Genetic correction of canine dystrophic epidermolysis bullosa mediated by retroviral vectors

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We have assessed the suitability of retroviral vectors for gene therapy of recessive dystrophic epidermolysis bullosa (RDEB) in dogs expressing a mutated collagen type VII. Isolation and analysis of the 9kb dog collagen type VII cDNA identified the causative genetic mutation G1906S and disclosed the interspecies conservation of collagen type VII. Highly efficient transfer of the wild-type collagen type VII cDNA to both dog RDEB and human primary RDEB collagen type VII-null keratinocytes using recombinant vectors derived from LZRS-Ires-zeo and MSCV retroviruses achieved sustained and permanent expression of the transgene product. The expression and post-translational modification profile of the recombinant collagen type VII was comparable to that of the wild-type counterpart. The recombinant canine collagen type VII in human RDEB keratinocytes and dog cells corrected the observable defects caused by RDEB keratinocytes in cell cultures and in vitro reconstructed skin. Hypermotility was fully reverted in human RDEB keratinocytes, and strongly reduced in the dog RDEB cells. This observation suggests that not only infection efficiency but also high expression levels are required to ensure therapeutic efficacy in the presence of mutated gene products. Our results set the basis for preclinical gene therapy assays in the first immune-competent large animal model for an inherited skin disease and broaden the spectrum of preclinical and clinical applications of retroviral vectors in the transfer of large recombinant genes in epithelial cells.

INTRODUCTION

Gene transfer to keratinocytes is an attractive method for the treatment of genetic disorders because of the ease with which the epidermal cells can be biopsied, expanded in culture and grafted back to the patients following procedures used in the treatment of burn injuries and chronic ulcers (1). Cultured epithelia made with genetically modified epidermal stem cells after transplantation on immunodeficient SCID mice generate self-renewing skin that replaces defective tissue in inherited skin diseases or functions as a source of secretable gene products that are flawed in inherited disorders of circulating proteins (2). The two most important factors that assure the persistent expression of transgenes in human skin implants are the vector design and the use of protocols that allow efficient targeting of the self-renewing epidermal stem cells. The use of Moloney murine leukemia (MMLV) retroviral vectors allowed persistent expression of sustained levels of lacZ or diffusible factors, such as clotting factor IX, for more than one year in human epidermis grafted onto immunodeficient mice (3,4). Efficient transduction of epidermal stem cells has been achieved either by co-culture with packaging cell lines or by the use of high-titer vector preparations (4). Both methods allow a sustained expression of the transgene in vivo without immune reaction of the host to the viral vector (5,6).

The first studies on gene therapy of genodermatoses have been mainly restricted to epidermolysis bullosa (EB), lamellar and X-linked ichthyosis and xeroderma pigmentosum (2). These conditions offer unique opportunities to develop models for corrective gene delivery because (i) their recessive transmission requires delivery of just one copy per cell of the curative transgene, (ii) the involved proteins are expressed by short cDNAs efficiently shuttled by existing viral vectors, and (iii) spontaneous animal

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models suitable for preclinical trials are available (2). Progress in the genetic correction of other dermatoses, including dystrophic epidermolysis bullosa (DEB), has been hampered by the relatively large size of the cDNA encoding the curative protein.

DEB is associated with mutations in the gene (COL7A1) for collagen type VII, a molecule consisting of three alpha chains each comprising a 145 kDa central collagenous segment flanked by two non-collagenous NC1 (145 kDa) and NC2 (34 kDa) domains (7). The collagen type VII homotrimers associate to form the anchoring fibrils (AF) of the papillary dermis that contribute to the attachment of the basal keratinocytes to the mesenchyme. Mutations in collagen type VII result in an abnormal synthesis of the AF and intradermal splitting of the dermal–epidermal junction. Collagen type VII-null patients suffering from severe recessive inherited DEB (RDEB) present generalized blistering and mutilating scarring that with advancing age develop into squamous cell carcinomas. The majority of DEB patients expresses a mutated collagen type VII and suffers from milder clinical manifestations that are inherited in either a dominant (DDEB) or recessive mode (8). Collagen type VII cDNA (8.8 kb) has been recently transferred into collagen type VII-null RDEB keratinocytes and fibroblasts using the ϕC31 bacteriophage integrase and a lentiviral vector (9,10). The reverted RDEB cells express the recombinant collagen type VII and restored the synthesis of AF after implantation onto SCID mice, which demonstrates the feasibility of gene transfer in collagen type VII-null RDEB cells.

