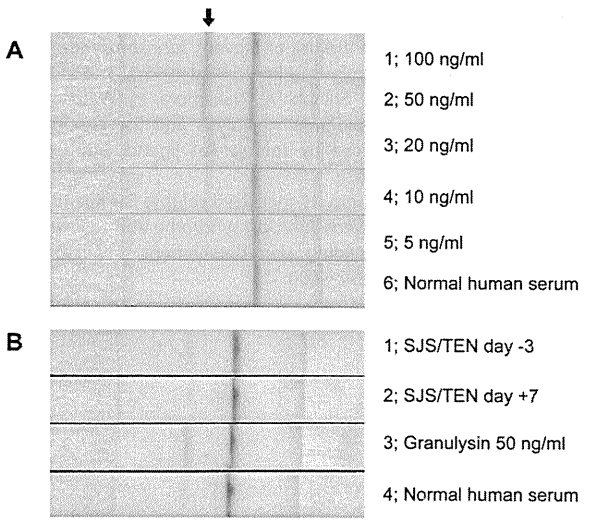

Fig 1. A, Immunochromatographic test strip detects elevated granulysin. *1 to 5*, Diluted recombinant granulysin is applied. *6*, Normal human serum as negative control (1.4 ng/mL). Positive results are shown as a band (indicated by the arrow). Approximately 10 ng/mL of granulysin is considered a positive result. **B**, Detection of serum granulysin by immunochromatographic assay. *1*, Serum taken from patient 1 with early Stevens-Johnson syndrome (*SJS*)/toxic epidermal necrolysis (*TEN*) 3 days before blister formation. Although patient showed only edematous erythema and papules without mucosal manifestations, serum granulysin was 52.1 ng/mL. *2*, Seven days after blister formation in same patient with SJS/TEN. No bands are observed, and serum granulysin has decreased to 5.7 ng/mL. *3*, Recombinant human granulysin as positive control. *4*, Normal human serum as negative control (3.5 ng/mL).

DISCUSSION

We succeeded in developing a rapid immunochromatographic test for the detection of high-level serum granulysin that puts our previous findings to practical use. Although 20% of the cases could be missed, it would be a useful adjunct in diagnosing SJS/TEN. It would not be necessary for every morbilliform drug eruption. We suggest that the test be applied when clinical findings hinting at SJS/TEN, such as target lesions, are seen. However, two biopsies should be done as soon as SJS/TEN are suspected, for hematoxylin-eosin processing and immediate frozen sections, in order to look for necrotic keratinocytes, which is another sensitive test.[8] If the results of either method are negative, careful daily and hourly monitoring of the patient for a few days should take place. Furthermore, to assess the severity of illness and to predict mortality, we should use the mathematical tool called SCORTEN that has been developed.[9]

Granulysin, a member of the saposin-like protein family of lipid-binding proteins, exhibits potent cytotoxicity against a broad panel of microbial targets, including tumor cells, transplanted cells, bacteria, fungi, and parasites, damaging negatively charged cell membranes.[10] Granulysin plays important roles in host defense against pathogens, and it induces apoptosis of target cells in a mechanism involving caspases and other pathways.[11] Chung et al[4] reported that granulysin was identified as the most highly expressed cytotoxic molecule in blisters of patients with SJS/TEN. Very recently, we showed that granulysin levels of sera from patients with SJS/TEN are significantly elevated before the development of skin detachment or mucosal lesions.[5] The elevated serum granulysin levels decrease rapidly within 5 days after disease onset. This pattern is similar to that

observed with sFasL.[3] When granulysin levels for patients with SJS/TEN in the early stage were compared with those levels for patients with ODSRs and healthy control subjects, the differences were statistically significant.[5]

This novel test enables the early diagnosis of SJS/TEN in patients with cutaneous adverse drug reactions that are otherwise indistinguishable from ODSRs.

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Special Issue "Epithelial regeneration in inflammatory diseases"

Mini Review

Regenerative medicine for severe congenital skin disorders: restoration of deficient skin component proteins by stem cell therapy

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Some congenital skin disorders lacking structure proteins in the basement membrane zone carry severe prognosis because of severe erosion and skin dysfunction on the whole body. So far, several therapeutic strategies have been emerging for such disorders: 1. gene therapies, 2. protein therapies and 3. cell therapies. Cell therapies have a potential to affect skin systemically, and stem cell transplantation is one of the most hopeful candidates for treating severe congenital skin disorders such as epidermolysis bullosa, from a perspective of transdifferentiation and re-programming of stem cells. We review here the recent strategies and progress of stem cell transplantation for epidermolysis bullosa.

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basement membrane zone, bone marrow transplantation, epidermolysis bullosa, stem cell therapy, type XVII collagen

Introduction

The skin is the human body's largest organ and accounts for approximately 16% of an adult's body weight. Several critical roles owe to the skin, including moderation of body temperature, prevention from electrolyte loss and protection from physical stimuli. In order to resist mechanical stress, the skin has complicated structures connecting epidermis and dermis, called basement membrane zone (BMZ) or dermal-epidermal junction. The BMZ consists of more than 30 structure proteins to strengthen the adhesion (Fig. 1)[1], and one defect of these proteins by congenital abnormality or acquired autoimmunity cause skin fragility and blister formation immediately after mild mechanical stimuli. Blistering on the whole body extremely worsens the quality-of life and even causes death due to severe water loss and infections.

