six clinical features of AD disease intensity: erythema/darkening, edema/papulation, oozing/crust, excoriations, lichenification/prurigo, and dryness. Dryness was evaluated on noninflamed skin. The other features were assessed on a representative area for a given intensity item, also on a scale of 0–3. To measure AD clinical severity, we employed the objective SCORAD (OSCORAD; score range 0–83; Holm *et al.*, 2006). In the OSCORAD, which is often used in clinical trials, two subjective symptoms (itch and sleep loss) were excluded from the conventional SCORAD.

Measurement of stratum corneum hydration, TEWL, and stratum corneum thickness

Measurements were performed under standardized conditions, that is, at a room temperature of 22–25 °C and a humidity level of 40–55%. Before the measurements, patients were given time to adapt to room conditions without covering the measurement sites with clothes. All the measurements were performed by one investigator (INH). Almost all patients were taking anti-histamines, and treated by topical steroids, topical immunosuppressants, emollients (heparinoid), which were all kept maintained. However, from 2100 hours on the day before the investigation, nothing was applied to the skin to be examined. To exclude the bias of different dermatitis severities in the examination sites, three body sites, clinically normal areas in the extensor and flexor aspects of the forearm and on the back, were selected for examination. All measured values were expressed as the median of three recordings to avoid measuring inaccuracies. SC hydration was measured as (low-frequency susceptance) × (square root of electrode distance)/(square root of low frequency conductance) by using noninvasive methods (Yamamoto, 1994) with a Corneometer ASA-M2 (ASAHI BIOMED, Yokohama, Japan). ASA-M2 evaluated conductance of two different electric currents with low frequency and high frequency. The low-frequency current was limited to the superficial SC and the high-frequency current penetrated the highly moist region immediately below the SC. Thus, SC thickness was calculated from low-frequency susceptance and high-frequency admittance by the corneometer as (square root of low-frequency susceptance)/(high-frequency admittance; Yamamoto, 1994). TEWL was measured using Evaporimeter AS-TW1 (ASAHI BIOMED, Yokohama, Japan). AS-TW1 utilizes the ventilated chamber method of measuring TEWL. Its hygrometer measures the humidity of incoming air and of outgoing air that has passed over the test area of skin, and TEWL is calculated from the difference. TEWL measurements were done on the extensor and flexor sides of the forearm that were observed clinically normal. All the measurements were performed three times for each skin spot.

Immunohistochemical staining

Immunoperoxidase staining of paraffin-embedded sections was performed using the ChemMate Peroxidase/DAB system (Dako Cytomation, Hamburg, Germany). Mouse monoclonal antibody 15C10 (Novocastra, Newcastle, UK) was used to detect the human filaggrin repeat unit. Antigen retrieval was performed by heating sections under pressure for 10 to 15 minutes in 10 nmol l^{-1} citrate buffer, pH 6.0.

Laboratory tests

Peripheral blood EOS count (number × 100 per μl; normal 40–440), serum LDH (IU l^{-1}; normal 119–229), total serum IgE (IU ml^{-1}; normal 0.0–400.0), and allergen-specific IgE (SRL Inc., Tokyo, Japan) were measured. Allergen-specific IgE were estimated by fluoroenzyme immunoassays for house dust, mite allergen, grass pollen (*Tancy*), cedar pollen, fungal allergen (*Candida*), animal dander, and foods. Concerning to the sensitivity for detection of specific IgE, 100 lumicount and values greater than or equal to 100 lumicount were considered positive (+).

Statistical analysis

Statistical analysis was performed using Excel 2000 (Microsoft, Redmond, WA) with the add-in software Statcel 2 (OMS, Saitama, Japan) and JMP 6.0.3 (SAS Institute, Tokyo, Japan). Wilcoxon rank sum and Turkey–Kramer's honestly significant difference tests were used to compare the continuous variables between the group of total AD patients and normal controls, and between filaggrin-related AD and non-filaggrin AD. Data with *P*-values less than 0.05 were evaluated as significant. We interpreted *P*-values less than 0.01 as highly significant.

Simple regression analyses were also used to identify significant associations of SC hydration, thickness, or TEWL to OSCORAD. Data with *P*-values less than 0.05 were evaluated as significant. We interpreted *P*-values less than 0.005 as highly significant. Wilcoxon rank sum test and simple regression analyses were performed to assess the association or correlation between different biological markers including IgE, LDH, EOS, and the OSCORAD.

CONFLICT OF INTEREST

Irwin McLean has filed patents relating to genetic testing and therapy development aimed at the filaggrin gene.

ACKNOWLEDGMENTS

We thank Dr James R. McMillan for his critical reading of this paper. We thank Ms Akari Nagasaki, for her fine technical assistance on this project. Filaggrin/ichthyosis/eczema research in the McLean laboratory is supported by grants from the British Skin Foundation, National Eczema Society (UK), and the UK Medical Research Council (reference number G0700314) and donations from anonymous families affected by eczema in the Tayside Region of Scotland. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan to M. Akiyama (Kiban B 18390310 and Kiban B 20390304).

SUPPLEMENTARY MATERIAL

Table S1. Clinical information on the patients and FLG mutations.

Table S2. Patients' clinical severity (OSCORAD), SC hydration, TEWL, and SC thickness.

Figure S1. Box-whisker plots of OSCORAD in the two AD groups. Wilcoxon rank sum test and box-whisker plots revealed no significant difference in OSCORAD scores between filaggrin-related AD and non-filaggrin AD.

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Novel mutation p.Gly59Arg in *GJB6* encoding connexin 30 underlies palmoplantar keratoderma with pseudoainhum, knuckle pads and hearing loss

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Accepted for publication
31 January 2009

Key words
Bart–Pumphrey syndrome, Clouston syndrome, gap junction, pseudoainhum, Vohwinkel syndrome

Conflicts of interest
None declared.

DOI 10.1111/j.1365-2133.2009.09137.x

Summary

Background Connexins, components of the gap junction, are expressed in several organs including the skin and the cochlea. Mutations in connexin genes including GJB2 (Cx26), GJB3 (Cx31), GJB4 (Cx30.3), GJB6 (Cx30) and GJA1 (Cx43) are responsible for various dermatological syndromes and/or inherited hearing loss, frequently showing overlapping phenotypes.

Objectives To clarify the spectrum of clinical phenotypes caused by connexin mutations.

Methods We report a 32-year-old Japanese woman with mild palmoplantar keratoderma (PPK) with severe sensorineural hearing loss, knuckle pads and pseudoainhum of her toes.

Results Direct sequencing revealed no mutation in GJB2, but a novel heterozygous missense mutation p.Gly59Arg in GJB6. Electron microscopy revealed no apparent morphological abnormality of gap junctions in the patient's lesional epidermis.

Conclusions The patient harboured the novel GJB6 missense mutation p.Gly59Arg in the first extracellular loop of Cx30. Mutations in glycine 59 of Cx26 are associated with PPK–deafness syndrome, and the similar phenotype here supports the observed heteromeric channel formation; the dominant nature of the mutation suggests an effect on gap junctions similar to that of the comparable mutation in Cx26.

Gap junctions are cell-to-cell connecting structures containing clusters of intercellular channels that allow intercellular passage of ions and molecules of up to 1 kDa. These channels are oligomeric assemblies of family members of related proteins called connexins. Six connexin polypeptides assemble into a connexon, a hemichannel that interacts with its counterpart in an adjacent cell membrane to form a complete intercellular channel.[1] All the connexins in an individual connexon may be of the same type (homomeric), or heteromeric connexons may be formed by oligomerization between different connexins. Connexin 26 (Cx26) and connexin 30 (Cx30) are known to form heteromeric connexon hemichannels.[2]

Connexins are expressed in several organs including the skin and the cochlea. Mutations in connexin genes including GJB2 (Cx26), GJB3 (Cx31), GJB4 (Cx30.3), GJB6 (Cx30) and GJA1 (Cx43) are responsible for several hereditary skin disorders associated with hearing loss.[3] Cx30 mutations are typically associated with Clouston syndrome[4] in which palmoplantar keratoderma (PPK) is only occasional and not usual, although cases resembling keratitis–ichthyosis–deafness (KID) syndrome have also been reported.[5] Various mutations affecting Cx26 cause PPK–deafness syndrome.[6] PPK–deafness syndromes are typically GJB2 associated. However, as Cx26 and Cx30 interact, one might expect more phenotypic overlap as exemplified by the report of Jan *et al.*[5]

Here, we report a Japanese woman with clinical features resembling those of Vohwinkel syndrome and Bart–Pumphrey syndrome. We found a novel heterozygous missense mutation p.Gly59Arg in GJB6. As far as we know, this is the first reported patient with PPK with pseudoainhum, knuckle pads and hearing loss, harbouring a GJB6 gene mutation.

Patients and methods

Patient

The patient was a 32-year-old Japanese woman. She was diagnosed with congenital sensorineural hearing loss when she

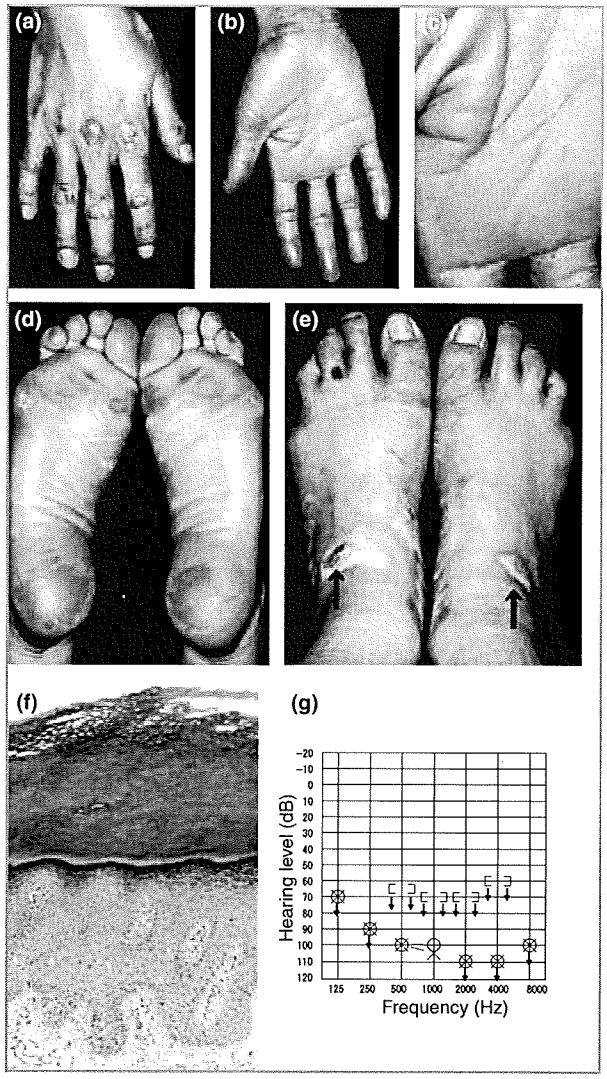

Fig 1. (a–e) Clinical features of the patient's skin. (a) Knuckle pads on the dorsa of the fingers of the patient; (b–d) diffuse palmar and plantar hyperkeratosis without honeycomb features; (d, e) amputation due to constriction bands on the fifth toe of each foot; extensive hyperkeratosis was seen on the ankle joints. (f) Skin biopsy from the sole revealed marked orthohyperkeratosis with hypergranulosis (haematoxylin and eosin; original magnification × 100). (g) Patient's pure tone audiogram showed pronounced sensory hearing loss: air conduction indicated by cross/round marks (cross, left ear; round, right ear); bone conduction indicated by bracket marks ([, right ear;], left ear); arrows pointing downwards indicate the loudest tone that was not heard.

