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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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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.

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Spontaneous Giant Aneurysm of the Superficial Temporal Artery

—Case Report—

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Abstract

A 78-year-old woman presented with preauricular superficial temporal artery (STA) aneurysm and scalp porocarcinoma, which had both increased in size over 2 years. She had no previous history of head trauma. Three-dimensional (3D) computed tomography (CT) angiography revealed a 4-cm diameter STA aneurysm arising from the main trunk of the left STA and located just lateral to the zygomatic arch. The scalp porocarcinoma was excised by dermatologists. The STA aneurysm was carefully dissected from the surrounding tissues, and was resected after ligation of the proximal STA. Histological examination showed the aneurysm consisted of intima, media, and adventitia, and the diagnosis was atherosclerotic fusiform aneurysm. 3D CT angiography is quite useful to plan surgical strategy for such an unusually large STA aneurysm.

Key words: superficial temporal artery, true aneurysm, giant aneurysm, three-dimensional computed tomography angiography

Introduction

Traumatic aneurysm of the superficial temporal artery (STA) is not unusual, with more than 400 reported cases,[13] including the first case in 1740.[4] Traumatic STA aneurysm usually develops in young adult men at 2 to 6 weeks after blunt head trauma,[11] and the majority are pseudoaneurysms. Spontaneous or non-traumatic STA aneurysms are quite rare, with only 16 cases described.[1,2,4,5,7,9,10,12,15] Histological examination confirmed "true" aneurysm in 7 of these cases,[1,2,5,7,9,10,12] and 3 were giant true STA aneurysm, with more than 25-mm diameter.[1,2,5]

Here we describe a case of giant true STA aneurysm of 47-mm diameter that gradually increased in size over 2 years.

Case Report

A 78-year-old female first noticed two masses in the left temporal region 2 years previously, but did not consult a physician because of the absence of pain or discomfort. However, both masses gradually increased in size over 2 years, so she visited our hospital. She had no history of head trauma. Physical examination found a painless, pulsatile mass in the left preauricular area, and a black, hairy non-pulsatile mass in the left temporal region (Fig. 1). First, she was admitted to the Department of Dermatology and underwent surgical resection of the non-pulsatile mass. The histological diagnosis was porocarcinoma. She was then admitted to our department.

The pulsatile mass was round and about 4 cm in diameter. The left external auditory meatus was almost obstructed by the mass. Facial nerve function was intact. Computed tomography (CT) with contrast medium demonstrated homogeneous enhancement. Three-dimensional (3D) CT angiography revealed that the aneurysm arose from the main trunk of the left STA, which was located medial to the mass. The distal STA was not visible, probably because the blood flow was delayed due to turbulent flow in the huge aneurysm (Fig. 2). Magnetic resonance (MR) imaging and ultrasonography showed turbulent flow within the aneurysm. Based on these physical and radiological findings, our diagnosis was a non-traumatic, giant STA aneurysm.

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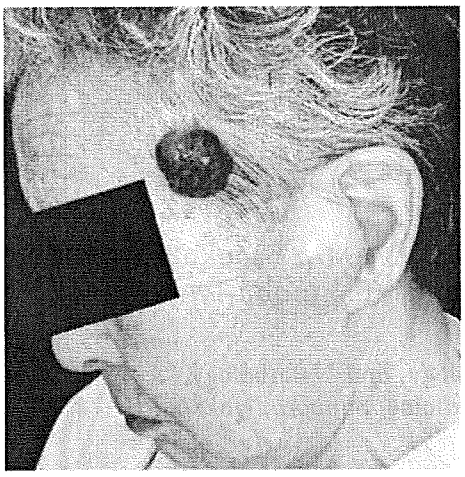

Fig. 1 Photograph showing a huge pulsating tumor in the left preauricular region (4 × 4 × 4 cm) and a hairy, black tumor in the left temporal region (3 × 3 × 2 cm).