Epidermolysis bullosa

One important example on the importance of BMZ proteins is epidermolysis bullosa (EB). EB comprises a group of inherited disorders in which the patient's epidermis can exhibit skin fragility caused by genetic abnormalities of a BMZ protein[2]. From the location of causative BMZ protein, EB is classified roughly into 3 categories: EB simplex (EBS), junctional EB (JEB) and dystrophic EB (DEB). Worldwide approximately 50 EB cases arise per a million live births and 92% accounts for EBS which is caused by cytokeratin 5/14 mutation with autosomal dominant inheritance[2]. Clinical manifestations vary broadly, from occasional mild erosion on the extremities to severe ulcers on the whole body or even stillbirth in Herlitz JEB and EBS/JEB with pyloric atresia. In recessive DEB, the most frequently recognized subtype in Japan, defect of type VII collagen (COL7) causes recurrent, deep erosions and ulcers on the extremities which results in mitten deformities and squamous cell carcinoma.

Emerging novel strategies for EB treatment

Most prevalent treatments for EB patients are skin protective care, wound dressing agents and antibiotics against local infections. There have been no established and fundamental treatments because EB arises from gene mutations of keratinocytes and fibroblasts on the whole body. However, several novel strategies have been emerging for EB treatment recently: 1. gene therapies, 2. protein therapies and 3. cell therapies.

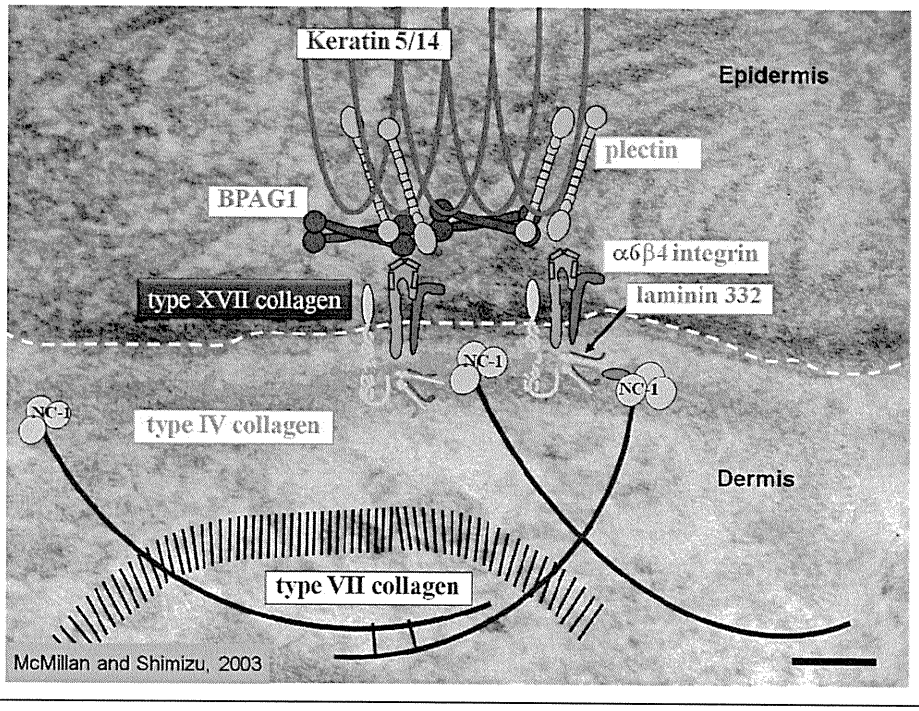

Fig. 1
Structure of basal membrane zone (BMZ) in the skin[1].

Gene therapies were performed by virus-mediated normal gene transfection into autologous keratinocytes, followed by cell culture to form epidermal sheet and grafting into the patients' skin. Such *ex vivo* gene-treated cultured autografting, reported by Mavilio *et al.*, is a promising therapeutic approach for junctional EB[3]. One of the merits of gene-mediated therapies is that autologous cells are fundamentally accepted without rejection response, except for the risk of immunoreactivity against the restored protein. Conversely, its effects are limited to the area of grafting and might be insufficient for systemic involvement of EB. Furthermore, the ethical and safety problems of using retroviruses for gene correction still exist[4]. Autologous induced pluripotent stem (iPS) cells are another source for gene therapies, since high proliferation potential provide enough number of differentiated cells without invasive techniques[5]. Successful treatment of sickle cell anemia model mice was recently reported by utilizing gene-corrected hematopoietic cell transplantation from autologous iPS cells[6]. Tolar *et al.* succeeded in the generation of autologous iPS cells from recessive DEB patient, which indicates that iPS-mediated therapies are theoretically possible by generation of epidermal/dermal sheets and hematopoietic stem cell transplantation[7]. However, ethical problems still lie on autologous iPS cells for the treatment of EB since gene correction by transfection is essential.