was 3 years old. She also had diffuse PPK without a honeycomb hyperkeratosis appearance and hyperkeratotic plaques over the knuckles (Fig. 1a–c). An audiogram obtained at 24 years of age showed pronounced sensorineural hearing loss (Fig. 1g). At the age of 26 years, the fifth toe on each foot was surgically amputated due to pseudoainhum (Fig. 1d,e). Her fingers did not show mutilation. Extensive hyperkeratosis was seen in areas exposed to mechanical stress, such as the extensor (ventral) aspect of her ankle joints (Fig. 1e) probably because of folding the legs in the Japanese sitting or kneeling style. She had no features of ichthyosis on her trunk or extremities. Her hair, teeth and nails were normal and she had no ocular involvement. There was no family history of skin disorders or auditory dysfunction, or consanguinity in her family. All members of the family, including her parents and her elder sister, were generally healthy and were without PPK or hearing loss.

Mutation detection

After fully informed, written consent, peripheral blood samples were obtained from the patient and genomic DNA was extracted (Qiagen, Hilden, Germany). The entire coding region and exon/intron boundaries of *GJB2* and *GJB6* were amplified by polymerase chain reaction (PCR) using the specific primers described previously.[7,8] PCR products were sequenced and mutation was confirmed by enzyme digestion with BtgZI restriction enzymes (New England Biolabs, Ipswich, MA, U.S.A.). Reference cDNA for *GJB6* was cDNA sequence GenBank accession number NT_009799.

Morphological observations

A skin biopsy was taken from the right sole of the patient with fully informed consent. The biopsy specimen was processed for routine histological analysis and for ultrastructural observations as previously described.[9]

Results

Mutation analysis

Analysis in *GJB2* revealed no mutation, although a common polymorphism p.Val27Ile was found. We identified a heterozygous 175G>C transversion in *GJB6* (Fig. 2a). This novel nucleotide alteration leads to the replacement of glycine 59 (neutral, hydrophilic residue) with a positively charged hydrophilic arginine residue (p.Gly59Arg) in the first extracellular loop. The mutation introduces a single BtgZI restriction site in the gene. We confirmed the presence of the mutation in the patient's genomic DNA by restriction enzyme BtgZI digestion (Fig. 2b). This nucleotide change was not detected in 100 unrelated, healthy Japanese individuals (200 alleles).

Histological evaluation of the patient's skin

A biopsy specimen from the patient's plantar skin revealed compact orthohyperkeratosis with hypergranulosis and acanthosis in the epidermis (Fig. 1f).

Electron microscopic findings

Ultrastructurally, keratinocytes in the epidermis of the patient's plantar skin assembled gap junctions showing normal morphology with a typical pentalaminar structure, 20 nm in width (data not shown).

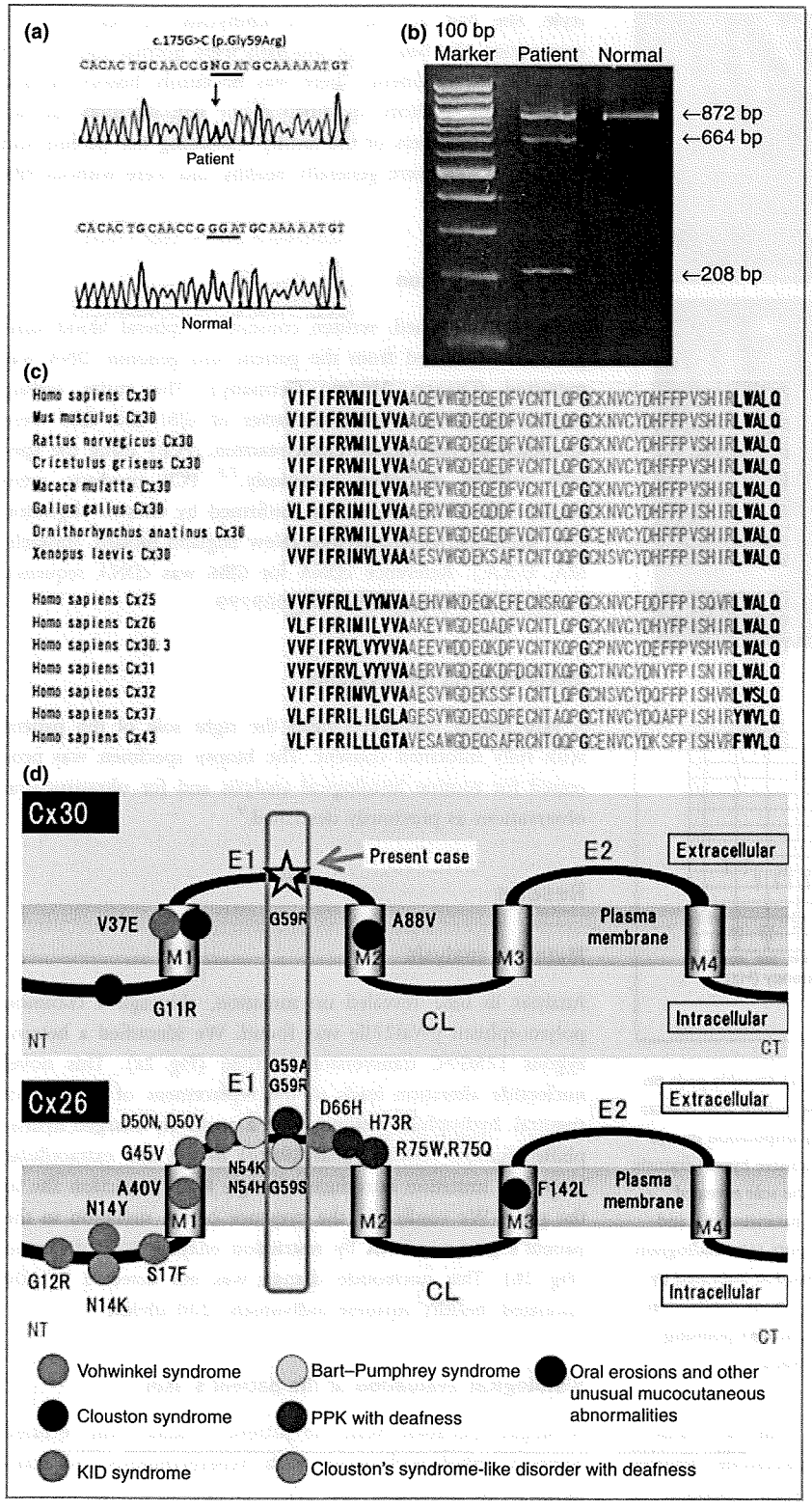

Fig 2. (a) Sequence chromatograms of GJB6 from the patient (upper) showed the heterozygous transition c.175G>C at codon 59 (p.Gly59Arg). (b) Confirmation of the presence of the mutation p.Gly59Arg in the patient by BtgZI restriction digestion. An 872 bp polymerase chain reaction fragment from the mutant allele was digested into 664 bp and 208 bp fragments, whereas that from the wild-type allele was not cut. Thus, 872 bp, 664 bp and 208 bp bands were seen in the patient harbouring the heterozygous p.Gly59Arg mutation, although only a 872 bp band was detected in the normal control. (c) Comparison of amino acid sequences of Cx30 from diverse species and other members of the human connexin family. Glycine residue at codon position 59 (red) is located in the centre of the first extracellular domain (blue) and is highly conserved in diverse species and other members of the human connexin family. (d) Cx30/Cx26-associated syndromes and reported causative mutations in Cx30/Cx26. M1–M4, transmembrane domains 1–4, respectively; E1 and E2, extracellular domains 1 and 2, respectively; CL, cytoplasmic loop; PPK, palmoplantar keratoderma; KID, keratitis–ichthyosis–deafness.

Discussion

We herewith report, as far as we know, the first case of PPK–deafness caused by a mutation affecting the E1 domain of Cx30. Glycine 59 in Cx30 is located in the first extracellular loop (Fig. 2c,d), which exhibits high sequence conservation in homologous proteins from different species (Fig. 2c). The first extracellular loop is thought to be essential for the

interaction between a connexon and its counterpart in an adjacent cell to form a complete intercellular channel. Three mutations in Cx26, p.Gly59Ala, p.Gly59Trp and p.Gly59Ser, occur at glycine 59 which is orthologous to glycine 59 in Cx30. These were reported in syndromes comprising sensorineural hearing loss and PPK[6,10] (Fig. 2d).

Cx30 and Cx26 form heteromeric junctions both in the skin and the inner ear and functional data suggest a dominant interaction between the two.[2,11] From these facts, we think it reasonable to speculate that missense mutations in the first extracellular loop domain in either Cx26 or Cx30 can lead to similar phenotypes. However, this hypothesis does not explain why the phenotypes look so similar or why, for example, p.Gly11Arg in Cx30 does not lead to KID syndrome whereas p.Gly12Arg in Cx26 does. There may be a genetic background effect which contributes to the phenotype in each patient.

The GJB2 keratoderma/deafness phenotypes are almost all caused by mutations clustering in the first extracellular loop domain.[12] The Cx26 mutation p.Gly59Arg results in a diffuse (although more severe) keratoderma, as does this mutation in Cx30 which is shown in the present patient. In contrast, mutations in the N-terminal cytoplasmic region in Cx26 are associated with KID syndrome and similar phenotypes. Likewise, p.Gly11Arg and p.Val37Glu in Cx30 in the N-terminal cytoplasmic region underlie KID-like syndrome or Clouston syndrome. We do not know the exact function of the N-terminal domain. There are two hypotheses: the first is that the N-terminus is involved in connexon assembly;[13] the second is that the N-terminal domain works as a plug in a vestibule of the connexon hemichannel, which physically blocks the channel (plug gating mechanism).[14] It was suggested that the mutation p.Asn14Tyr in Cx26, which is associated with a KID phenotype, causes the channel to be locked in a closed position by the plug.[15] Thus, if a similar phenomenon happens with the Cx30 mutation in a heteromeric channel composed of Cx30 and Cx26, Cx30 mutations in the N-terminus may lead to similar KID-like phenotypes. The present patient harbouring the Cx30 mutation in the first extracellular loop domain showed a phenotype distinct from KID syndrome.