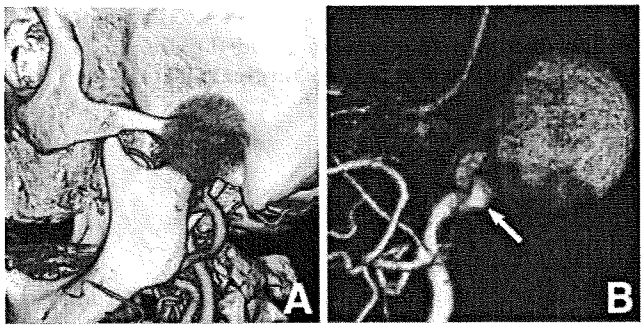

Fig. 2 Three-dimensional computed tomography angiograms demonstrating a giant aneurysm arising from the main trunk of left superficial temporal artery (STA), and located lateral to the zygomatic arch (A), and the proximal STA located medial to the aneurysm (B, *arrow*).

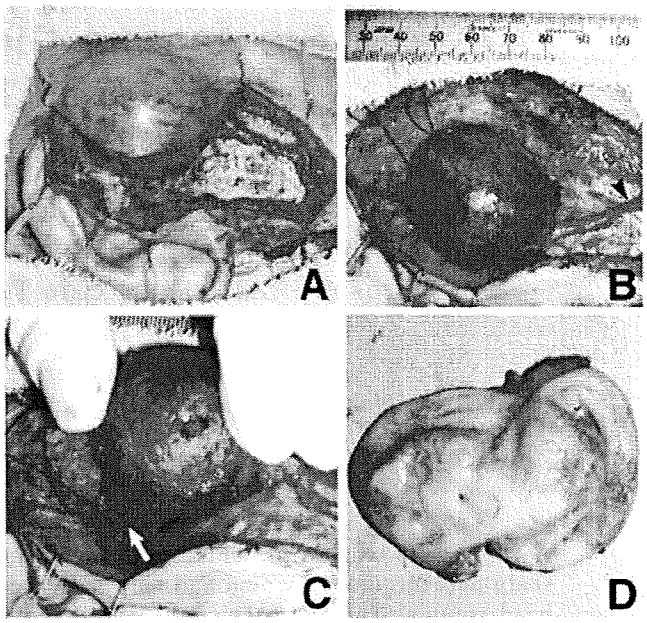

Fig. 3 Intraoperative photographs showing the parietal branch of the superficial temporal artery (STA) and the aneurysm covered by the skin (A). The aneurysm and the parietal branch of the STA (*arrowhead*), seen at the distal end of aneurysm, were completely exposed (B). The proximal STA medial to the aneurysm was dissected and ligated (C, *arrow*). The resected aneurysm had thickened wall around the orifice (D).

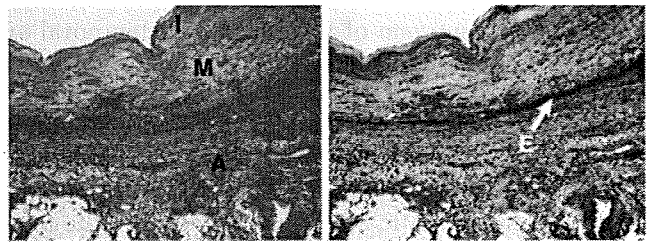

Fig. 4 Photomicrographs of the resected aneurysm showing preservation of the intima (I), media (M), and adventitia (A), and fragmentation of the elastic membrane (E, *arrow*). Hematoxylin and eosin stain (*left*) and elastica Masson stain (*right*), original magnification ×200.

She underwent surgical excision under general anesthesia. After exposure of the distal STA, the aneurysm was carefully dissected from the surrounding tissues, including the temporal fascia, parotid gland, and zygomatic arch. The dissected aneurysm was rotated and the proximal STA was ligated. Finally, the aneurysm was resected (Fig. 3). The aneurysm was 47 mm × 45 mm × 35 mm with thickened wall. Histological examination revealed that the aneurysm consisted of the intima, media, and adventitia, and the elastic membrane was extended and fragmented. No malignant or inflammatory cells were found in the specimen. The histological diagnosis was a true aneurysm (Fig. 4). She was discharged without neurological deficits.