Conversely, few reports have been published as to *in vivo* gene therapies for EB[8]. As one candidate, several drugs have been reported to read through the specific stop codons of nonsense mutations, resulting in producing full-length proteins [9-11]. Therefore such "read-through" drugs might ameliorate severe congenital skin disorders if they are caused by the specific nonsense mutations. Since some subtypes of junctional EB have "hot spots" of nonsense mutations[12], there seems to be a space of novel gene-therapeutic agents in the future.

Congenital disorders that lack secretory proteins could be ameliorated by supplying the recombinant proteins systemically or locally. Several congenital metabolic disorders such as Fabry's disease have been already treated with enzyme replacement therapy[13]. Woodley and colleagues succeeded in the deposition of COL7 at the BMZ of artificially-constructed DEB skin by injecting recombinant COL7[14]. The same group later reported the amelioration of RDEB mice by injecting human COL7[15]. Other than secretory proteins like COL7, laminin beta-3, a structural protein in the BMZ, is found to be provided with protein therapy by protein transfection technique[16]. Protein therapies are safer than other novel therapies in the way that patients can attempt the therapy with lower dose of protein and that no gene correction is needed. Conversely, its effects are limited to the area of injection. The safety of the recombinant protein should be alarmed since bovine serum is generally essential for the culture of transfected cells. Efficient purification of large amount of protein is another challenge. The risk of immunoreactivity might weaken the effect of protein therapy and even cause exacerbation. In recessive DEB-generalized other type, the mutated COL7 protein partially function to form incomplete anchoring fibrils. Therefore, protein therapy-induced autoimmunity in such patients might inhibit the residual COL7 functions, resulting in exacerbation of blistering on the whole body.

Considering the clinical application of congenital disorders, the easiest source of normal proteins is allografts. Therefore, utilizing allogenic normal cells could be the fundamental therapeutic strategy. Applying allogenic keratinocytes, or allo-skin graft could treat congenital skin disorders, but allogenic keratinocytes are generally rejected because of their high immunogenicity. In order to overcome rejection, less immunogenic cells such as fibroblasts have been attempted to treat DEB. Intralesional injection of allogenic fibroblasts into DEB patients caused the deposition of COL7 for more than 3 months with matured anchoring fibrils[17]. Furthermore, intravenous injection of human fibroblasts into nude mice introduced human COL7 deposition in the BMZ of wound-healed skin[18]. Mesenchymal stem/stromal cells (MSCs) are another candidate for cell therapies; Conget and colleagues reported COL7 deposition at the site of intradermal injection of allogenic MSCs[19] in RDEB patient.

Another strategy of cell therapy is stem cell transplantation such as bone marrow transplantation (BMT) and cord-blood stem cell transplantation. If such stem cells engraft completely and provide functional stem cell-derived skin component cells from peripheral blood flow, systemic amelioration of EB will be accomplished for a long time without immunological rejection. Since stem cell transplantation has already performed widely for hematologic disorders and some congenital metabolic disorders, ethical and technical hurdles are much lower than gene/protein therapies.

Differentiation from bone marrow cells into functional keratinocytes

Stem cells in the bone marrow were recently found to have a pluripotency; a potential to differentiate into various cell lineages other than hematocytes. This pluripotency or transdifferentiation are observed more frequently in the injured organs such as damaged liver, ischemic heart, injured nerve tissues and wounded skin[20,21]. However, it had been unknown what causes efficient differentiation from bone marrow stem cells into injured skin, and whether these differentiated cells actually function like other normal organ cells.

Our group first revealed that a chemokine CTACK/CCL27 from the injured skin tissue accelerates the differentiation from bone marrow stem cells into epidermal keratinocytes[22]. Murine GFP-positive bone marrow cells were transplanted into normal mice, and the acceleration of wound healing and GFP-positive epidermal keratinocytes were investigated with or without local injection of CTACK/CCL27. Interestingly, CTACK/CCL27 enhanced the bone marrow-derived keratinocytes approximately 4 times, which was inhibited by anti-CTACK/CCL27 antibodies. Another chemokine SLC/CCL21 are similarly found to enhance wound healing via differentiating MSCs into various skin component cells including keratinocytes[23].

We also revealed that these differentiated keratinocytes actually function and provide BMZ component proteins. Focused on one basal keratinocyte-specific structural protein type XVII collagen (COL17), we prepared mice expressing normal murine Col17 (mCol17), transgenic mice expressing both murine and human COL17 (hCOL17) and COL17-humanized mice that express only hCOL17[24]. Interestingly, the expressions of donor bone marrow-derived COL17 in the skin were confirmed after performing BMTs among these mice of different COL17 expression patterns[25]. Since only keratinocytes express COL17 among skin-component cells and peripheral blood, bone marrow-derived keratinocytes are found to function and produce a BMZ component COL17.

