Development and Characterization of A Recombinant Truncated Type VII Collagen “Minigene”

IMPLICATION FOR GENE THERAPY OF DYSTROPHIC EPIDERMOLYSIS BULLOSA*

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Dystrophic epidermolysis bullosa (DEB) is an inherited mechano-bullous disorder of skin caused by mutations in the type VII collagen gene. The lack of therapy for DEB provides an impetus to develop gene therapy strategies. However, the full-length 9-kilobase type VII collagen cDNA exceeds the cloning capacity of current viral delivery vectors. In this study, we produced a recombinant type VII minicollagen containing the intact noncollagenous domains, NC1 and NC2, and part of the central collagenous domain using stably transfected human 293 cell clones and purified large quantities of the recombinant minicollagen VII from culture media. Minicollagen VII was secreted as correctly-folded, disulfide-bonded, helical trimers resistant to protease degradation. Purified minicollagen VII bound to fibronectin, laminin-5, type I collagen, and type IV collagen. Furthermore, retroviral-mediated transduction of the minigene construct into DEB keratinocytes (in which type VII collagen was absent) resulted in persistent synthesis and secretion of a 230-kDa recombinant minicollagen VII. In comparison with parent DEB keratinocytes, the gene-corrected DEB keratinocytes demonstrated enhanced cell-substratum adhesion, increased proliferative potential, and reduced cell motility, features that reversed the DEB phenotype toward normal. We conclude that the use of the minicollagen VII may provide a strategy to correct the cellular manifestations of gene defects in DEB.

Dystrophic epidermolysis bullosa (DEB) is a group of heritable mechano-bullous skin diseases characterized by skin fragility, separation of the epidermis from the dermis (blister formation), milia and scarring (1). The pathogenesis of DEB involves a basement membrane zone (BMZ)-specific collagen called type VII collagen, the major component of structures within the BMZ called anchoring fibrils. DEB patients have defects in the gene, designated COL7A1, that encodes for type VII (anchoring fibril) collagen (2). The BMZ of patients with DEB is characterized by a paucity or diminutive size of anchoring fibrils (3). Earlier genetic linkage studies identified the human COL7A1 as the gene responsible for DEB (2, 4–6), and then numerous COL7A1 gene defects were directly identified in families of patients with DEB (2).

Type VII collagen is composed of three identical α chains, each consisting of a 145-kDa central collagenous triple-helical segment characterized by repeating Gly-X-Y amino acid sequences, flanked by a large 145-kDa amino-terminal, non-collagenous domain (NC1), and a small 34-kDa carboxy-terminal non-collagenous domain (NC2) (7, 8). Within the extracellular space, type VII collagen molecules form antiparallel, tail-to-tail dimers stabilized by disulfide bonding through a small carboxy-terminal NC2 overlap between two type VII collagen molecules. A portion of the NC2 domain is then proteolytically removed (9). The antiparallel dimers then aggregate laterally to form anchoring fibrils with large globular NC1 domains at both ends of the structure. NC1 domains have been suggested to interact at one end with BMZ components, and at the other ends with type IV collagen in “anchoring plaques” (7). Sequence analysis of the NC1 domain revealed the presence of multiple submodules with homology to adhesive proteins (10). These include a segment with homology to cartilage matrix protein, nine consecutive fibronectin type III-like repeats, and a segment with homology to the A domain of von Willebrand factor. Therefore, the NC1 domain may facilitate binding of type VII collagen to other BMZ and matrix components. These matrix interactions are thought to stabilize the adhesion of the BMZ to the underlying dermis. Our recent study using recombinant NC1 demonstrated that NC1 interacts with various extracellular matrix (ECM) components including fibronectin, laminin 5, type I collagen, and type IV collagen (11). Therefore, structural alterations in type VII collagen may result in functional disruption of its interactions with ECM components, leading to the epidermal-dermal disadherance seen in DEB.