Discussion

Eight cases of histologically verified, spontaneous true STA aneurysm have been reported, including

Table 1 Previous cases of histologically confirmed spontaneous true superficial temporal artery aneurysm

Author (Year)	Age (yrs)	Sex	Symptoms and signs	Location of aneurysm	Diagnosis	Size of aneurysm (mm)	Treatment	Outcome
Brown (1942)[1]	34	M	pulsatile mass	preauricular	physical examination	32 × 21 × 21	surgical excision	excellent
Martin and Shoemaker (1955)[9]	60	M	pulsatile mass	frontal branch	physical examination	not described	surgical excision	excellent
Buckspan and Rees (1986)[2]	70	M	pulsatile mass	preauricular	angiography	30 × 40	surgical excision	excellent
Locatelli et al. (1988)[7]	10	M	pulsatile mass	parietal branch	angiography	10 × 40	surgical excision	excellent
Nishioka et al. (1988)[10]	14	M	pulsatile mass	parietal branch	angiography	15 × 10	surgical excision	excellent
Endo et al. (2000)[5]	85	M	two pulsatile masses	preauricular and frontal branch	angiography	30 × 30, 10 × 10	surgical excision	excellent
Porcellini et al. (2001)[12]	24	F	pulsatile mass	parietal branch	physical examination	not described	surgical excision	excellent
Present case	78	F	pulsatile mass	preauricular	3D CT angiography	47 × 45 × 35	surgical excision	excellent

3D CT: three-dimensional computed tomography.

the present case (Table 1).[1,2,5,7,9,10,12] The 6 male and 2 female patients were aged between 10 and 85 years old. The aneurysm arose in the main trunk of STA in 4 cases and in the distal branch of STA in 4. All patients safely underwent surgical excision. The age range suggests that atherosclerosis is not the common and only cause, although atherosclerotic change of the STA and/or hemodynamic stress to the arterial wall might be important in its development.[5] Congenital vulnerabilities of the arterial wall, such as defect of the elastic membrane, may contribute to the development of a true STA aneurysm.[10] In the present case, both aneurysm and scalp tumor simultaneously increased in size for about 2 years. Therefore, we speculate that increased blood supply to the scalp tumor may have caused hemodynamic stress in the proximal STA and promoted the unusual growth of the aneurysm. The histological findings in the present case support this mechanism (Fig. 4). Three previous cases of giant true STA aneurysm of more than 25 mm of diameter have been reported.[1,2,5] All cases were located in the preauricular region, as in the present case. The aneurysm size varied from 30 to 40 mm.

Spontaneous true STA aneurysm usually occurs as a pulsatile scalp mass (Table 1). Any pulsatile, preauricular mass requires careful diagnosis. STA aneurysms often display arterial pulsation and thrills corresponding to systole, whereas STA arteriovenous fistula displays continuous thrill and bruit.[11,12,14] The differential diagnosis also includes aneurysm of the middle meningeal artery with temporal bone erosion, abscess, inflammatory lesion, epidermal inclusion cyst, angiofibroma, meningocele, encephalocele, parotid tumor, and lipoma.[3,11,14]

Cerebral angiography is the gold standard for diagnosis of spontaneous true STA aneurysm.[2,5,7,10,11] In the present case, MR imaging and ultrasonography were useful to visualize the turbulent flow within the aneurysm. Recent developments of multi-detector CT can non-invasively visualize intra- and extracranial vascular lesions with high spatial resolution.[6] Especially in the present case, 3D CT angiography provided important information on the anatomical relationship between the aneurysm and the surrounding tissue, including the external auditory meatus and zygomatic arch. Furthermore, 3D CT angiography could clearly visualize the topographical relationship between the aneurysm and the proximal STA. Based on the findings, we could avoid intraoperative massive bleeding by ligating the proximal STA.

The natural history of spontaneous true STA aneurysm is not well documented. However, we selected surgical resection because the giant aneurysm had gradually increased in size to almost obstruct the left external auditory meatus. Although local anesthesia is preferred for surgical excision, the aneurysm was resected under general anesthesia because of the large size and apparent location near the facial nerve.[8] STA aneurysm in the preauricular region should carefully be dissected from the surrounding tissue to avoid postoperative facial nerve paresis.

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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.