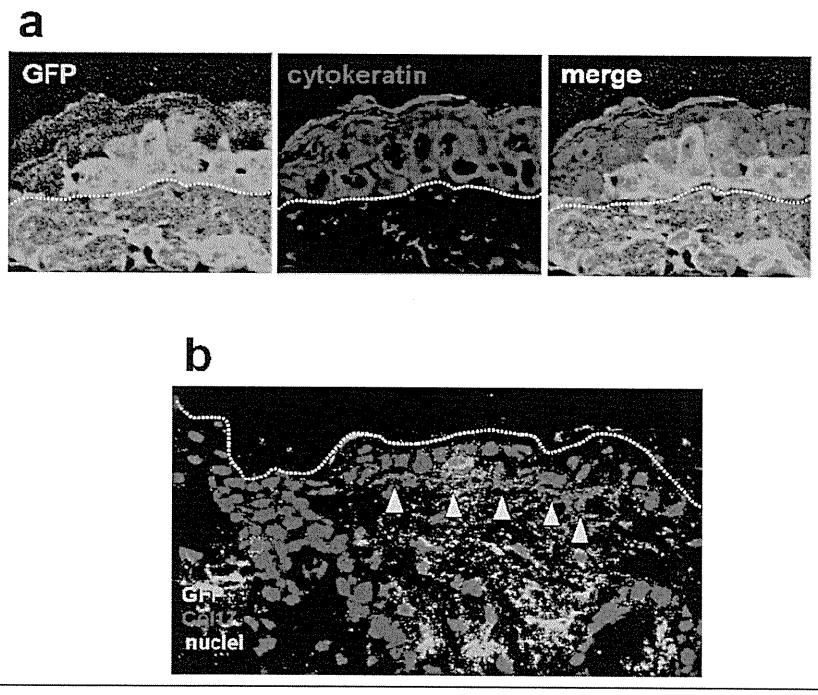

Fig. 2
Bone marrow transplantation into Col17 knockout JEB mice. (a) Donor-derived, GFP+ cytokeratin+ cells are aggregated in the basal cell layer of the epidermis, indicating bone marrow cells re-programmed into epidermal keratinocytes. (b) Immunofluorescence revealed GFP+ cells in the epidermis and dermis, with linear expression of Col17 in the BMZ.