Type VII collagen cDNA has been isolated. The full-length cDNA sequence contains 8,833 nucleotides encoding 2,944 amino acids (10, 12). Cloning of type VII collagen facilitated the molecular analysis of mutations underlying DEB (2). The identification of specific mutations in different parts of COL7A1 has enhanced the understanding of the structural and functional roles of various type VII collagen domains. Over 100 distinct mutations in COL7A1 have been identified, and these muta-
tions have occurred within NC1, NC2, and the helical domain (13, 14). The most severe form of DEB is recessive dystrophic EB (the Hallopeau-Siemens type, HS-RDEB), characterized by mutilating scarring of the hands and feet, joint contractures, strictures of the esophagus, and the development of aggressive squamous cell carcinomas in affected areas of the skin. These skin cancers frequently shorten the life of patients to two or three decades. In the most severe clinical subtype of recessive DEB (RDEB), the COL7A1 mutations involve the creation of premature termination codons which predict the synthesis of a very truncated type VII collagen (15). The main effect of any premature termination codon, however, is to create an unstable mRNA transcript and, consequently, little if any functional protein is generated from the mutant allele. In the homozygous or compound heterozygous state, these mutations result in the absence of anchoring fibrils. Identification of mutations in COL7A1 provides the foundation for the development of gene therapy strategies for DEB.

Gene therapy for heritable diseases through genetic engineering holds considerable promise, particularly for diseases where no effective treatment is currently available. The potential use of gene transfer is particularly well suited to RDEB patients who carry null alleles which are not expressed, leading to the absence of type VII collagen production and the absence of anchoring fibrils. Retroviral vectors are the most utilized vehicles in human gene therapy trials because of their capacity for high efficiency transduction and stable gene transfer (16). Retroviral vectors, however, have insert size limitations of 7–8 kilobases and therefore cannot accommodate the entire 9-kilobase type VII collagen cDNA (17). In order to use highly efficient retroviral vectors, we selectively deleted regions of the type VII collagen cDNA to produce a truncated type VII collagen minigene construct. Our hypothesis was that a smaller chain created by deletion of selected helical segments that are not functionally critical could still form functional anchoring fibrils. This approach has been used successfully in the development of gene therapy for Duchenne muscular dystrophy (18).

In the current study, we used an efficient eukaryotic expression system to obtain large quantities of recombinant minicollagen VII in condition medium from human kidney 293 cells. Functional analyses showed that the minigene product, a truncated type VII collagen α chain, retained the function and characteristics of a full-length type VII collagen α chain. In addition, our data demonstrated that the expression of mini-collagen VII in RDEB keratinocytes (in which type VII collagen was absent) induced reversion of the RDEB cellular phenotype.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human embryonic kidney cell line 293 (ATCC, Rockville, MD) was routinely cultured in Dulbecco’s modified essential medium/Ham’s F-12 (1:1) supplemented with 10% fetal bovine serum. Primary and immortalized RDEB keratinocytes (gift of Dr. Scott Herro, Stanford University) were cultured in low calcium, serum-free keratinocyte growth medium supplemented with bovine pituitary extract and epidermal growth factor (SFM; Life Technologies, Inc., Gaithersburg, MD) as described by Boyce and Ham (19) and modified by O’Keefe and Chiu (20). RDEB keratinocytes were immortalized using the E6 and E7 genes of human papillomavirus type 6 as described (21).

**RDEB Patients and Cells**—Patients with RDEB (defined by clinical, immunohistochemical, and ultrastructural criteria) were selected from the National Epidermolysis Bullosa Registry site of Stanford University.