Light microscopic observation of the lesional skin showed orthohyperkeratosis with hypergranulosis without any specific findings for our present patient. The mutation we found did not affect connexin morphology. Likewise, some Cx26 mutations in the E1 domain were examined for their effects on connexin morphology and apparently did not affect it.[11,16]

In conclusion, we present a novel *GJB6* missense mutation p.Gly59Arg in a patient who had PPK with pseudoainhum, knuckle pads and hearing loss. The present case expands the clinical spectrum of GJB6 mutations and shows that, in PPK–deafness cases where we do not find GJB2 mutations, we should check GJB6. Furthermore, our results suggest an interaction between Cx30 and Cx26 E1 domains.

Acknowledgments

We thank Ms Kaori Sakai, MS, Ms Megumi Sato and Ms Akari Nagasaki for their technical assistance on this project. We also thank Prof. James R. McMillan and Dr Heather Ann Long for proofreading this manuscript. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan to M.A. (Kiban B 20390304).

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Current Advances in Gene Therapy for the Treatment of Genodermatoses

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Abstract: Gene therapy provides the possibility of long term treatment for the severest of congenital disorders. In this review we will examine the recent advances in gene therapy for genodermatoses. Congenital diseases of the skin exhibit a wide range of severity and underlying causes and there are many possible therapeutic avenues. Gene therapy approaches can follow three paths-*in vivo*, *ex vivo* and fetal gene therapy, though the later is currently theoretical only it can provide potential results for even the most severe congenital diseases. All approaches utilize the many different vector systems available, including viral and the emerging use of non- viral integrating vectors. In addition, the use of RNAi based techniques to prevent dominant mutant protein expression has been explored as a therapy for specific dominant disorders such as keratin mutation disorders. Progress has been rapid in the past few years with some initial successful clinical trials reported. However, there are still some issues surrounding long term expression, transgene sustainability and safety issues that need to be addressed to further shift from experimental to clinically therapeutic applications.

With the continuing development, merger and refinement of existing techniques there is an ever increasing likelihood of gene therapies becoming available for the more severe genodermatoses within the next decade or shortly thereafter.

1. INTRODUCTION

Human skin provides the first line of defense between the internal and external environments and protects against external insults. Skin is composed of a multilayered epidermal sheet of stratified epithelia on top of a thick fibrous underlying dermis. The multilayered epidermal sheet comprises 4 distinct cell layers. The basal layer contains the partially characterized epidermal keratinocyte stem cell population. The basal keratinocytes divide and terminally differentiate forming the suprabasal and upper layers of the epidermis. The thicker spinous (suprabasal) layers lie above the basal layer, and as they move up during differentiation the cells gradually become flattened as they enter the granular layer where the cells collapse and active lipid and protein secretion commences. This protein and lipid secretion together with cell flattening helps to maintain the barrier function of the upper, cornified layers that are later shed. This cornified layer is composed of flattened keratinocytes and lipid/protein complexes which provide a barrier against water loss and external assaults.

Mutations in many epidermal-associated genes lead to a variety of skin diseases, affecting the ability of the skin to form a proper barrier against the external environment and withstand constant insults such as trauma [1]. There are currently three main areas of research into genodermatoses therapeutic treatment; protein therapy involving direct injection of specific polypeptides to correct specific protein defects, cell therapy where normal or gene corrected cells are applied to produce normal, functional molecules and gene therapy, targeting the mutated cells directly with a functional transgene.

In this review we will examine recent developments in skin gene therapy, examining the methods used and novel methods being developed that may prove beneficial in the future, particularly for the more severe genodermatoses. In many ways, skin is an ideal target for gene therapy - easy to monitor and identify results and easily accessible with the epidermis being largely avascular where gene therapy targets are localized. However progress on gene therapy has been slow. Many genes responsible for skin diseases have only recently been identified which has delayed the development of gene therapy. Other problems have been correct gene targeting and poor expression levels.

Current gene therapy techniques have focused on the use of viral vectors, lipid-DNA complexes or naked DNA alone for the delivery of transgenes and a variety of techniques for overcoming the stratum corneum barrier and introducing DNA directly to the skin have been developed. These techniques fall into three main gene therapy categories– *in vivo*, ex vivo and fetal gene therapy. Some disorders described have already benefited from at least one standard form of gene therapy using viral vectors and *in vitro* application of gene corrected keratinocytes to patients or animal models, for instance in the treatment of JEB and DEB. In the following section we will examine the current advances in specific disorders and discuss emerging areas that may benefit gene therapy approaches for other genodermatoses.

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1566-5232/09 $55.00+.00

2. MAJOR CANDIDATE DISEASES FOR GENE THERAPY

Genodermatoses exhibit a broad range of disease severity, from relatively minor disorders affecting the nails and hair to those which lead to a widespread failure of skin integrity which can be potentially fatal or severely impede normal function or quality of life. Gene therapy skin research has focused on diseases at the severe end of the spectrum as current clinical treatments are restricted to palliative treatment where the disruption to skin integrity is so severe that death can occur at any time after birth.

Bullous disorders are typically caused by a failure in cell anchorage or intracellular integrity due to mutations in proteins involved in cell structure, junctions and extracellular matrix interactions. These failures lead to disruption of skin integrity or a loss of adhesion in response to minor trauma. The congenital bullous disorders can be classified into 3 groups depending on the position or level of the skin separation and are often associated with mutations in proteins with mechanical or anchoring functions.

Mutations in the collagen VII gene lead to dystrophic epidermolysis bullosa (DEB) and occur as either dominant or recessive mutations (DDEB and RDEB respectively)[2, 3]. Collagen VII is a large protein which is expressed by both dermal fibroblasts but mostly by epidermal keratinocytes and is assembled into the extracellular matrix beneath the basal lamina forming the major component of the anchoring fibrils seen by electron microscopy that provides a structural link between the dermis and epidermis[4]. Mutations in the collagen VII gene lead to a reduction or loss of collagen VII expression [5, 6] which facilitates widespread loss of adhesion and dermal-epidermal separation in response to minor trauma. In the milder dominant DEB subtype, the blistering tends to subside over time and, in the more severe RDEB (the Hallopeau-Siemens variant) the blistering is more widespread and accompanied by digit fusion on the hands (syndactyly) and feet as a result of scarring and excess granulation tissue formation. The blistering occurs throughout life and is often accompanied by lesions in the oral mucosal and esophageal membranes and a tendency to develop malignant skin tumors, particularly squamous cell carcinoma.

Junctional EB (JEB), where separation of the dermis and epidermis occurs at the lamina lucida, is caused by mutations in either one of the hemidesmosomal complex proteins – laminin 332 and collagen XVII. Laminin 332 (formerly laminin-5) is a complex of 3 laminin polypeptide chains (three distinct genes products) and is thought to be involved in linking the NC-1 domain of collagen VII to the integrin complexes ($\alpha6\beta4$) [1, 7]. Mutations cause a loss or reduction in complete laminin 322 expression and loss of adhesion between the dermis and epidermis, leading to separation at the lamina lucida junction of these two tissues. The Herlitz type of JEB (H-JEB), where there is defective expression of laminin 332 [8], often leads to premature death within the first few weeks of life. In the milder form of non-Herlitz (nH)-JEB, there is partial expression of mutated or truncated forms of laminin 322 that leads to recurrent separation and scarring which severely impairs the quality of life of these patients [9]. Generally there is also incomplete alopecia and dental problems, including enamel hypoplasia and dental caries associated with this disorder (reviewed in [10]). Mutations in collagen XVII also lead to nH-JEB. Collagen XVII is a transmembrane constituent of hemidesmosomes, where it is thought to play a role in direct cell matrix adhesion and in maintaining structure and stability of anchoring filaments traversing the lamina lucida. Collagen XVII may also interact with the $\alpha6\beta4$ integrin and BPAG1 (BP230) within the hemidesmosomal complex. $\alpha6\beta4$ mutations have been mapped to two forms of EB junctional and simplex subtypes that are associated with pyloric atresia (EBS-PA or JEB-PA) that is often fatal, though milder variants occur with some missense mutations have been identified [11, 12].

Within the basal keratinocytes, mutations in a number of genes lead to epidermolysis bullosa simplex (EBS). These proteins are involved in cell-cell contacts and mechanical stability such as the keratins and plectin. Keratin 5 and 14 mutations lead to EB simplex forms (EBS), ranging in severity depending on mutation location and defect type. Keratins are cytoskeletal proteins, members of the intermediate filament super-family, with many types of keratins expressed in tissue specific and developmentally regulated manners. The mutations are usually dominant negative and can disrupt keratin assembly and structure leading to a loss of epithelial integrity in the basal keratinocytes and blister formation [13-15]. Mutations in the highly conserved residues lead to the most severe form Dowling-Meara (DM) EBS characterized by widespread blistering in response to minor trauma or friction, in less severe forms this blistering is restricted to hands or feet with few blisters at other sites. The Köbner form of EBS lies between these two in terms of disease severity and is associated with recessive mutations in the keratin genes. Plectin mutations also lead to forms of EBS, often with other organ involvement including muscle symptoms due to the wide expression of plectin and its isoforms. Plectin is a large cytoskeletal linker protein, linking intermediate filaments to actin, and the microtubule system. Its tissue wide expression means that EBS symptoms often occur along with other disorders, namely muscular dystrophy (EBS-MD) [16, 17]. Recently, two families were described with JEB-PA in which mutations in the plectin gene were discovered [18].Out of the three cases, two patients died demonstrating the seriousness of the combination of EBS and other complications.

Other serious disorders of the skin keratinization process can lead to hyperkeratinization of the skin. One of the most severe forms of this is Harlequin Ichthyosis (HI), which is often fatal and is characterized by the formation of large thick plate-like scales covering the body during development, often leading to flattened ears, ectropion (inside-out-turning of the eyelids) and eclabium (outward turning of the lips). These symptoms lead to massive fluid loss and infection shortly after birth and it is often fatal within the first few weeks after birth. It is only recently that the underlying mutations have been identified and reported. The lipid transporter protein ABCA12 was previously implicated in a milder form of ichthyosis, type 2 lamellar ichthyosis, as minor missense mutations in ABCA12 were found associated with this disorder [19]. More serious, truncation or splice site mutations in ABCA12 have been mapped to HI [20-23]. These mutations lead to loss of ABCA12 expression causing loss or reduction in lipid secretion and loss of normal trafficking of lamellar granules from the upper epidermis and

thickening of the cornified layer. This thickened epidermis lacking in extracellular lipids that help prevent excessive moisture loss then cracks in response to movement leading to deep fissures allowing moisture and fluid loss and a route for infections to enter the body. The current treatment for HI is the use of retinoids namely- etretinate or acitretin and these drugs have been shown to be effective in controlling excessive epidermal thickening the main symptom affecting HI patients. Long term use of these drugs has side significant effects on the development of individuals such as raised cholesterol levels and pregnancy problems [24] and these retinoids can only be applied after birth since they are strong and are likely to have potentially devastating teratogenic effects during development. Initial *in vitro* (*ex vivo*) studies have already shown that ABCA12 defects in cultured keratinocytes can be corrected by transient transgene expression [22].