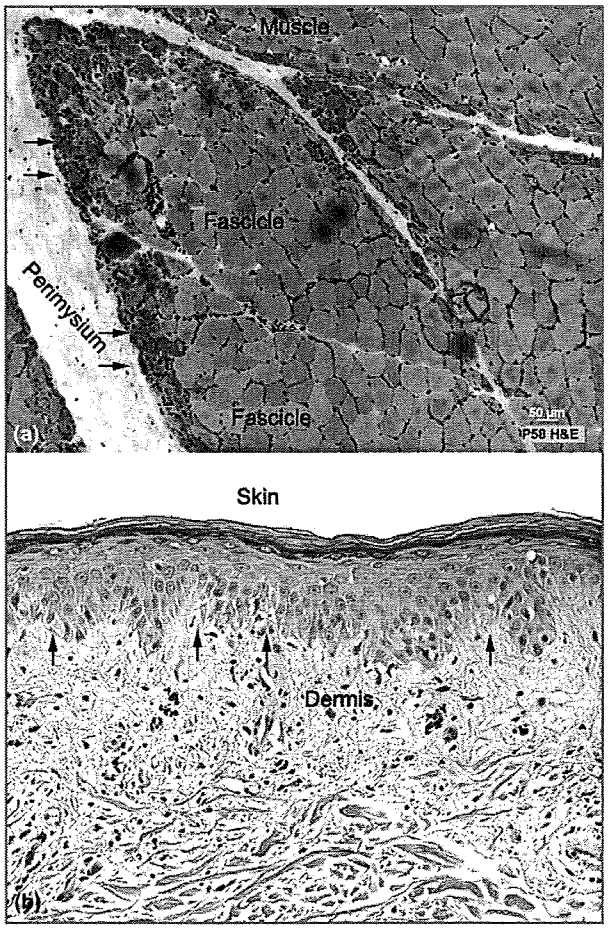

Fig 1. (a) Perimysial perifascicular atrophy in muscle. Small, basophilic fibres at the interface between myofibres and perimysial connective tissue (black arrows). (b) Interface dermatitis in skin. Vacuolated and degenerating keratinocytes at the interface between keratinocytes and loose connective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

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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

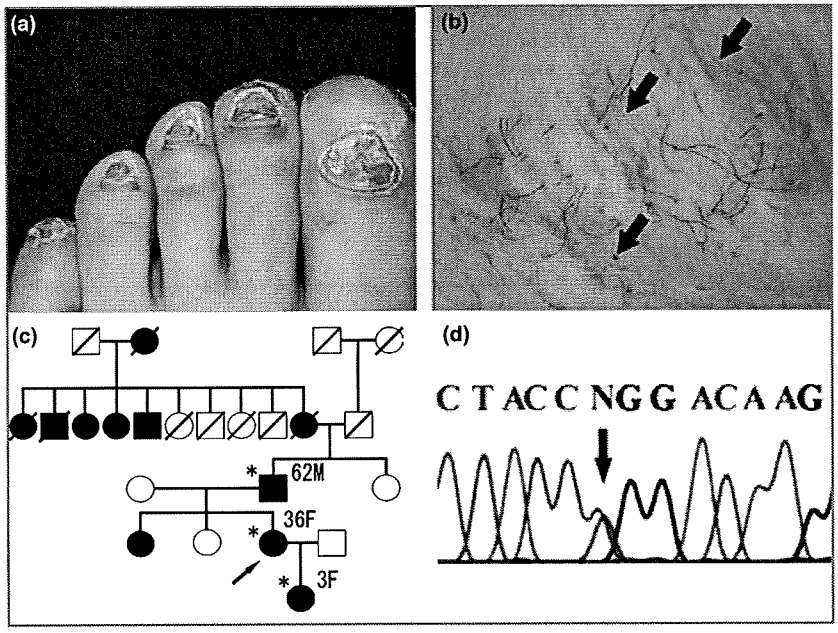

Fig 1. Clinical features of the proband, pedigree of the present family and mutation analysis of KRT17. (a) The proband's toenails showed severe dystrophy. (b) There were several steatocystomas on the proband's axillae (arrows). (c) The family history indicated strong penetrance. Squares indicate males, 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.