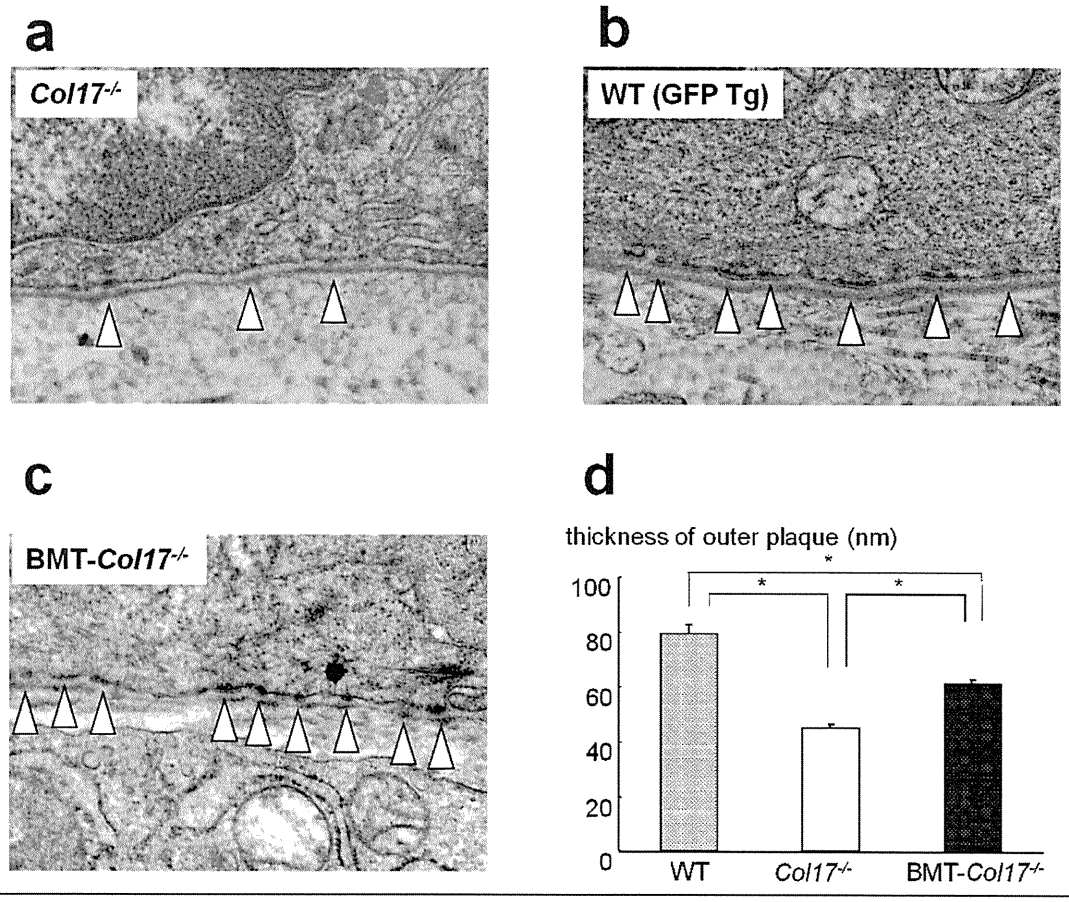

Fig. 3

Electron microscopy analysis in the skin after BMT into JEB mice. (a) Untreated Col17 knockout mice have thin, immature hemidesmosomes in the bottom of basal cell layer (arrowheads). (b) Normal C57BL/6 mice have mature, apparent hemidesmosomes. (c) Thick and matured hemidesmosomes are observed in the skin of BMT-treated Col17 knockout mice. (d) Thickness of the outer plaques of hemidesmosomes shows statistical improvement after BMT.

Stem cell therapy for epidermolysis bullosa

As mentioned previously, stem cell therapy is a promising strategy for systemic amelioration of EB for a long time. So far, a few investigations of BMT to treat RDEB have been published. Tolar *et al.* reported that hematopoietic stem cells contributed to life prolongation in RDEB model mice[26]. Chino *et al.* reported that treatment of embryonic BMT into RDEB model mice induced the expression of type VII collagen[27]. These reports proved the existence of donor-derived fibroblasts by immunohistochemistry and cell culture, and these fibroblasts are thought to produce type VII collagen. Based on these findings, hematopoietic stem cell therapies recently performed for RDEB patients in the US as a phase I/II clinical trial[28]. Five out of seven patients survived after the treatment, and less frequent dressings into the wound skin have achieved probably due to restoration of type VII collagen. These reports implied the benefit of stem cell transplantation in patients with deficient type VII collagen, which is produced by both epidermal keratinocytes and dermal fibroblasts[29]. Then, how is the clinical effect of stem cell transplantation in other subtype of EB, in which keratinocyte-specific skin component protein is lacked?

In order to answer the question we performed stem cell transplantation into adult Col17 knockout JEB model mice[25]. These treated mice expressed the lacked Col17 protein in the BMZ of the eroded skin around donor-derived GFP+ keratinocytes, with mature hemidesmosomes on the basal cells (Fig. 2, 3).

Clinical manifestations such as skin fragility and survival rates were also improved after stem cell transplantation (Fig. 4). Not only conventional BMT technique but hematopoietic stem cells transplantation and MSC infusion improved the expression of Col17. Furthermore, human hematopoietic stem cells also have a potential to restore epidermal component proteins by investigation of human-murine xeno-transplantation model, which implies stem cell transplantation might be a promising and fundamental therapeutic strategy for the treatment of severe EB patients.

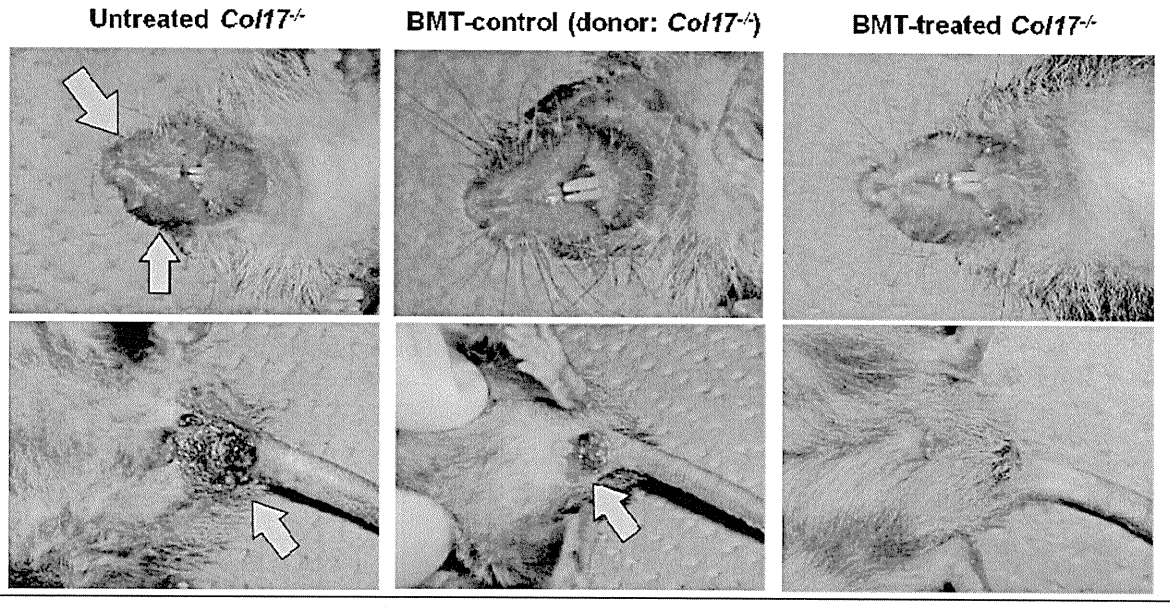

Fig. 4
Clinical manifestations of Col17 knockout mice after 120 days after birth (90 dayes after BMT). (left) Untreated Col17 knockout mice show erosions and ulcers on the perioral and perianal areas, which is compatible with clinical manifestations of JEB. (middle) As a control, BMT was performed from Col17 knockout mice into Col17 knockout mice. Severe erosions still appear as untreated mice. (right) Therapeutic BMT from GFP+ mice caused less severe erosions.