Gene therapy for other genenodermatoses has recently been discussed, for example xeroderma pigmentosum disease which is characterised by an increased frequency of skin cancer [25]. Here, we will discuss gene therapy options for the previously discussed diseases in the context of current advances and future areas to consider.

3. RECENT ADVANCES AND NOVEL THERAPEUTIC APPROACHES

3.1. *Ex-Vivo* Gene Therapy; Advances for Epidermolysis Bullosa; Herlitz Junctional EB (Laminin 332 (Formerly Laminin-5) Defects

The discovery of a mutational hotspot (p. Arg635X) in the beta 3 chain of laminin 332 that affects approximately 50% of all HJEB patients has cleared the way for groups to develop transgene vectors that target this specific gene defect [8, 26]. Ortiz-Urda *et al.*, have reported the successful integration of LAMB3 encoding the laminin beta 3 chain into primary keratinocytes from severe Herlitz JEB patients using a plasmid encoding ϕC31 integrase, containing the recombination sequence attP [27]. These keratinocytes were transplanted onto immune-deficient SCID mice and human skin was produced from the grafts that expressed normal laminin 322 with no evidence of sub-epidermal blistering or dermal-epidermal junction adhesion and normal hemidesmosome assembly. Using canine keratinocytes from a canine JEB model, keratinocytes were transfected with laminin α3 chain cDNA using murine retroviral vectors [28]. The group achieved long term, stable expression, with high transfection efficiency and the corrected cells acquired normal keratinocyte morphology and adhesive behavior when grafted onto the back of SCID mice.

In a phase I/II clinical trial, primary keratinocytes were cultured from a patient suffering from non-lethal junctional epidermolysis bullosa (JEB) affected by a point mutation in the LAMB3 gene (encoding laminin 332-β3 chain [29, 30]. These primary keratinocytes were corrected using LAMB3 cDNA under the control of a moloney leukemia virus promoter (MLV-LTR). These corrected keratinocytes were transplanted as grafts back onto the patient where they demonstrated a rescued, non blistered phenotype and continued expression of the transgene for at least one year. The authors suggested that the transgene was present in the epidermal stem cell population allowing the expression to continue during prolonged epidermal renewal. Furthermore, they were unable to detect any clonal expansion or selection of integration events *in vivo* which is a neoplastic risk associated with the use of MLV-derived retroviruses. Though still in the early stages, this apparently successful but limited trail has demonstrated the powerful potential of targeting stem cells in the maintenance of long term expression of transgenes in gene therapy.

Other gene mutations underlie typically less severe forms of JEB (so called non-Herlitz JEB subtypes). Collagen XVII mutations result in an absence or deficiency in collagen XVII expression (reviewed in [31]). Retroviral gene transfer of collagen XVII into nHJEB patient keratinocytes resulted in expression of protein at the dermal/epidermal junction with no evidence of blistering in reconstituted epidermis [32] demonstrating the feasibility of gene therapy for this disease. The production of a collagen XVII-null mouse has recently been reported and will prove to be an essential tool for developing novel therapies for non-Herlitz JEB [33].This mouse has been used in research into the autoimmune disease bullous pemphigoid, which is typified by production of autoantibodies against collagen XVII resulting in blistering in patients. Using these mouse-collagen XVII null model mice, a humanized mouse expressing only the human collagen form (not mouse) was produced and autoantibodies against this protein were induced via injection into the tail vein [27, 33].

In this section we discussed *ex-vivo* gene therapy in the context of using either gene corrected autologous cells or transfected keratinocytes for gene therapy where these cells are applied to patient or animal models in the form of grafted cells or tissues. In the next section gene therapy using cell suspensions for direct or indirect application to patient or model animal skin is discussed.

3.2. Cell Therapy: Progress for Severe Recessive Dystrophic EB

Cell therapy, the use of cells as protein factories, can be readily attempted in the skin and reports have already been shown to have some therapeutic benefit with collagen VII. Collagen VII is secreted into the region below the dermal epidermal junction. Nonsense mutations in collagen VII lead to severe recessive dystrophic epidermolysis bullosa (SRDEB). Both keratinocytes and fibroblasts produce collagen VII, though in the steady state the expression in keratinocytes is greater than in fibroblasts [27, 34].

Studies using lentivirus [35] and retrovirus [36] and phi C31 integrase [27] have been employed to transfer collagen VII into keratinocytes, however, the expression of this transgene was relatively low, perhaps related to the large size of this specific transgene. Other approaches have targeted fibroblasts, using gene-transfected autologous DEB fibroblast dermal injections [37-39]. Genetically altered fibroblasts have been shown to produce sufficient levels of collagen VII and may be more clinically beneficial than gene transferred keratinocytes [39] especially in skin grafts on SCID mice and so be a more attractive target for gene therapy in dystrophic EB patients [40].

Recently, allogeneic fibroblasts have been used with RDEB patients in a small clinical trail [41]. Intramuscular injections of allogeneic fibroblasts were given as a single injection and the progress followed in 5 patients of RDEB, all of whom still maintained some partial mutant collagen VII expression. The results were encouraging as an increase in collagen VII was detected at the dermal- epidermal junction and a reduction in blistering was also noted. The fibroblasts themselves did not survive long in the patients and the main effect appeared to be an increase in the patients own collagen VII mRNA levels, suggesting that the mutant protein, still expressed in these cases, was capable of partial adhesion capabilities. These patients were only followed up for 3 months so it is not clear whether this effect was sustainable for longer and it might only benefit those RDEB patients who maintain a low level of expression due to the principle effect of the patients own mRNA level increase.

The use of cells as protein factories is a strategy that is likely to benefit those disorders where a gene product is exported from cells after synthesis, as in the case of DEB and collagen VII where this occurs from both fibroblasts and keratinocytes. For the majority of other genodermatoses the gene product is required within the cell or is limited to one specific cell type, therefore other methods or approaches for introducing and regulating transgene expression have to be employed and explored.

3.3. RNAi Technology to Prevent Mutant Protein Translation and Aminoglycosides to Promote Mutant Protein Expression

Mutations in either the intermediate filament proteins keratins 5 or 14 lead to epidermolysis bullosa simplex forms of human bullous diseases. The theory of gene therapeutic approaches to these keratin 5 and 14 diseases is not restricted to this specific disease subtype, but also applies to other keratin and intermediate filament genodermatoses. These mutations are generally dominant and can be severe but are also affected by significant phenotypic variation in the extent of disease severity. Gene therapy approaches for these diseases differ from other genodermatoses due to the dominant negative effect of the mutations on the remaining, wild type, paired keratin bundles. Transgenic mouse studies have suggested that over-expression of the normal keratin copy can overcome these dominant mutations to a significant extent, but only for specific keratins [42, 43]. Another intermediate filament protein, desmin (usually present in mesoderm derived tissues including muscle), has also been used to restore the function of keratinocytes containing dominant negative mutations in K5 and K14. The introduction of an alternative replacement protein restores a cells' response to physical stresses such as scratch wounding, heat and osmotic shock. [43]. This technique could benefit a number of mutations as it is independent of any site specific mutation or particular keratin gene.

A separate approach is to target the mutant keratin directly using RNA and DNA specifically targeted against the mutant form of DNA, this approach provides probably the most successful gene therapy technique for dominant gene disorders as this allows the normal gene product to function. Targeting mRNA using siRNA can be effective[44], along with other techniques such as spliceosome-mediated RNA trans-splicing (SMaRT). SMaRT uses the endogenous spliceosome machinery to effectively excise mutant exons knocking out the mutant protein from the cells and is thus potentially beneficial in dominant negative disorders. This technology has been shown to correct mutations in keratinocyte collagen XVII [45], plectin mutations in a fibroblast model of epidermolysis bullosa simplex with muscular dystrophy (EBS-MD) [46]. The transfection of the fibroblasts with the pre-trans-splicing molecule (PTM) corrected the mutant transcript restoring wildtype plectin expression. These initial experiments demonstrate the potential for this technique for dominant gene disorders.

Another potential therapy is the use of molecules which cause defective gene mRNA transcripts to be read, allowing some mutant protein to be expressed. Potentially, this mutant protein could provide limited function and alleviate some of the symptoms (reviewed in [47]). Aminoglycosides, commonly used as antimicrobial agents, can cause translational mis-reading (at low concentrations) in eukaryotic ribosomes leading to translation of normal or mutant proteins. These compounds are useful for gene disorders with premature termination codons as they can cause readthrough of transcripts and expression of the truncated protein. The use of these agents have been examined with respect to cystic fibrosis and muscular dystrophy with varying success, but this emerging technology may have some role in therapy for those genodermatoses carrying premature termination codon mutations and should be further explored.

3.4. Targeting Epidermal Stem Cells for Sustained Transgene Expression

Stem cell targeting is widely regarded as the key to achieving long term transgene expression. However in the epidermis, there is a lack of clear markers identifying the interfollicular epidermal stem cell population. Currently those cells that are thought to be stem cells are selected on the basis of self-renewal potential and low terminal differentiation rates. The identification of p63 as a potential marker for keratinocyte stem cells may be of benefit [48]. p63 is a p53 homologue transcription factor, but has been shown to be expressed in early development stages and in the epidermis and was shown to be highly expressed in transit amplifying cells in stratified epithelia [48]. However there is some controversy as to whether p63 truly functions as a stem cell marker [48, 49] or is simply a lineage commitment or differentiation marker [50-52] as p63 has been implicated in a number of developmental roles- p63 knockout mice lack limbs suggesting a role of p63 in limb formation [50] and also in the stratification of the epidermis [51]. Cell surface markers such as α6 integrin [53, 54] transferrin receptor (CD71, in mice) [54], Lrig1 [55] have been also shown to be putative adult epidermal stem cell markers. It is becoming more obvious that a combination of these markers helps in identifying epidermal stem cell populations. For example, high expression of α6 integrin and low expression of CD71 identified a small sub population of cells with high regenerative capacity and quiescence which suggested they may represent an epidermal stem cell population [53], Other, more general hematopoietic stem cell markers such as CD34 in mice [56, 57] and CD90 [58] have also been identified on

putative epidermal stem cell populations. Considerable efforts are being made to definitively identify epidermal stem cell markers and their relationship with each other (particularly for adult interfollicular stem cells) for both mice and human epidermal stem cells which will help in isolation or targetting for therapy.

Many previously discussed therapies have already indiscriminately or inadvertently targeted epidermal stem cells as transgene expression has persisted throughout subsequent rounds of epithelial turnover.