**Expression Vector Construction and Transfection**—The eukaryotic expression vector pRC/CMV (Invitrogen, San Diego, CA) which contains a cytomegalovirus (CMV) promoter and enhancer was used to express the truncated human type VII collagen minigene (Fig. 1). The cDNA comprised the sequences coding for the NH2-terminal 1253 amino acids of the noncollagenous NC1 domain, 880 amino acids of the central collagenous helical domain, and 161 amino acids of the COOH-terminal NC2 domain. The construct has a 683-amino acids in-frame deletion from amino acids 1920 to 2603 within the central collagenous domain. The truncated human type VII cDNA was assembled from CMV/NC1 and three cDNA clones previously described (11) by standard subcloning techniques and recombinant PCR. The construct contains an internal restriction site, including BamHI, Xhol, and SacII from each cDNA fragment. The correct ligation and in-frame insertion of various DNA fragments were confirmed by DNA sequence analysis.

The expression vector was used to transfect the human embryonal kidney cell line 293 (ATCC, Rockville, MD) and immortalized RDEB keratinocytes using Lipofectin (Life Technologies, Inc., Gaithersburg, MD) as described previously (11). Stable clones were selected using 500 μg of G418/ml (11).

**Retroviral Expression Vector and Gene Transfer**—The mini-type VII collagen cDNA was subcloned into a MFG-based LZRS vector and high titer virus-producing cells were generated from human BING 293 packing cells by transfection with calcium-phosphate co-precipitation followed by selection in the presence of 1 μg/ml puromycin as described (22). Cells were shifted to serum-free medium for 24 h and retroviral supernatants were collected (22). For retroviral infection, keratinocyte cultures were trypsinized and seeded onto 100-mm plates and incubated for 24 h. Viral supernatant (2.5 ml) was diluted to 50% with SFM (2.5 ml) and supplemented with 5 μg/ml Polybrene and added to keratinocyte cultures which were 40–60% confluent. Plates were then incubated for 6 h, 5 ml of fresh SFM was added, and plates were incubated for an additional 16 h at 37 °C, after which the medium was changed with 8 ml of fresh SFM.

**Protein Purification and Analysis**—For immunoblot analysis, clonal cell lines resistant to G418 were grown to confluence, the medium was changed to serum-free medium, and the cultures were maintained for an additional 24 h. The media were collected, equilibrated to 5 mM EDTA, 50 μM N-ethylmaleimide, and 50 μM phenylmethylsulfonyl fluoride and concentrated 10–15-fold (Centricron-100, Amicon, Beverly, MA) and subjected to 6% SDS-PAGE. Proteins were then electrotransferred onto a nitrocellulose membrane. The presence of recombinant minicollagen VII was detected with a mouse monoclonal antibody, LH7.2 (Sigma), and polyclonal antibodies to the NC1 and NC2 domains of type VII collagen, respectively (23), followed by a horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG and enhanced chemiluminescence detection reagent (Amersham Pharmacia Biotech).

For large-scale purification of recombinant minicollagen VII, serum-free media were equilibrated to 5 mM EDTA, 50 μM phenylmethylsulfonyl fluoride, and 50 μM N-ethylmaleimide and precipitated with 300 mg/ml ammonium sulfate at 4 °C overnight with stirring (24). Precipitated proteins were collected by centrifuging at 1.2 × 10^6 g/min for 1 h, resuspended and dialyzed in Buffer A (65 mM NaCl, 25 mM Tris-HCl, pH 7.5). Following dialysis, insoluble material was collected by centrifugation at 8,600 × g for 20 min, and the pellet redissolved in Buffer B (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 2 mM N-ethylmaleimide, 2 mM phenylmethylsulfonyl fluoride). The solution was clarified as above, and the supernatant, S1, stored at −20 °C. The pellet was re-dissolved in Buffer C (50 mM Tris-HCl, pH 7.5, 2 M urea, 5 mM EDTA, 2 mM N-ethylmaleimide, and 2 mM phenylmethylsulfonyl fluoride). The solution was clarified as above and the supernatant containing purified minicollagen VII, S2, stored at −20 °C.