There still have problems to overcome on the stem cell transplantation for severe EB patients; *e.g.* risk of infection, conditioning regimens and donor supply. Although stem cell transplantation is prevalent, treatment-related deaths do occur due to severe infection, regimen-related toxicity and graft-versus-host disease (GVHD). Since EB patients have severe erosion and blisters on the whole body, severe cutaneous infections during the treatment could be fatal[28,30]. Conditioning regimens and the consideration of mini-transplantation should be determined carefully to avoid severe GVHD; both GVHD and regimen-related toxicity could cause severe erosions that are indistinguishable from EB symptoms. The donor is another challenge. Related HLA-matched siblings without EB phenotype are ideal for donors, but few cases meet the condition[28]. Unrelated HLA-matched stem cells from donor coordination programs, T-cell depleted haploidentical stem cell transplantation and iPS cell-bank projects might open the door to stem cell therapies in the future[31,32].

Concluding remarks

Stem cell therapies have been emerged as a promising strategy for congenital severe skin disorders such as EB. Although merits and demerits should be considered compared to gene therapies and protein therapies, novel treatments from the view of regenerative medicine will be one of the main streams to provide fundamental answers for severe disorders.

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CASE REPORT

Autosomal dominant bullous dermolysis of the newborn associated with a heterozygous missense mutation p.G1673R in type VII collagen

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ABSTRACT

Bullous dermolysis of the newborn is an inherited mechano-bullous disorder classed as a rare subtype of dystrophic epidermolysis bullosa. Fewer than 30 cases of bullous dermolysis of the newborn have been reported in the literature and the pathogenesis of the disease is poorly understood. Only a minority of cases have had pathogenic mutations identified. We present a case of a neonate born to non-consanguineous Caucasian parents with an exon 54 (c.5017G > A, p.G1673R) mutation reported as one mutant allele in a case of recessive dystrophic epidermolysis bullosa (generalized other).

Key words: bullous dermolysis of the newborn, genodermatoses, type VII collagen.

INTRODUCTION

Bullous dermolysis of the newborn (BDN) is an inherited mechano-bullous disorder classed as a rare subtype of dystrophic epidermolysis bullosa (DEB) and classified according to its inheritance as an autosomal dominant DEB-BDN or recessive DEB-BDN disorder.[1,2] It is characterized by blistering of the skin at birth in the setting of mechanical trauma, with subsequent improvement or complete resolution of symptoms over the ensuing months of life (OMIM 131705). Initial neonatal blistering can be widespread, involving the trunk and extremities. It occasionally affects the nails and mucous membranes and typically resolves by the end of the first year of life. Healing typically occurs without scarring, although permanent scarring has been reported.[2,3]

Fewer than 30 cases of BDN have been reported in the literature since the identification of the disease in 1985.[4] The pathogenesis of the disease is poorly understood; however, mutations in the collagen VII gene (*COL7A1*) have been implicated. Only a minority of cases have had pathogenic mutations identified.[2–8] The poor understanding of the disease process has detrimental implications in the accurate prognostication of neonatal blistering disorders.

We present a recent case of BDN involving a novel pathogenic *COL7A1* mutation. This mutation has also been described[6] in what was previously known as non-Hallopeau–Siemens recessive RDEB, now known as generalized other RDEB (RDEB-O).[1]

Main text

A male infant (Australasian EB Registry Patient Number: 234)[9] was born at 37 weeks gestation in November 2006 after an uncomplicated pregnancy to a mother of gravida 2, parity 0. The mother's first pregnancy underwent a

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Abbreviations:

BDN	bullous dermolysis of the newborn
DEB	dystrophic epidermolysis bullosa
RDEB-O	generalized other recessive DEB

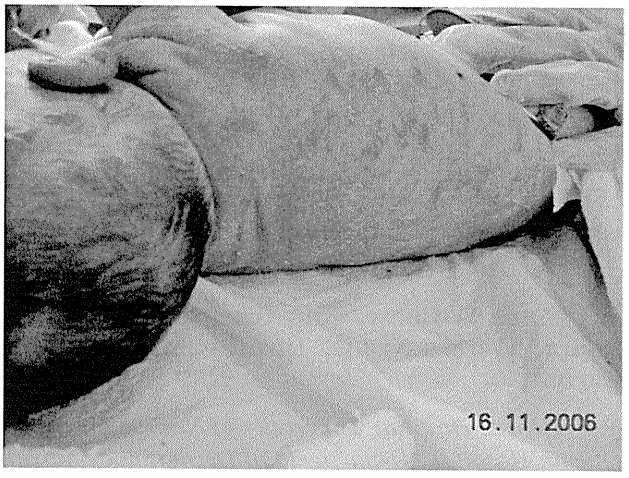

Figure 1 Neonate demonstrating blisters at birth.