Stem cells have been used in enhancing wound healing following severe injury. For example in burns or chronic ulcers, using hematopoietic stem cells from bone marrow that have transdifferentiated into epithelial cells [59] or follicular stem cells, help to regenerate or repopulate interfollicular keratinocytes, or may aid areas of poor wound healing where the epidermis has been lost as in chronic ulcers [60] and burns. A similar therapy could be applied to the EB diseases where blistering and skin fragility often lead to poor wound healing. The use of either gene corrected stem cells or non-mutant stem cells from normal donors is being examined with respect to genodermatoses treatment and this area is likely to be rapidly developed as a potential therapeutic treatment in the next few years.

4. FETAL GENE THERAPY

Still in the theoretical stages and currently under development in various animal models, fetal or in utero gene therapy has the potential to provide significant benefits in the treatment of severe congenital diseases including the most severe genodermatoses. Due to the predicted increase in pluripotent or stem cell populations and higher stem cell density presumed to be present in fetal tissue, targeting tissue in utero, is thought to improve the chances of sustained transgene expression.

Previous fetal gene therapy studies have employed viral vectors for gene transfer and thus far, the results have been reported in mouse or sheep models of human diseases. Fetal gene therapy for Cystic fibrosis (caused by mutations in the Cystic Fibrosis Transmembrane Regulator gene, CFTR) has been explored and intra-amniotic application of the CFTR gene using both mouse [61] and sheep models [62] have been used with moderate success and have demonstrated therapeutically relevant levels of transgene expression. Other groups using different defective genes to target different diseases have shown positive responses after fetal gene therapy; Criglar-Najar disease type I [63, 64] Leber's congenital amaurosis [65], Pompe's disease [66] and hemophilia B [67]. The rare glycogen storage disease, Criglar-Najar disease type I, affects the liver. Using a modified lentivirus expressing the corrected form of the defective gene, UDP-glucuronyl bilirubin transferase, was injected into mouse fetal liver, Seppen *et al.* reported a 45% reduction in harmful bilirubin levels for up to a year [63, 64]. Side effects were observed including an increase in liver tumors in mice treated with a lentiviral vector in utero [68]. Other studies have successfully transduced specific cells in early mouse embryos which were then implanted into the mouse to then develop normally. For skin treatment, simple intra-amniotic injections are thought the easiest and most direct methods for transgene introduction.

Recently, intra-amniotic delivery of LAMB3, the beta chain gene of laminin 332 has been tested in a mouse model of Herlitz JEB ([69]. Using an adeno-associated virus and adenoviral vectors carrying the murine LAMB3 cDNA, these vectors were intra-amniotically injected at 14 days post-coitum (full term 20 days). Although both viruses targeted the fetus, the adenovirus preferentially targeted the epidermis whereas the AAV targeted to the mucous membranes of the upper airway and digestive tract. The gene was expressed and lead to a reduction in basement membrane separation. Disappointingly, using only combinations of these two viruses there was only a moderate synergistic increased survival of these mice, although the poor survival was partly attributed to the rejection (attack) of the abnormal pups by the mother (as is frequently seen in genodermatoses knock-out model mice). Despite this initial failure, fetal gene therapy is an area that could benefit those severe genodermatoses with high mortality from birth.

There are many factors to consider within this particular area of gene therapy for example the timing of treatment to optimize the gene expression to attain the optimal expression levels in particular cell types. This will obviously depend on the baseline expression of the causative gene involved and would require studies into the specific developmental expression of all the genes involved. In addition, the ethical considerations of fetal gene therapy need to be carefully considered since the risks of premature labor, miscarriage or gross infection are thought to be higher [70] and obviously prior knowledge of the severity of a disorder (from an affected proband) may mean parents opt for a prenatal test and termination instead of treatment [71]. Nevertheless, it is an important area for future focus particularly for the most severe genetic diseases. Further studies may benefit genodermatoses such as HI, in which the window for treatment is short immediately after birth and as early treatment with retinoids cannot be given before birth due to their teratogenic effects. The relatively large size of the ABCA12 gene means that retroviruses, for example, would be unsuitable as vectors due the limits of packaging size. Other viral vector systems, adenoviruses for example, could support the size but this expression is typically short term, whether this treatment would be sufficient to allow newborn survival until retinoid treatment could become effective needs to be explored. The development of non-viral vectors and their use in fetal gene therapy has also yet to be explored. However, if the ethical and technical difficulties involved in fetal gene therapy could be addressed the benefits for the treatment of severe genodermatoses would be significantly improved.

5. PHYSICAL METHODS OF INTRODUCTION; POTENTIAL FOR *IN-VIVO* THERAPY

A number of groups are looking at in-vivo gene therapy options that require no use of cells and thus are likely to have less complicated procedures and less ethical problems for future therapy. One main focus is looking at the direct introduction of the genes into the skin using a wide range of physical techniques. There are numerous methods for introducing DNA into the skin, whether as naked DNA, in complexes with lipids or in viral form.

Perhaps the simplest of therapies is the direct injection of naked DNA into the skin. This technique is particularly beneficial for cytokine gene expression which can be used in the treatment of skin ulcers [72] and malignant melanoma [73] or for congenital blistering disorders such as epidermolysis bullosa [74] for example. Plasmid DNA is injected intradermally, where is diffuses through the dermal epidermal junction before uptake by a keratinocyte and subsequent expression. There have been many trials in keratinocytes and with mouse, rat, pig and human skin using interleukin 8 [75] and interleukin 6 [76]. One of the main limitations appears to be the short expression of the transgene using this delivery method and the small number of cells that often take up the DNA. There are no antibodies raised against the naked DNA itself, though this technique can also be employed as a vaccine against tumor agents [77].

Electroporation of DNA is an established method for improving the delivery of DNA into many tissues *in vivo*. In the case of skin, DNA is applied in plasmid form, which has been introduced intradermally or biolistically. The skin is then subjected to electric pulses applied over the skin surface or through penetrating electrode needles reviewed in [77, 78]. Recently, this application was applied to malignant melanoma tumors that showed significant reductions in the outgrowth and metastases after RNAi transcription factors were directly electroporation into the tumor mass [78].

A biolisitic approach, using ballistics to deliver biological agents to cells and tissues, was originally designed for introducing genes into plants and has been subsequently adapted for mammalian cell use. Genes incorporated into a mammalian expression plasmid are coated onto micrometer-sized gold particles and accelerated towards the target tissue using a so-called gene gun-using gun-powder [79], or compressed gases [80]. The particles penetrate the cell membrane or even the nucleus directly and the plasmids are stripped off the gold particles and become incorporated and subsequently expressed. The advantage of this technique is that specific cell layers can be targeted, after controlling the impact momentum per unit area for each dose of microparticles [81, 82]. Typically, however, the expression of these plasmids is only transient. However this technique has been successfully used for genetic immunization, and targeting tumors with "suicide genes", making the use of the gene gun relatively wide ranging.

Engineered needles of micrometer size have had tests of their ability to introduce transgenes into the skin particularly the epidermal layers These microneedles are usually in an array and are sufficiency small to cause little or no pain when applied [83] and in array formats transgenes can be applied to large areas of skin, however the expression is still low and limited to the short term in most cases. This is perhaps, the main and most common current limitation with many *in vivo* gene therapy approaches in the skin.

6. PROBLEMS AND FUTURE BENEFITS

There may be a number of complications arising from gene therapy in the skin. One major complication includes the immune-mediated loss of transgene expression. This has been seen using a lacZ retroviral vector in mouse skin as a model [84], though this a common problem for adenoviral vectors too. The transduced mouse skin became infiltrated with CD4 (+) and CD8 (+) cells and cytotoxic T-Lymphocytes were also observed. This immune response likely leads to loss of LacZ transgene expression directly through the action of both the CD4 (+) and CD8 (+) cells on the virally transduced cells. More recently, one group showed that different gene therapy approaches are likely to stimulate different immune responses that lead to transgene loss. *In vivo* transfer gene transfer in epidermis induced Th-1 (T-helper 1-type) responses leading to transgene loss, whereas *ex vivo* transduced keratinocytes elicited a Th2/eosinophilic response and subsequent rejection of the transfected keratinocytes [85]. The Th2 response could be prevented with pre-treatment with an interleukin 5 (IL-5) antibody, as both IL-4 and IL-5 were produced by T-cells in response to the ex-vivo keratinocytes in the mice models. The types of cells targeted or applied in gene therapy can also elicit different immune reponses. Zhang *et al.* have shown that grafted transgenic keratinocytes were rejected in mouse models through a dominant Th-2 response [86], but transgenic fibroblasts failed to induce acute rejection although Th-1 mediated inflammation was seen in the grafted area. Also they noted that in a small number of animals, the transgenic fibroblasts persisted for more than 20 weeks despite Th-1 immune responses being generated [86]. This data could perhaps explain why, in SRDEB, the fibroblast cell therapy was more effective than the use of keratinocytes not least from an expression point of view, but also with respect to immune responses. These immune responses are an important area to consider in any future gene therapy trial and pre-treatment with interleukin or other immunosuppressant drugs may help in attenuating or preventing immune responses and careful selection of cells for targetting may help in reducing immune responses against gene therapy treated skin.

Newly modified viral vectors and other vector systems and techniques are being developed which have been designed and have been shown to overcome these problems and as the field continues to develop further, these potential risks should become easier to assess and overcome.

7. CONCLUSIONS AND PROSPECTS FOR THE FUTURE

The severe nature of some of the genodermatoses means that current treatment is very limited. The use of gene therapy for these severe disorders provides an opportunity for improving the outcome for many patients.

Current research is working towards developing treatments for specific genodermatoses and a number of studies have already employed small scale clinical trials with limited success particularly for junctional and dystrophic EB. New techniques or combinations of techniques which improve transgene expression, delivery and safety should provide further opportunities to develop approaches that will pay off for the treatment of severe congenital human skin disorders. It is likely to be several years (perhaps 10 or more) before these treatments become more widely or routinely used due to issues of ensuring safety and implementing standard procedures, but the initial promise and prospects for epidermal gene therapy are now already tangible.

ACKNOWLEDGEMENTS

This work was supported by a grant-in-aid from the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO) (H.A.L). Health and Labor Sciences Research Grant (research into Human Genome, Tissue Engineering) H17-Saisei-12 (J.R.M.) and by a grant-in-aid of Scientific Research A (17209038, H.S.) from the Japanese Society for the Promotion of Science. This work was also supported by a Health and Labor Sciences Research Grants (Research into Measures treating Intractable Diseases) from the Ministry of Health, Labor and Welfare (H16-Nanchi-05, to H. S. and a Project for Realization of Regenerative Medicine from the Ministry of Education, Science, Sports, and Culture of Japan (to H.S.).

DISCLOSURE STATEMENT

No competing financial interests exist.