**Protein Binding Assay**—Binding of soluble minicollagen VII and NC1 to immobilized ligands followed by a colorimetric enzyme-linked antibody reaction was performed as described previously (11). Briefly, multwell plates (96 wells, Dynach Laboratory Inc., Alexandria, VA) were coated overnight with ECM proteins (2 μg) in 100 μl carbonate buffer, pH 9.3. The wells were then blocked with 1% bovine serum albumin in phosphate-buffered saline, 0.05% Tween 20. Coated wells were subsequently incubated with 2 μg of purified recombinant minicollagen VII or NC1 overnight at 4 °C. The binding of minicollagen VII to ECM proteins was detected using an affinity-purified goat anti-mouse or goat anti-rabbit IgG and enhanced chemiluminescence detection reagent (Amersham Pharmacia Biotech). The ligands used for binding included purified type I and type IV collagens (Collaborative Biomedical Product, Bedford, MA). Laminin 1 was routinely prepared from the EHS tumor as described (25). Additionally, laminin 5 was purified from collagenase-solubilized EHS tumor matrix using the method described (26). Laminin 5 was the generous gift of Dr. Peter Marinkovich, Stanford University. Human laminin 5 was purified from collagenase-solubilized EHS tumor matrix using the method described (26). Laminin 5 was the generous gift of Dr. Peter Marinkovich, Stanford University.
human amniotic membranes by antibody affinity chromatography using monoclonal antibody K140-Sepharose as described (27).

Proteases Digestion—Purified recombinant minicollagen was incubated with chymotrypsin (Sigma) at an enzyme-to-substrate ratio of 1:10 by weight in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl or with pepsin in 0.1 M acetic acid at 37 °C for 2 h and then analyzed by SDS-PAGE followed by immunoblot analysis with a polyclonal antibody against the collagenous domain of type VII collagen.

Cell Migration Assay—Keratinocyte migration was assayed by the method of Albrecht-Buehler (28), as modified by Woodley et al. (29). Briefly, colloidal gold salts were immobilized on coverslips and covered with type I collagen (15 μg/ml). Keratinocyte cultures were suspended, plated on the coverslips, and allowed to migrate for 16–20 h. The cells were fixed in 0.1% formaldehyde in phosphate-buffered saline and examined under dark field optics with a video camera attached to a computer equipped with a frame grabber. The computer analyzes 15 nonoverlapping fields in each experimental condition with NIH Image 1.6 and determines the percentage area of each field consumed by cell migration tracks, a so called Migration Index. Confirmation of a difference in migration as statistically significant requires rejection of the null hypothesis of no difference between mean migration indices obtained from replicate sets at the p = 0.05 level with a Student’s t test.

Cell migration was also assessed in an in vitro scratch assay as described by Cha et al. (30). Confluent monolayer cultures of keratinocytes were scratched in a standardized manner with a plastic instrument to create a 2-mm wide cell-free zone in each culture. The medium was then aspirated and replaced with fresh SFM containing type I collagen (15 μg/ml). The keratinocytes from the cut edge of the scratch were then allowed to migrate for 16 h and photographed.

Cell Adhesion Assay—To measure cell-substrate adhesion, a standardized cell adhesion assay according to Varani et al. (31) was used. Briefly, keratinocytes grown in 60-mm dishes were washed two times and treated with 0.05% trypsin, 0.01% EDTA, 37 °C. At various times, detached cells were gently harvested and counted. A total cell count was made when all of the cells had been released, and the percentage of cells released at each time point was determined.

Proliferation Assay—Keratinocyte proliferation was measured by seeding 35-mm (diameter) culture dishes with 7.5 × 10^4 cells per dish in keratinocyte growth medium (SFM). The cultures were then incubated at 37 °C and 5% CO_2 for 1 to 6 days. After the incubation, the cells were harvested with trypsin/EDTA and counted with hemocytometer.

RESULTS

Recombinant Production of Type VII Minicollagen—We constructed a type VII collagen “minigene” as diagrammed schematically in Fig. 1, comprising the sequences coding for the NH_2-terminal 1253 amino acids and the COOH-terminal 161 amino acids of the noncollagenous domains (NC1 and NC2, respectively) and 880 amino acids of the central collagenous domains minus a 683-amino acids in-frame deletion (deletion from amino acids 1920 to 2603). The cDNA insert was cloned into a HindIII/XbaI-digested eukaryotic expression vector, pRC/CMV.