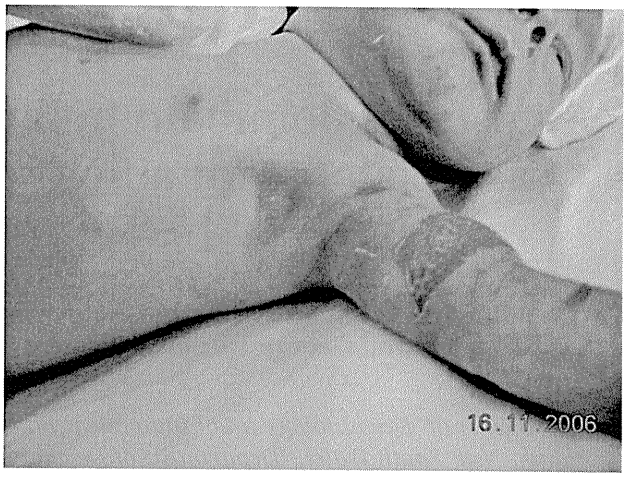

Figure 2 Blister caused by adhesive tape.

termination for reasons unrelated to the present case. The parents were non-consanguineous Caucasian Australians of British descent and had no previous children. The neonate was born with multiple erosions and erythematous patches that subsequently developed into confluent bullae on the trunk and extremities over the following hours (Fig. 1). The infant displayed neither mucosal involvement nor dysmorphic features and was otherwise systemically well.

Initial investigations of the proband included swabs of the eroded areas for microscopy, culture and sensitivity, Blood cultures were taken and intravenous flucloxacillin and gentamicin were initiated. On the second day of life, bullae were evident on the site where the cannula was secured to the arm with adhesive tape, and on other sites of mild mechanical trauma (Fig. 2). Subsequently, skin biopsies, light microscopy, immunofluorescence mapping (Fig. 3) and electron microscopy (Fig. 3) were undertaken.

Over the next 2 months, the severity and number of blisters and erosions decreased and previous blisters resolved without scarring. At 18 months of age, blistering had ceased and the only manifestations seen were milia on the palmar surface of the hands.

Differential diagnoses included Staph scalded skin syndrome, impetigo, toxic epidermal necrosis and epidermolysis bullosa.

Investigative results

Skin biopsies of the blisters showed sub-basal dermoepidermal separation below the level of the lamina densa. Immunofluorescence mapping showed reduced intensity staining with the LH7.2 antibody to collagen VII and basal as well as suprabasal collections of collagen VII (Fig. 3). Electron microscopy demonstrated a paucity of dermal anchoring fibrils and highly dilated endoplasmic reticula, forming stellate bodies within basal keratinocytes (Fig. 3).

The infant's genomic DNA from blood was found to be heterozygous for a missense mutation (Fig. 3) on exon 54 of *COL7A1* (c.5017G > A, p.G1673R) not previously described in BDN.[6,10] A second sequence variant on exon 5 was identified (c.592G > A, p.V198I); however, this was considered unlikely to be pathogenic. Both of these mutations were paternal in origin. In order to clarify the nature of the second sequence variant a polymorphism study was undertaken, screening 145 wildtype samples from a normal Caucasian European population for the c.592G > A, p. V198I sequence variant using polymerase chain reaction and direct sequencing. It was found in one of the 145 samples.

DISCUSSION

Bullous dermolysis of the newborn is characterized histologically by blister formation at the dermoepidermal junction, just below the level of the lamina densa. Dermal anchoring fibrils are reduced in number and quality, while epidermal collections of collagen VII are evident.[2] Electron microscopy reveals grossly dilated endoplasmic reticula of basal and suprabasal keratinocytes, known as 'stellate bodies'. The pathogenesis of BDN involves a defect in the intracytoplasmic packaging or transport of collagen VII in basal keratinocytes;[2] however, the underlying explanation of why specific mutations in *COL7A1* result in a transient as opposed to a permanent deficiency of anchoring fibrils is unclear. Only four *COL7A1* mutations have been previously described in BDN and none of the aforementioned mutations have been described in cases of recessive or dominant DEB.[10]

Previously described mutations in BDN include an acceptor splice site mutation in intron 35 of COL7A1 (4120-1G > C)8 and two cases involving glycine substitution mutations; p.G1522E[2] and a heterozygote for p.G1519D/p.G2251E.[5] In the latter case, p.G2251E was presumed to be the pathogenic mutation, as carriers for this polymorphism also displayed mild DEB characteristics such as nail dystro-