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Received: June 04, 2009 **Revised: September 21, 2009** **Accepted: September 23, 2009**

dermatomyositis muscle, myofibre injury is apparent in two forms: large areas of apparent infarction visible as the loss of myofibrillar uptake of multiple histochemical stains, seen occasionally in juvenile dermatomyositis and rarely in adult dermatomyositis, and the presence of perifascicular atrophy (PFA). PFA is a term used to describe small, basophilic myofibres around the edges of muscle fascicles. PFA does not generally affect all myofibres around the edge of the fascicle, but rather usually affects the specific myofibres that border the loose connective tissue (the perimysium), sparing myofibres that border other myofibres in neighbouring fascicles. The lesion is better described by the term perimysial PFA,[1,2] or even perimysial myofibre atrophy.

In dermatomyositis skin, the characteristic pathology is an interface dermatitis, in which abnormalities are most prominent at the boundary between the epidermis and dermis. It is characterized by the presence of dying keratinocytes centred mostly on those in the basal layer, in opposition to the underlying basement membrane. The more differentiated cells that are located higher in the stratum spinosum or stratum granulosum are rarely affected. This cell death is manifested by cells with pyknotic nuclei and eosinophilic cytoplasm as well as by vacuolization of the basal layer. Curiously, the dying cells are often not confluent, and are present in patchy foci often in association with infiltrating mononuclear cells. Epidermal atrophy is also seen in long-standing lesions.

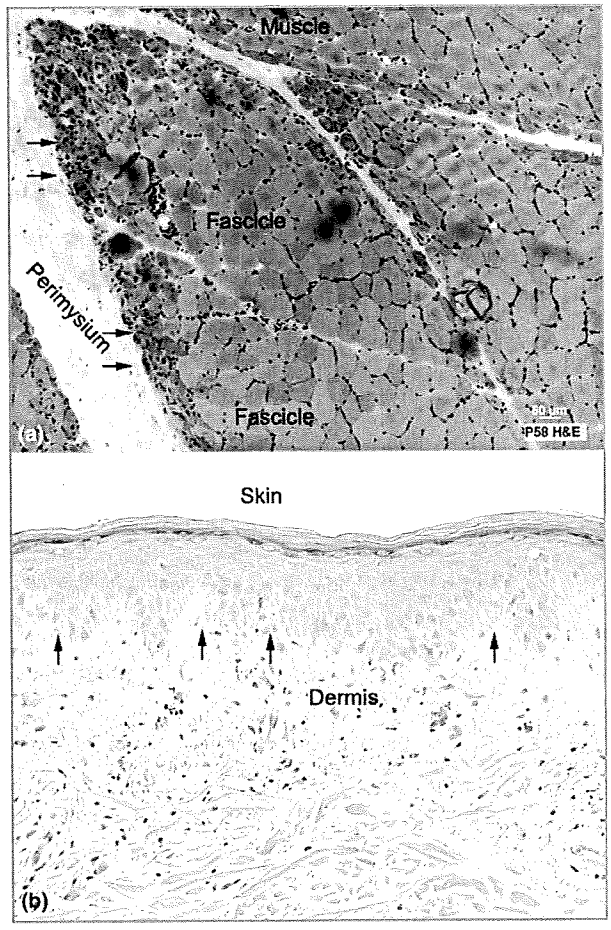

Fig 1. (a) Perimysial perifascicular atrophy in muscle. Small, basophilic fibres at the interface between myofibres and perimysial connective tissue (black arrows). (b) Interface dermatitis in skin. Vacuolated and degenerating keratinocytes at the interface between keratinocytes and loose connective tissue (black arrows). H&E, haematoxylin and eosin.

We point out here that the topology of the injury to both myofibres and keratinocytes is similar in dermatomyositis muscle and skin (Fig. 1). In both cases, injury preferentially affects the cells that border the loose connective tissue adjacent to them, with relative sparing of cells that are entirely surrounded by other cells. In cases with greater severity of changes, the pathology extends into deeper regions of muscle fascicles and more superficial layers of keratinocytes. Why such muscle and skin cells bordering loose connective tissue are more susceptible to injury is unknown. One speculative possibility is that these bordering cells are closer to cells of the immune system and their secreted products. It is possible that the pattern of muscle inflammation in dermatomyositis represents the 'muscle equivalent' of a lichenoid tissue reaction in the skin. Indeed, in this disease the inflammatory cells are usually much more abundant in muscle in the perimysial connective tissue than within fascicles, as they are more commonly seen in the papillary dermis and dermoepidermal junction than within the more superficial keratinocyte layers.

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Key words: dermatomyositis, interface dermatitis

Conflicts of interest: none declared.

Morphological and genetic analysis of steatocystoma multiplex in an Asian family with pachyonychia congenita type 2 harbouring a *KRT17* missense mutation

DOI: 10.1111/j.1365-2133.2008.08983.x

Sir, Pachyonychia congenita (PC) is a rare, autosomal dominant keratin disorder. PC can be classified into two main clinical subtypes: PC type 1 (PC-1, OMIM 167200) and PC type 2

(PC-2, OMIM 167210). PC-1 is associated with mutations in *KRT6A* or *KRT16*, and PC-2 corresponds to mutations in *KRT6B* or *KRT17*.[1,2] Almost all mutations detected in patients with PC occur in the helix boundary motifs of each keratin gene.[3] Common clinical features of both PC subtypes are hypertrophic nail dystrophy, and focal hyperkeratosis of the palms, soles, knees and elbows.[4] Among clinical manifestations in patients with PC, the development of steatocystoma multiplex is one of the most characteristic features for differentiating PC-2 from PC-1. Typically, patients with PC-2 exhibit 100–2000 round or oval cysts widely distributed on the back, anterior trunk, arms, scrotum and thighs.

We report an Asian PC-2 family with a missense mutation in *KRT17*. In this study, we present histological and ultrastructural features of a steatocystoma from the proband. Furthermore, comparative analysis of genomic DNA (gDNA) extracted from steatocystomas and peripheral blood of the family was performed. These observations could provide significant information for understanding the pathomechanisms of cyst formation in patients with PC-2.

The proband was a 36-year-old Asian woman with the chief complaint of nail dystrophy. Natal teeth were observed at birth. During childhood, nail hypertrophy was seen on the toenails and fingernails (Fig. 1a). Follicular hyperkeratosis on the knees and elbows was also noted at puberty, although the symptom disappeared as she grew older. She also complained of focal hyperkeratosis on the soles. On the axillae, several subcutaneous cysts were observed (Fig. 1b). The proband's 3-year-old daughter had follicular keratosis on the knees, nail deformity, pilosebaceous cysts on the face, and focal hyperkeratosis on the soles. The proband's 62-year-old father had had nail dystrophy, numerous steatocystomas on the trunk and hyperkeratosis on the soles since his adolescence. The family has a strong genetic background of nail hypertrophy and steatocystoma multiplex (Fig. 1c).

gDNA was extracted from whole blood samples of the proband, her father and her daughter. *KRT6B* and *KRT17* were amplified from their gDNA by polymerase chain reaction (PCR) using specific primers to amplify the helix boundary motifs of each gene without coamplification of the pseudogenes and isogenes.[5,6] Mutation analysis for *KRT6B* showed no mutations of the gDNA, and analysis of *KRT17* indicated that the proband was a heterozygote for a recurrent mutation of c.296T>C transition (p.Leu99Pro) in *KRT17* (Fig. 1d). The father and daughter were also heterozygotes for the same mutation in *KRT17*. Restriction enzyme digestion of PCR products by *NciI* was carried out to confirm the mutation (data not shown). The mutation was not found in 50 normal control individuals. This mutation was previously reported elsewhere.[2,7]

Histopathological findings of skin specimens from a steatocystoma of the proband showed that the cyst wall consisted of several thin epithelial cell layers without granular layers (Fig. 2a,b). There were sebaceous glands near the cyst wall (Fig. 2b). Large basophilic granules were scattered in the cytoplasm of the uppermost-layer cells in the cyst walls (Fig. 2c). Immunohistochemically, upper layer cells in the cyst wall expressed keratin 17 (Fig. 2d). Ultrastructural observation revealed keratin clumps in the cytoplasm of epithelial cells in the cyst wall (Fig. 2e). The keratin clumps were large and irregularly shaped (Fig. 2f).

We excised one steatocystoma and overlying epidermis from the proband and three steatocystomas from the proband's father, and we removed the normal tissue of the steatocystomas and intracystic materials as much as possible. DNA was extracted from both the cyst wall of steatocystomas and the overlying epidermis. Direct sequencing of gDNA from all

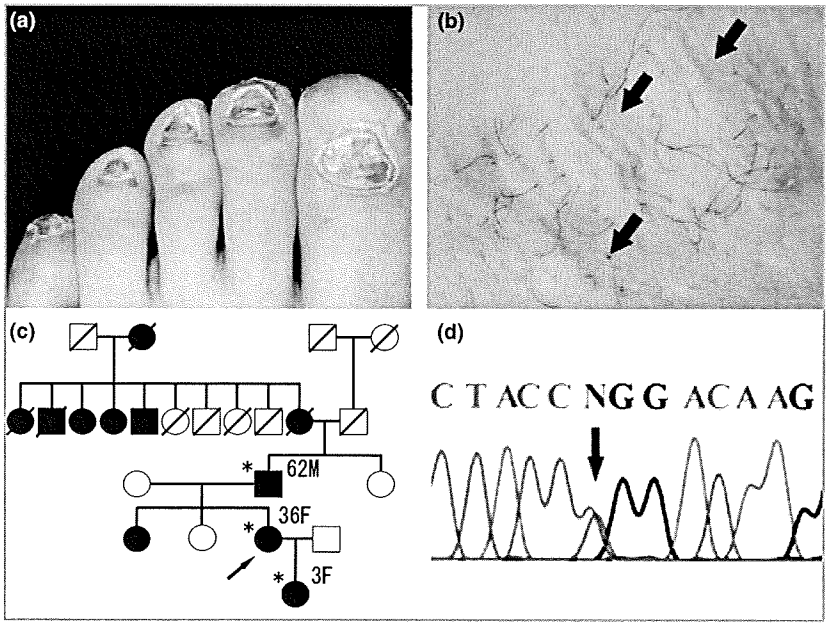

Fig 1. Clinical features of the proband, pedigree of the present family and mutation analysis of *KRT17*. (a) The proband's toenails showed severe dystrophy. (b) There were several steatocystomas on the proband's axillae (arrows). (c) The family history indicated strong penetrance. Squares indicate males, and circles, females. Blackened symbols are individuals with pachyonychia congenita type 2. The proband is indicated by an arrow. The asterisks indicate individuals who underwent mutation analysis. (d) Direct DNA sequence analysis of the helix initiation motif in *KRT17*; the c.296T>C transition mutation (p.Leu99Pro) in one allele of *KRT17* was found in the proband's blood.