![Fig. 1. Domain organization and cDNA construct for expression of the type VII minicollagen. A, the deduced 2944 amino acid sequence of type VII collagen (C7) consists of a central triple-helical domain (TH), flanked by a large amino-terminal noncollagenous domain, NC1, and a smaller carboxy-terminal noncollagenous domain, NC2. The TH domain contains a non-collagenous 39-amino acid hinge region. B, the minigene cDNA construct (C7M) contains intact NC1 and NC2 domains and a truncated TH domain with an in-frame deletion region.](Image 281x729)

![Fig. 2. Immunoblot of C7M transfected 293 cells. A, conditioned media from control vector CMV transfected 293 cells (lanes 1 and 3) or CMV/C7M transfected 293 cells (lanes 2 and 4) were concentrated and subjected to 6% SDS-PAGE followed by immunoblot analysis using either a polyclonal antibody to NC1 (lanes 1 and 2) or a polyclonal antibody to NC2 (lanes 3 and 4). B, immunoblot analysis of unreduced conditioned media from cells transfected either in the absence of ascorbate (lane 5) or in the presence of ascorbate (lane 6) with a polyclonal antibody to NC1. The positions of the molecular mass marker, 230-kDa minicollagen VII (C7M) and 700-kDa trimer of minicollagen VII (C7M-T) are indicated.](Image 535x729)

The demonstration that minicollagen apparently assembled to form S–S bonded trimers suggests, but does not prove, that these composites are stable and have a proper triple helical conformation in their collagenous domain. To examine this question further, purified minicollagen VII was treated with chymotrypsin and pepsin and then subjected to immunoblot analysis using a polyclonal antibody against the triple helical
domain. As shown in Fig. 4, digestion of the 230-kDa minicollagen VII with two enzymes produced a 115-kDa protease-resistant fragment under reducing conditions (Fig. 4, lane 2 and lane 3). Under nonreducing conditions, the 700-kDa trimer disappeared to give rise to a 350-kDa fragment (lane 5 and lane 6), the expected molecular mass of the disulfide-bonded collagenous domain. The resistance to protease digestion demonstrates the triple helical conformation of the minicollagen VII (32).

Interaction of Minicollagen with ECM Proteins—We previously showed that the NC1 domain of type VII collagen interacts with fibronectin, laminin 5, type I collagen, and type IV collagen. Therefore, we examined the ability of purified minicollagen VII to bind immobilized ECM components. In order to compare the binding affinity between NC1 and minicollagen VII, purified recombinant NC1 was also included in the binding assay. As shown in Fig. 5, like NC1, minicollagen VII binds to fibronectin, laminin 5, type I collagen, and type IV collagen. Little or no binding occurred with laminin-1. The ECM binding affinities appeared the same when NC1 and minicollagen VII were compared with the exception of the affinity for fibronectin. In this case, minicollagen VII appears to bind more avidly than NC1. This is consistent with our previous study that mapped a second fibronectin-binding site within the collagenous domain (33).

Expression of Minicollagen in RDEB Keratinocytes—To examine if the genetic defect of RDEB keratinocytes could be corrected by minicollagen VII expression, the minigene cDNA construct was introduced into RDEB keratinocytes by retroviral-mediated gene transfer using an amphotropic retroviral expression vector (22). This vector was used to transduce keratinocytes immortalized from two RDEB patients. The patients in this study lacked type VII collagen expression, but expressed other BMZ proteins, including BP230, BP180, type IV collagen, and laminin 5 (data not shown). Western analysis of the secreted proteins detected the expression of 290-kDa type VII collagen in the medium of normal keratinocytes (Fig. 6A, lane 1). This band was completely absent in the RDEB keratinocytes (Fig. 6A, lane 2). Transduction of minigene construct into RDEB keratinocytes resulted in the expression of a 230-kDa minicollagen VII (lane 4) while this band was completely absent in the control virus-transduced cells (lane 3). Similar results were obtained with keratinocyte from the other patient. The expression of minicollagen VII was sustained at least 8 months in vitro (Fig. 6B).