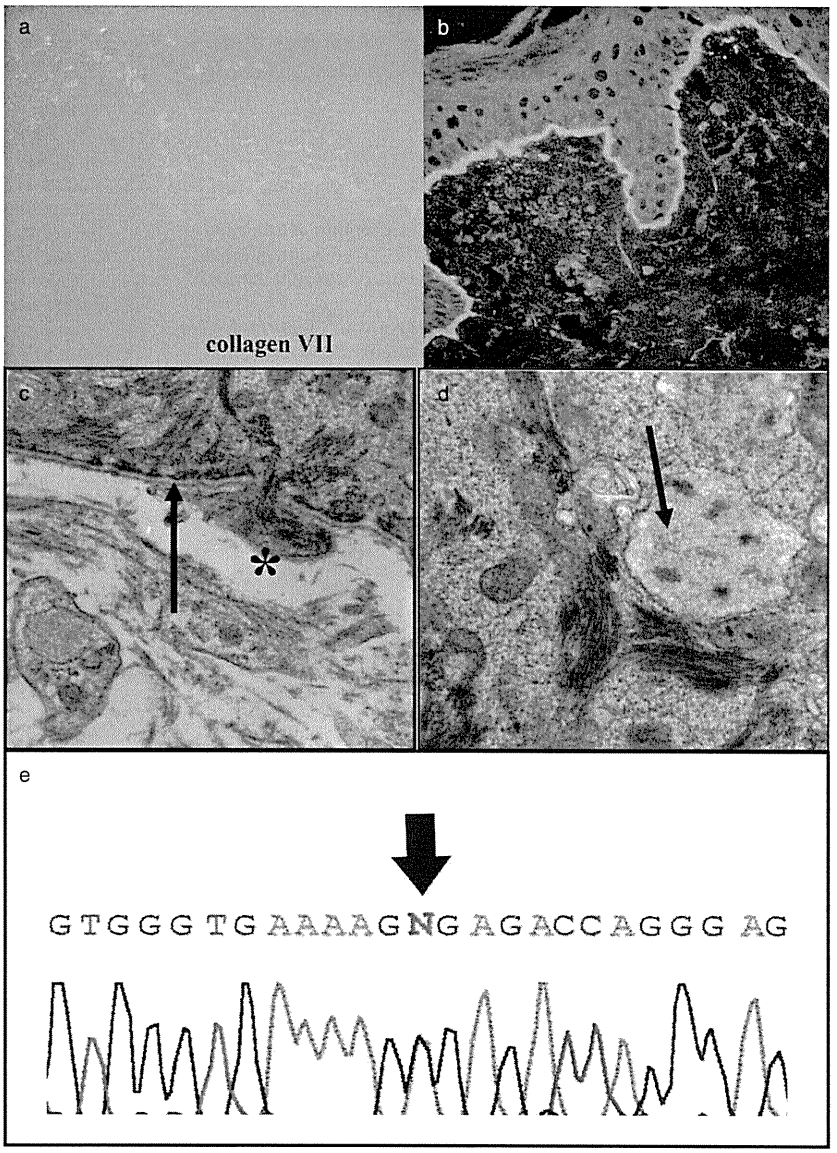

Figure 3 (a) Immunofluorescence mapping: stippled staining in the epidermis and lack of linear staining at the dermoepidermal junction, consistent with bullous dermolysis of the newborn (×20). (b) Control sample. (c) Electron microscopy showing the plane of cleavage (*) the basal lamina (arrow) and (d) typical keratinocyte 'stellate bodies' (arrow) (×10 000). (e) Genomic sequence data showing the missense mutation in exon 54 of *COL7A1*.

phy. The missense mutation in exon 54 (c.5017G > A, p.G1673R) has been previously described in another Caucasian family with RDEB-O;[6] however, the second mutation was not identified. Direct sequencing of exon 54 in 70 unrelated wildtype samples failed to identify the mutation (c.5017G > A, p.G1673R), indicating that it is not a neutral polymorphism. However, it may be possible that this glycine substitution is both a dominant and recessive mutation.

With regards to the exon 5 sequence variant, our polymorphism studies detected this genetic variation (V198I) once in 145 wildtype samples taken from a Caucasian European population. This suggests that the sequence variant is a rare single nucleotide polymorphism that is unlikely to be pathogenic. We postulate that this polymorphism is likely to lie on the same paternal allele of COL7A1, as the father and son both have the two mutations. Unfortunately, the mother declined genetic testing.

CONCLUSION

In summary, we present a case of autosomal dominant BDN in whom a linked *COL7A1* sequence variant has been identified which has been previously described in RDEB-O. Although the mechanism and pathogenesis of BDN is poorly understood, the fact that this mutation is also present in a previously described case of RDEB-O gives scope for hypotheses into how these two different diseases with different prognoses can coexist whilst sharing a pathogenic mutation. An increased understanding of the genotype–phenotype correlations in BDN may lead to a deeper

understanding of the mechanisms underlying defects in collagen VII production and transportation, which may in the future lead to therapeutic advancements for these disabling and disfiguring diseases.

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