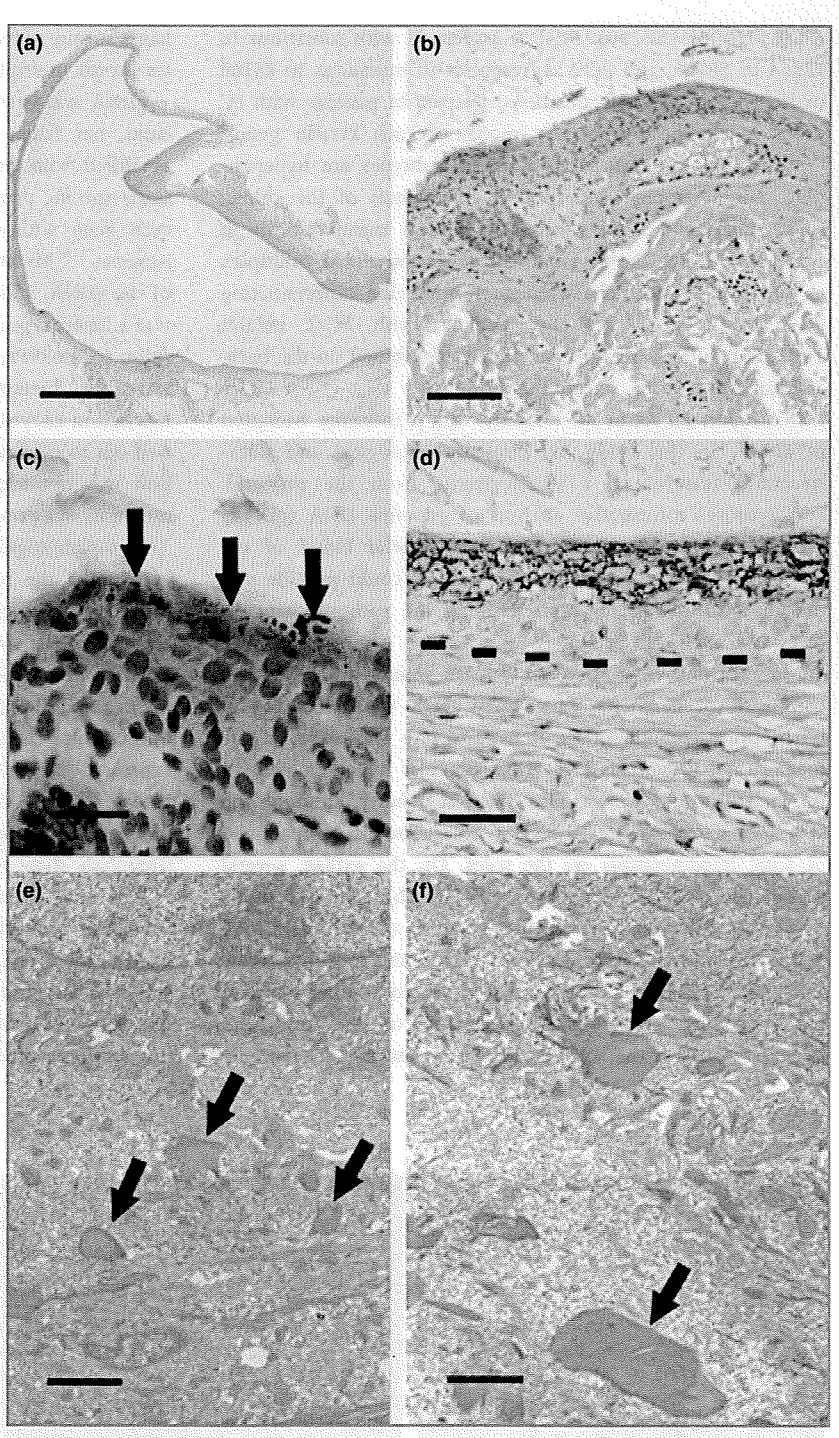

Fig 2. Histological and ultrastructural findings of a steatocystoma from the proband. (a) There were sebaceous glands near the cyst wall (haematoxylin and eosin; bar = 1·2 mm). (b) The cyst wall was composed of several epithelial cell layers (haematoxylin and eosin; bar = 100 μm). (c) Large basophilic granules (arrows) were present in upper layer cells in the cyst wall (haematoxylin and eosin; bar = 20 μm). (d) Immunohistochemical examination was performed using primary antibody, mouse monoclonal antibody E3 recognizing keratin 17 (K17). K17 was expressed in the upper layer cells in the cyst wall. Dotted line: epithelial–mesenchymal junction (bar = 50 μm). (e) There were keratin clumps in the cytoplasm of epithelial cells in the cyst wall (arrows) (bar = 2 μm). (f) The keratin clumps (arrows) were large and irregularly shaped (bar = 1 μm).

samples identified the same *KRT17* mutation in one allele as seen in the family's peripheral blood (data not shown). Comparative sequence analyses for helix boundary motifs of *KRT6B* and *KRT17* on gDNA extracted from the cyst wall and overlying epidermis vs. gDNA isolated from whole blood samples revealed neither sequence deviations indicative of loss of heterozygosity (LOH) nor second-hit mutations (data not shown).

Our results for four steatocystomas from patients with PC-2 suggest that cyst formation does not require a complete functional loss of keratin. The absence of LOH or second-hit mutations indicates that steatocystoma multiplex comprises benign cysts rather than tumours. Notably, the cyst wall of the steatocystoma from the proband had large basophilic granules. Ultrastructural observation confirmed that the granules were keratin clumps, which resulted from the conformational change in keratin filaments due to the *KRT17* mutation. Dominant negative effects from a mutation in *KRT17* may be sufficient to cause steatocystomas in patients with PC, although the exact mechanisms of steatocystoma formation remain unclear.

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Key words: autosomal dominant disorders, heterodimer, intermediate filament, keratin disease, pilosebaceous cysts

Conflicts of interest: none declared.

Metastatic prostate cancer presenting as paraneoplastic pemphigus: a favourable clinical response to combined androgen blockade and conventional immunosuppressive therapy

DOI: 10.1111/j.1365-2133.2008.08982.x

Sir, Paraneoplastic pemphigus (PNP), first described in 1990, is an autoimmune mucocutaneous blistering disease which is associated with an underlying malignancy and is characterized by polymorphic clinical signs.[1] Pathogenesis is due to an aberrant autoimmune response against the proteins of the plakin family such as plectin, envoplakin, periplakin, desmoplakin I and II, and bullous pemphigoid antigen I (BP230),[2] although several cases of PNP with antibodies to desmoglein (Dsg) 1 and 3 have been described.[3]

A 77-year-old man was admitted to our Oral Medicine Unit because of recalcitrant severe oral bullous/erosive mucositis with crusting lesions of the lips (Fig. 1a), accompanied by marked conjunctivitis of both eyes (Fig. 1b), with cutaneous bullous lesions of the abdomen and bilaterally of the hip and inguinal area (Fig. 1c). Nikolsky's sign, performed on the oral mucosa and skin, was positive.

Oral biopsy revealed suprabasal epithelial detachment with an eosinophilic and neutrophilic infiltrate. Direct immunofluorescence showed positive fluorescence in the intercellular cement substance (ICS) of IgG and complement 3c, while IgA and IgM were negative. Indirect immunofluorescence, using normal human skin as substrate, showed an intercellular signal confined to the ICS with a titre of 1 : 360. Enzyme-linked immunosorbent assay gave a value of 54 U mL^{-1} for Dsg1 (normal 0–14) and a value of 162 U mL^{-1} for Dsg3 (normal 0–14), confirming a diagnosis of pemphigus vulgaris.

PNP was suspected due to the severe and polymorphic mucocutaneous involvement, in particular of the conjunctiva and labial mucosa, which resembled erythema multiforme-like lesions. Routine haematological tests, serum tumour markers [β_2-microglobulin, prostate-specific antigen (PSA), alpha-fetoprotein, carcinoembryonic antigen, Ca 19-9, Ca 72-4, Ca 125, acid phosphatase, Bence-Jones proteinuria], chest X-ray, echocardiogram, colonoscopy and oesophagogastroduodenoscopy were negative except for microhaematuria and an elevated level of PSA (49·1 ng mL^{-1}; normal 0–4). A total body computed tomography (CT) scan revealed enlargement of the prostate, while bone scintigraphy revealed multiple foci of increased uptake (L2–L3, D8–D10). An ultrasound-guided needle biopsy of the prostate revealed a diffuse infiltration of adenocarcinoma. The prostate cancer grading (Gleason scale) was 8 (4 + 4). Immunoblotting analysis revealed the presence of antibodies to 250-, 210-, 190-, 160- and 130-kDa proteins (Fig. 2). So, in line with the criteria previously proposed,[2] a diagnosis of PNP was confirmed. Investigations by an internist and an otorhinolaryngologist were negative. High-resolution CT scan and tests for pulmonary function ruled out bronchiolitis obliterans.

The patient received conventional immunosuppressive therapy (CIST) comprising prednisone 100 mg daily and azathioprine 150 mg daily, and, at the same time, was referred to a nearby urological unit where he received combined androgen blockade (CAB) therapy comprising bicalutamide 150 mg and tamsulosin chlorohydrate 0·4 mg daily, goserelin acetate 10·8 mg every 75 days, alendronic acid 70 mg once weekly, and calcium carbonate/cholecalciferol 500 mg/440 IU every other day.

After 6 months, he was in complete clinical (Fig. 1d–f) and immunological remission on therapy (prednisone 50 mg twice weekly and azathioprine 50 mg daily), although still taking CAB, alendronic acid and calcium carbonate/cholecalciferol. The PSA level was 0·446 ng mL^{-1} and bone scintigraphy revealed only two foci with weak hypercaptation (areas of increased uptake).

It has been postulated that the autoimmune response in PNP may be twofold: (i) humoral, via cross-reaction of foreign tumour antigens to epidermal antigens, or production of plakin proteins induced by the tumour, or an epitope spreading phenomenon,[4] and (ii) cell mediated, via activation of CD8+ cytotoxic T lymphocytes, CD56+ natural killer

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 28, pp. 18942–18952, July 10, 2009
Printed in the U.S.A.