Expression of Minicollagen Reduced Cell Motility—We previously observed that cultured RDEB keratinocytes display a different morphology than normal keratinocytes. Compared with normal human keratinocytes, RDEB keratinocytes show decreased cell-substrate adhesion, a decreased proliferative po-
tential and hypermotility on collagen matrices. To determine if minigene transfer exerts any functional effects on RDEB cells, we first assessed motility by the in vitro scratch assay. In this assay, the keratinocytes were plated densely in tissue culture wells and after 24 h a standardized scratch was made through confluent monolayers of cultured keratinocytes. The keratinocytes from the cut edge of the scratch were then allowed to migrate for 16 h and photographed. As shown in Fig. 7A, RDEB keratinocytes were found to migrate into the scratch much more rapidly than normal keratinocytes (compare panel RDEB to panel NHK). Thus, RDEB keratinocytes display hypermotility. Compared with parental RDEB or control virus transduced RDEB keratinocytes, minigene transduced RDEB keratinocytes demonstrated reduced motility (compare panel RDEB/C7M to panel RDEB and RDEB/LZR), that was similar to the motility of normal keratinocytes. No appreciable difference in cell motility was noted between RDEB keratinocytes and RDEB keratinocytes transduced with control virus (compare panel RDEB and RDEB/LZR).

The migration capacity of RDEB keratinocytes was also evaluated using the colloidal gold track assay in which keratinocytes migrate on immobilized type I collagen. Fig. 7B shows representative microscopic fields of the keratinocytes migrating on type I collagen. Compared with RDEB keratinocytes (RDEB), which migrated leaving long tracks behind them, gene corrected RDEB keratinocytes (RDEB/C7M) demonstrated reduced motility to the level similar to normal keratinocytes (NHK) and normal immortalized keratinocytes (IKC). The migration index is the percentage of the field taken up by the motility track. As shown in Fig. 7C, the migration index values were 40.2 for RDEB keratinocytes, and 22.9, 24.6, and 25 for normal human keratinocytes, immortalized keratinocytes, and gene corrected RDEB keratinocytes, respectively. Expression of the minicollagen VII in RDEB keratinocytes induced a reversal of the hypermotility phenotype and the cells migrated like normal cells.

Expression of Minicollagen Enhanced Cell Adhesion—We next evaluated cell-substratum adhesion by measuring the rate of trypsin-mediated cell release from substratum. As shown in Fig. 8, RDEB keratinocytes detached from the substratum more rapidly than normal keratinocytes. Eight minutes was required to detach the normal keratinocyte against 6 min in the case of RDEB keratinocytes. Expression of minicollagen VII in RDEB keratinocytes enhanced their adhesion and decreased their sensitivity to trypsin to a similar level as that of normal keratinocytes.

Expression of Minicollagen Increased Cell Proliferation—The proliferative potential of keratinocytes was evaluated by counting the number of cells in culture over time (Fig. 9). Cell counts of RDEB keratinocytes were significantly lower than that of normal keratinocytes for all days analyzed. Minigene transfer is associated with a restoration of proliferation potential to normal levels, suggesting a functional correction of this abnormality.

DISCUSSION

DEB is an incurable potentially fatal skin disease in which gene therapy may hold promise. Lack of expression of type VII collagen and the resulting paucity of anchoring fibrils in DEB
NC2 and adjacent 167-amino acid of collagenous domains initi-
ate the triple-helical assembly of type VII collagen and direct antiparallel dimer formation. All of these domains have been preserved in the minigene construct.