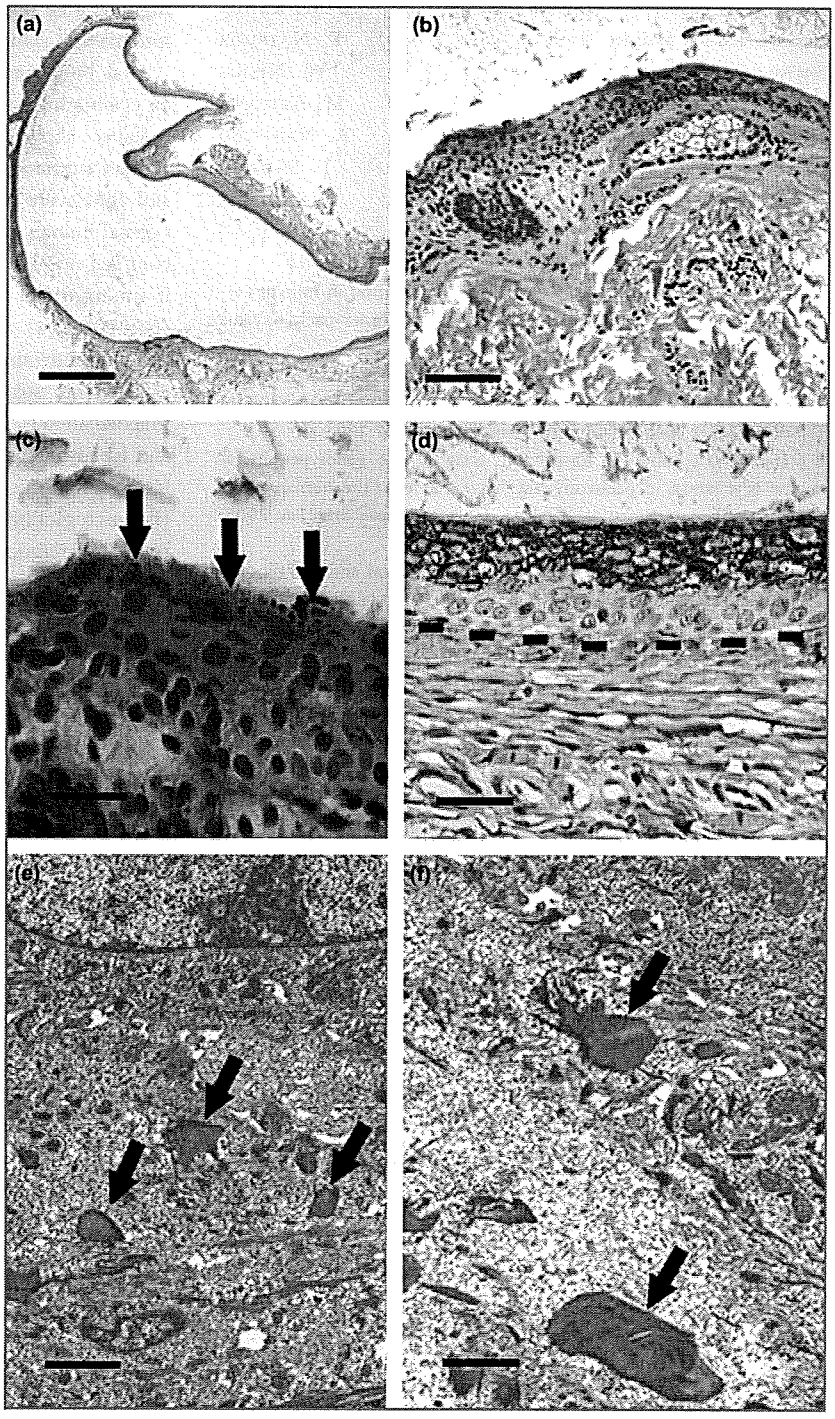

Fig 2. Histological and ultrastructural findings of a steatocystoma from the proband. (a) There were sebaceous glands near the cyst wall (haematoxylin and eosin; bar = 1·2 mm). (b) The cyst wall was composed of several epithelial cell layers (haematoxylin and eosin; bar = 100 μm). (c) Large basophilic 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

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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

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Molecular Pathogenesis of Genetic and Inherited Diseases

Keratinocyte-/Fibroblast-Targeted Rescue of *Col7a1*-Disrupted Mice and Generation of an Exact Dystrophic Epidermolysis Bullosa Model Using a Human *COL7A1* Mutation