Ceramide Stimulates ABCA12 Expression via Peroxisome Proliferator-activated Receptor δ in Human Keratinocytes*

Received for publication, April 13, 2009 Published, JBC Papers in Press, May 8, 2009, DOI 10.1074/jbc.M109.006973

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ABCA12 (ATP binding cassette transporter, family 12) is a cellular membrane transporter that facilitates the delivery of glucosylceramides to epidermal lamellar bodies in keratinocytes, a process that is critical for permeability barrier formation. Following secretion of lamellar bodies into the stratum corneum, glucosylceramides are metabolized to ceramides, which comprise ~50% of the lipid in stratum corneum. Gene mutations of *ABCA12* underlie harlequin ichthyosis, a devastating skin disorder characterized by abnormal lamellar bodies and a severe barrier abnormality. Recently we reported that peroxisome proliferator-activated receptor (PPAR) and liver X receptor activators increase ABCA12 expression in human keratinocytes. Here we demonstrate that ceramide (C_2-Cer and C_6-Cer), but not C_8-glucosylceramides, sphingosine, or ceramide 1-phosphate, increases ABCA12 mRNA expression in a dose- and time-dependent manner. Inhibitors of glucosylceramide synthase, sphingomyelin synthase, and ceramidase and small interfering RNA knockdown of human alkaline ceramidase, which all increase endogenous ceramide levels, also increased ABCA12 mRNA levels. Moreover, simultaneous treatment with C_6-Cer and each of these same inhibitors additively increased ABCA12 expression, indicating that ceramide is an important inducer of ABCA12 expression and that the conversion of ceramide to other sphingolipids or metabolites is not required. Finally, both exogenous and endogenous ceramides preferentially stimulate PPARδ expression (but not other PPARs or liver X receptors), whereas PPARδ knockdown by siRNA transfection specifically diminished the ceramide-induced increase in ABCA12 mRNA levels, indicating that PPARδ is a mediator of the ceramide effect. Together, these results show that ceramide, an important lipid component of epidermis, up-regulates ABCA12 expression via the PPARδ-mediated signaling pathway, providing a substrate-driven, feed-forward mechanism for regulating this key lipid transporter.

The outermost layer of mammalian epidermis, the stratum corneum, is essential for permeability barrier function and critical for terrestrial life. The stratum corneum consists of terminally differentiated, anucleate keratinocytes, or corneocytes, surrounded by lipid-enriched lamellar membranes composed of three major lipids, ceramides, cholesterol, and free fatty acids (1). These lipids are delivered to the extracellular spaces of the stratum corneum through exocytosis of lamellar body contents from outermost stratum granulosum cells (2). Mature lamellar bodies contain primarily cholesterol, phospholipids, and glucosylceramides (3). Following lamellar body secretion, the secreted phospholipids and glucosylceramides are converted to free fatty acids and ceramides by phospholipases and β-glucocerebrosidase, respectively (1, 4). ABCA12 (ATP binding cassette transporter, family 12), a lipid transporter predominantly expressed in epidermis, has been shown to play a vital role in the formation of mature lamellar bodies (5, 6), although how this transporter is regulated remains unresolved.

ABCA12 is a member of the ABCA subfamily of transporters, which are involved in the transport of a variety of lipids (7). Mutations in *ABCA1* cause Tangier disease, which is due to a defect in transporting cholesterol and phospholipids from intracellular lipid stores to apolipoproteins, particularly apolipoprotein A-I (8–11). Mutations in *ABCA3* cause neonatal respiratory failure due to a defect in surfactant transport from alveolar type II cells into the alveolar space (12). Mutations in *ABCA4* cause Stargardt's macular degeneration, with visual loss due to a defect in transporting phosphatidylethanolamine-retinylidene out of retinal pigment cells (13).

Recently, mutations in *ABCA12* have been shown to cause harlequin ichthyosis and a subgroup of lamellar ichthyosis, two disorders of keratinization (5, 14, 15). *ABCA12* mutations lead to an abnormality in lamellar body formation, a decrease in lamellar membranes in the extracellular spaces of the stratum corneum, an accumulation of glucosylceramide in the epidermis with a reduction in ceramide (16), and ultimately loss of permeability barrier function (17), which in harlequin ichthyosis can result in neonatal lethality (5, 15). Strikingly, genetic correction of ABCA12 deficiency in patients' keratinocytes by gene transfer normalized loading of glucosylceramides into lamellar bodies (5). These studies demonstrate a critical role for ABCA12 in epidermal physiology, specifically in the formation of mature lamellar bodies and subsequent permeability barrier homeostasis. Hence, it is crucial to understand how ABCA12 is regulated.

* This work was supported, in whole or in part, by National Institutes of Health Grants AR39448 and AR049932. This work was also supported by the Medical Research Service, Department of Veterans Affairs at San Francisco.

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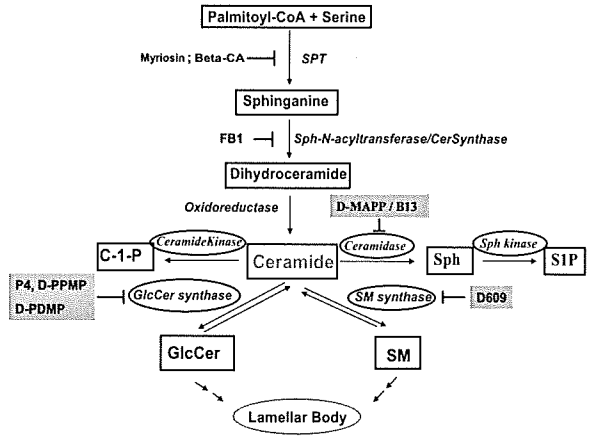

FIGURE 1. **The central role of ceramide in sphingolipid metabolism in keratinocytes.** *C1P*, ceramide 1-phosphate; *Sph*, sphingosine; *S1P*, sphingosine-1-phosphate; *GlcCer*, glucosylceramide; *SM*, sphingomyelin.

Our laboratory recently demonstrated that activation of peroxisome proliferator-activated receptor (PPARδ and PPARγ) or liver X receptor (LXR) stimulates ABCA12 expression in cultured human keratinocytes (18). Both PPARs and LXR are important lipid sensors that stimulate keratinocyte differentiation and enhance permeability barrier function (19). Additionally, PPARα and -δ as well as LXR activators stimulate ceramide synthesis in keratinocytes (20, 21). Likewise, ceramide synthesis increases in keratinocytes during differentiation, foreshadowing the formation of lamellar bodies (22, 23).

In addition to serving as structural membrane components, ceramides are also important signaling molecules that can induce growth arrest, differentiation, and apoptosis in various cells, including keratinocytes (24–26). Moreover, distal ceramide metabolites, sphingosine and sphingosine-1-phosphate (Fig. 1), are also important signaling molecules (27).

It is well established that the expression of ABCA1 is regulated by cellular cholesterol levels in many cell types, including keratinocytes (28). Cholesterol, if metabolized to certain oxysterols, can activate LXR, which then stimulates ABCA1 expression and the transport of cholesterol out of cells (29). This example of feed-forward regulation leads us to hypothesize that either ceramide or a metabolite of ceramide might stimulate ABCA12 expression, thereby leading to an increase in the transport of glucosylceramides into maturing lamellar bodies. Here, we provide evidence that ceramide stimulates ABCA12 expression in keratinocytes via a mechanism involving PPARδ signaling.

EXPERIMENTAL PROCEDURES

Materials—D609 was purchased from BIOMOL International (Plymouth Meeting, PA). Fumonisin B1 and β-chloro-D-alanine hydrochloride (βCA) were purchased from Sigma. Myriocin was from Calbiochem. D-MAPP, L-MAPP, sphingosine, and sphinganine were from EMD Biosciences, Inc. (La Jolla, CA). Synthetic ceramides, *N*-hexanoyl-D-*erythro*-sphingosine (C_6-Cer) and *N*-acetyl-D-*erythro*-sphingosine (C_2-Cer), D-*threo*-P4 (P4), and D-*threo*-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol·HCl (D-PPMP) were purchased from Matreya Inc. (Pleasant Gap, PA). C_8-β-D-glucosyl ceramide was purchased from Avanti Polar lipids (Alabaster, AL). Molecular grade chemicals such as TRI reagent were obtained from either Sigma or Fisher. The iScript™ cDNA synthesis kit for first strand cDNA synthesis was purchased from Bio-Rad. All reagents and supplies for real time PCR were purchased from Applied Biosystems (Foster City, CA). All other reagents for Western blot, including NuPAGE® Novex precast gradient gels (3–8% Tris-acetate), buffers, protein standards, and detection kits were purchased from Invitrogen.

Keratinocyte Culture—The second passage of human neonatal foreskin keratinocytes was seeded and maintained in 0.07 mM calcium chloride, serum-free 154CF with growth supplement (Cascade Biologics, Inc., Portland, OR). Once the cells were attached, the culture was switched to medium containing 0.03 mM calcium chloride, as described (18).

Trypan Blue Staining and TUNEL Assay—Trypan blue staining (Sigma) and TUNEL assay (TdT-FragELTM DNA fragmentation detection kit; Calbiochem) were carried out following the manufacturer's protocol.

Real Time PCR—Total RNA was isolated using TRI reagent, and first strand cDNA for PCR was synthesized using an iScript™ cDNA synthesis kit, following the manufacturer's protocol. Relative mRNA levels of hABCA12 (full length), two major transcripts (hABCA12-L and -S), and an invariant transcript, cyclophilin, were determined as previously described (18). The primers used for human PPARα, -δ, and -γ, LXR-α, and LXR-β are listed in Table 1. Relative mRNA levels of ABCA1 were determined as described previously (28). The expression levels of each gene were normalized against cyclophilin using the comparative C_T method and expressed as a percentage of control, with the control as 100%.

siRNA Transfection—A control siRNA and human alkaline ceramidase 1-specific siRNA (sequences listed in Table 1) were used for transfecting cultured human keratinocytes. Human

TABLE 1
Oligonucleotide primer sequences for real time PCR

Gene	Sequence of forward and reverse primers	GenBank™ accession number
PPARα	GGTGGACACGGAAAGCCCAC GGACCACAGGATAAGTCACC	NM_005036
PPARδ	CACACGGCGCCCTTTG CCTTCTCTGCCTGCCACAA	NM_006238
PPARγ	CTCATATCCGAGGGCCAA TGCCAAGTCGCTGTCATC	U79012
LXR-α	CGCACTACATCTGCCACAGT TCAGGCGGATCTGTTCTTCT	NM_005693
LXR-β	CGCTACAACCACGAGACAGA GAACTCGAAGATGGGGTTGA	NM_007121
Control siRNA	UAAGGUAUGAAGAGAUACUU GUAUCUCUUCAUAGCCUUAUU	
CER1 siRNA	GGCCUGUUCUCCAUGUAUUUUU AAUACAUGGAGAACAGGCCUU	

[2] The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; LXR, liver X receptor; βCA, β-chloro-L-alanine hydrochloride; C_2-Cer, *N*-acetyl-D-*erythro*-sphingosine; C_6-Cer, *N*-hexanoyl-D-*erythro*-sphingosine; D-MAPP, D-*erythro*-2-tetradecanoylamino-1-phenyl-1-propanol; L-MAPP, L-*erythro*-2-tetradecanoylamino-1-phenyl-1-propanol; P4, D-*threo*-1-phenyl-2-palmitoyl-3-pyrrolidinopropanol; D-PPMP, D-*threo*-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol·HCl; TUNEL, terminal dUTP nick-end labeling; siRNA, small interfering RNA.