Minicollagen VII migrated as doublet on SDS-PAGE. The nature of the doublet is not clear, but could be due to a post-translational modification such as glycosylation, hydroxylation, and/or phosphorylation. cDNA sequence analysis of type VII collagen reveals three putative N-linked glycosylation sites all within the NC1 domain and four potential phosphorylation sites by casein kinase I and II within the NC2 domain. Deglycosylation of the purified minicollagen VII with peptide N-glycosidase F converted it to two lower molecular weight species while digestion by bacterial collagenase released a single specie of NC1. This suggests that N-linked glycosylation is not responsible for the two species of minicollagen VII. Whether phosphorylation or hydroxylation is responsible for the two species of minicollagen VII is under further investigation. It is also possible that the two species of minicollagen VII represent the procollagen and collagen. The polyclonal antibody used in our study reacts with both procollagen and collagen. An antibody that recognizes only procollagen would be needed to address this issue.

Type VII collagen is synthesized and secreted by both human keratinocytes and fibroblasts, the two principal cell types in the skin (35–37). Therefore, both DEB keratinocytes and fibroblasts could be used for gene correction. We show here that RDEB keratinocytes lacking type VII collagen expression can be genetically corrected in vitro by the introduction of a minigene using retroviral-mediated high-efficiency gene transfer. This is accomplished by generating retroviral vectors at high titer using a replicating, episomal approach in human 293 packaging cells. The level of minicollagen VII expression in gene-corrected DEB keratinocytes was similar to or greater than that of normal human keratinocytes. Furthermore, the minicollagen VII production was sustained for at least 8 months in vitro. However, with other human genes, long terminal repeat-mediated gene expression in primary human keratinocytes could not be maintained in vivo for longer than 4 weeks (38–42). In these systems, the loss of expression of the transgene was due to promoter inactivation rather than the death of genetically engineered cells. Keratinocytes used in our study were immortalized with the E6 and E7 gene of HPV16. Immortalized keratinocytes have been used to achieve stable and prolonged expression of transgenes in vivo and generate an epidermis with normal morphology (43). It was suggested that processes that lead to immortalization inhibit mechanisms that switch off transgenes inserted in the host genome by retroviral vectors in vivo, and/or inhibit in vivo apoptosis of cells that have been stably transduced ex vivo (43).

After restoring the ability to express minicollagen VII in RDEB keratinocytes, we wished to determine if these restored RDEB cells had a reversal of the RDEB cellular phenotype to the phenotype of normal keratinocytes. During the course of this study, we observed that keratinocytes cultured from 8 RDEB patients demonstrated a slow growth potential, reduced attachment to matrix, and enhanced cell motility. The expression of minicollagen VII in RDEB keratinocytes was associated with enhanced growth potential, increased matrix adhesion, and reduced cell motility to levels similar to those of normal human keratinocytes. Our study was performed using immortalized keratinocytes. Immortalization of keratinocytes may have utility, provided that the immortalized cells maintain critical biological features of the parent cells. In vitro, we could not detect any significant differences between normal and im-

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2 M. Chen and D. T. Woodley, unpublished observation.
mortalized human keratinocytes with respect to cellular adhesion, migration, and proliferative potential.

In summary, our studies demonstrated that the minicollagen VII has many of the same functional characteristics as a full-length type VII collagen chain. We also demonstrated that efficient minigene delivery to cultured RDEB keratinocytes can produce a population of phenotypically corrected RDEB cells. Human keratinocytes in culture can be expanded from a 1-cm² skin biopsy to a surface area equivalent to or greater than the entire body surface area in less than 3 weeks. Cultured keratinocytes have been used to make autografts to cover burn wounds and chronic skin ulcers (44, 45). With successful correction of gene-deficient human keratinocytes in vitro, the foundation is laid to use gene-corrected RDEB cells in organotypic cultures and a human skin/mouse xenograft model and demonstrate that the corrected RDEB cells expressing the minicollagen VII can actually make anchoring fibril structures. Our data may provide a basis for future ex vivo gene therapy for DEB.

REFERENCES

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