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Recessive dystrophic epidermolysis bullosa (RDEB) is a severe hereditary bullous disease caused by mutations in *COL7A1*, which encodes type VII collagen (COL7). *Col7a1* knockout mice ($COL7^{m-/-}$) exhibit a severe RDEB phenotype and die within a few days after birth. Toward developing novel approaches for treating patients with RDEB, we attempted to rescue $COL7^{m-/-}$ mice by introducing human *COL7A1* cDNA. We first generated transgenic mice that express human *COL7A1* cDNA specifically in either epidermal keratinocytes or dermal fibroblasts. We then performed transgenic rescue experiments by crossing these transgenic mice with $COL7^{m+/-}$ heterozygous mice. Surprisingly, human COL7 expressed by keratinocytes or by fibroblasts was able to rescue all of the abnormal phenotypic manifestations of the $COL7^{m-/-}$ mice, indicating that fibroblasts as well as keratinocytes are potential targets for RDEB gene therapy. Furthermore, we generated transgenic mice with a premature termination codon expressing truncated COL7 protein and performed the same rescue experiments. Notably, the $COL7^{m-/-}$ mice rescued with the human *COL7A1* allele were able to survive despite demonstrating clinical manifestations very similar to those of human RDEB, indicating that we were able to generate surviving animal models of RDEB with a mutated human *COL7A1* gene. This model has great potential for future research into the pathomechanisms of dystrophic epidermolysis bullosa and the development of gene therapies for patients with dystrophic epidermolysis bullosa. (*Am J Pathol 2009, 175:000–000; DOI: 10.2353/ajpath.2009.090347*)

Dystrophic epidermolysis bullosa (DEB) is clinically characterized by mucocutaneous blistering in response to minor trauma, followed by scarring and nail dystrophy. The blistering occurs along the epidermal basement membrane zone (BMZ) just beneath the lamina densa at the level of the anchoring fibrils. The inheritance of DEB can be autosomal dominant (DDEB) or autosomal recessive (RDEB), each comprising subtypes of different clinical presentations and severities.[1] Both DDEB and RDEB are known to be caused by mutations in the *COL7A1* gene encoding type VII collagen (COL7), the major component of anchoring fibrils.[2] The most severe RDEB subtype, the Hallopeau-Siemens subtype, shows a complete lack of expression of type VII collagen, whereas a less severe RDEB subtype, the non-Hallopeau-Siemens subtype, shows some collagen expression. The clinical fea-

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tures of DDEB are, in general, milder than those of RDEB and tend to improve with age. The molecular mechanisms of DEB have been thoroughly investigated, and precise diagnosis and estimation of prognosis is now possible. There is no specific treatment for different forms of DEB, and the current focus of research is to develop more effective treatments for this group of blistering disorders.

Corrective gene therapy whereby normal COL7 is introduced into the patients' cells, has great potential as a treatment for DEB. However, several obstacles must be overcome before its clinical therapeutic application. First, there have been no useful DEB animal models that reproduce the human mutated gene for experiments. Although COL7 knockout mice have been generated, most of such mice die within a few days of birth, and none survive more than 2 weeks.[3] A surviving DEB mouse that was reported recently was the DEB hypomorphic mouse model.[4] These mice, which had about 10% of the normal mouse COL7, did not show the abnormal form and function of anchoring fibrils seen in human patients of RDEB. Second, no studies have examined in detail whether the introduction of the human COL7 gene into DEB mouse cells can rescue the DEB phenotype without causing adverse effects in a living DEB model. Third, there is controversy over which cells may serve as optimal targets in gene therapies for DEB. Several studies have targeted keratinocytes, because the cells that secrete COL7 are mainly keratinocytes and to a lesser extent fibroblasts.[5,6] However, we and others have recently reported that injection of gene-transferred fibroblasts into the skin can efficiently restore COL7 expression in the dermal-epidermal junction *in vitro*.[6–8] Furthermore, intradermal injection of allogeneic fibroblasts into skin of patients with RDEB skin was shown to result in enhanced COL7 expression in selected patients.[9] Therefore, we need to compare keratinocytes and fibroblasts to clarify their efficacy as target cells in an *in vivo* model system of RDEB.

To address these issues, we generated transgenic mice with human *COL7A1* under different promoters and performed transgenic rescue experiments on the Col7a1$^{m-/-}$ background using those transgenic mice. Furthermore, to develop a DEB model that accurately reproduces human DEB not only in terms of clinical manifestations but also in terms of gene mutation, we also introduced a mutated human *COL7A1* gene into this mouse model system and created human mutant gene-expressing rescued mice corresponding to the surviving animal of DEB. Our results advance our understanding of the function and biology of COL7.

Materials and Methods

Generation of Transgenic Mice

Human full-length *COL7A1* cDNA was constructed from several overlapping cDNA clones (Sawamura et al, 2002). We used a pCMVβ expression vector (Invitrogen, Carlsbad, CA) that contained the cytomegalovirus (CMV) promoter, the simian virus 40 (SV40) splice donor/splice acceptor site, the *lacZ* gene, and the SV40 polyadenylation signal. We selected human keratin 14 (K14),[10] the mouse pro-α 2 chain of type I collagen (col1a2),[11] or the CMV promoter for epidermis-specific, dermis-specific, or ubiquitous expression of the transgene, respectively. We first modified pCMVβ by replacing LacZ with human full-length *COL7A1* cDNA, and the CMV promoter with the human K14 or the mouse col1a2 gene. Finally, we produced three *COL7A1* constructs for transgenic mice (Figure 1A). They were digested with appropriate restriction enzymes, purified, and introduced into BDF1 oocytes, which were subsequently transplanted into the recipient mice. Founders were bred to wild-type C57BL/6 females. To confirm germline transmission, PCR analyses on genomic DNA were performed (forward, 5′-CTCAGTGGATGTTGCCTTT-3′; reverse, 5′-TAAGAACACAATGTCAGCGG-3′) using specific primers and the following thermal cycling parameters: 94°C for 5 minutes, 94°C for 45 seconds, and 56°C for 45 seconds; followed by 35 cycles at 72°C for 45 seconds and 72°C for 7 minutes. The transgenic (Tg) mice with K14, col1a2, and CMV

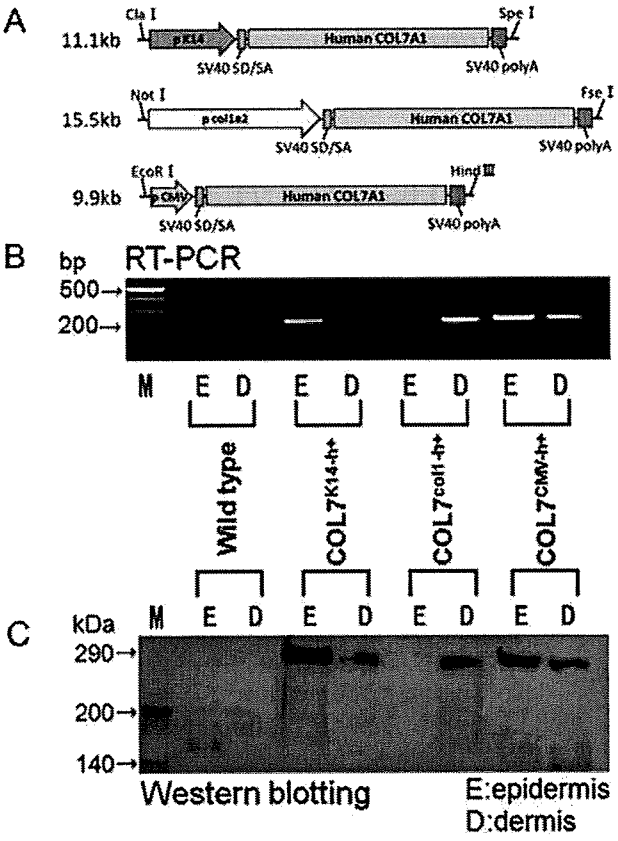

Figure 1. Epidermis- or dermis-specific expression of the human *COL7A1* full-length cDNA in the transgenic mice. **A:** Three expression vectors for transgenic mice were constructed using the promoters of human K14, mouse col1a2, and CMV. The vector contains the SV40 splice donor/splice acceptor (SD/SA) site and the SV40 polyadenylation (polyA) signal. **B:** We obtained epidermis and dermis from the K14Tg mice (COL7$^{K14\text{-}h+}$), col1a2Tg mice (COL7$^{col1\text{-}h+}$), and CMVTg mice (COL7$^{CMV\text{-}h+}$) and then examined human *COL7A1* mRNA expression by RT-PCR analysis. Molecular weight markers (M) are a 100-bp DNA ladder. **C:** Expression of COL7 was also investigated by Western blot analysis using anti-human monoclonal antibody LH7.2. Molecular weight markers are a biotinylated protein ladder.