At this stage, elaboration of gene therapy protocols for RDEB requires appropriate animal models, but the severity of congenital RDEB described in domestic animals or obtained by targeted inactivation of the COL7A1 gene in mice has compromised the establishment of such models. Recently, we reported the identification of a strain of golden retriever dogs with mild RDEB that constitutes the unique animal model suitable to validate the gene therapy approach for DEB (11). Similar to human patients with DEB, the diseased animals suffer from bullous eruptions inherited in an autosomal-recessive mode, with involvement of the oral and esophageal mucosa, and a generalized tendency of the skin to blister with generation of milium. The active blistering of the skin diminishes from the age of 8 months, whereas formation of blisters and erosions of the upper digestive tract persist, causing growth retardation.

Here we set out (i) to determine the genetic basis of the RDEB in dogs that express a mutated collagen type VII, (ii) to determine the suitability of retroviral vectors to transduce the collagen type VII cDNA into RDEB keratinocytes that synthesize abnormal collagen type VII, and (iii) to study the expression profile of the transferred dog collagen type VII gene in ex vivo reconstructed skin. Permanent expression of the dog collagen type VII transgene was observed both in human and dog RDEB keratinocytes with correction of the observable defects caused by RDEB.

RESULTS

Establishment of canine collagen type VII cDNA sequence

Preliminary studies on a breed of golden retriever dogs presenting skin fragility, diagnosed inherited RDEB associated with altered expression of collagen type VII (11). To define the genetic background of the condition, synthesis and secretion of collagen type VII in the affected animals was investigated by immunofluorescence staining of keratinocytes isolated from the skin and the oral mucosa using mAb LH7.2 directed against collagen type VII. Compared with the wild-type controls, the RDEB dog keratinocytes expanded in vitro exhibit an increased intracellular immunofluorescence signal for collagen type VII (Fig. 1A). Immunoblotting of cell extracts showed that the RDEB keratinocytes contain higher amounts of collagen type VII than the wild-type counterparts, which indicates accumulation of collagen type VII possibly associated with a genetic mutation in the dog COL7A1 gene (Fig. 1B). To verify this hypothesis, the cDNA for dog collagen type VII was isolated and characterized. Total RNA purified from wild-type dog keratinocytes was amplified by RT–PCR using sets of oligonucleotide primers homologous either to the cDNA sequence for the dog NC1 domain or the human collagens and NC2 domains (Supplementary Table 1). Direct sequencing of the amplification products identified the full-length nucleotide sequence of the dog collagen type VII cDNA. The nucleotide sequence (GenBank accession no. AY183408) comprises an open reading frame (8811 bp) encoding a polypeptide of 2936 residues 87.8 and 83.5% homologous to the human and mouse counterparts, respectively. The domain organization is identical in the three species (Supplementary Table 2). Specifically, the collagenous domain (1523 amino acids) presents the characteristic Gly–X–Y repeats with 19 non-collagenous imperfections, 16 of which are conserved in man. Positions of the cysteine residues, the potential N-linked glycosylation sites, the RGD/RGE adhesion peptides, and the BMP-1 cleavage site SYAA/DTAG are conserved. These observations attested to the high interspecies conservation of collagen type VII in mammals.

Identification of mutation G1906S

To search for genetic mutations in the COL7A1 gene, total RNA purified from dog RDEB keratinocytes was used for RT–PCR amplification of the collagen type VII cDNA. The 809 bp cDNA fragment (residues 5287–6095) obtained with primers 11L and 11R was found to contain a homozygous 5716G-to-A transition (Fig. 1C) that substitutes the codon for glycine 1906 (GGC) with codon AGC for a serine residue. No additional sequence variation was found. Because transition 5716G-to-A abolishes a restriction site for endonuclease HaeIII, its association with the RDEB phenotype and its mendelian inheritance in the dog strain was determined by enzymatic digestion of a 127 bp PCR fragment of COL7A1 gene carrying variation 5716G-to-A obtained from the proband dogs and their relatives. The electrophoretic analysis of the digestion products showed that the DNA samples of the RDEB dogs were uncleaved, which denotes homozygosity for the mutated COL7A1 allele. Two DNA fragments of 95 and 32 bp were found in the healthy dogs homozygous for the wild-type COL7A1 alleles, and an additional 127 bp fragment was detected in the healthy carriers, heterozygous for the mutated and wild-type allele (Fig. 1D).
Retroviral transduction of human collagen type VII-null keratinocytes

To verify that the genetic defect of collagen type VII-null keratinocytes can be complemented by transfection of a curative cDNA, the full-length dog collagen type VII cDNA was inserted into the retroviral vector pLZRS-Ires-zeo to generate plasmids LZRS-Col7 (Fig. 2B). LZRS-Col7, pseudo-typed by the Ampho envelop protein, expresses a bi-cistronic Col7-Zeo messenger RNA of 12.5 kb and allows selection of the transduced cells. In addition, because retroviral vectors expressing a resistance gene are immunogenic in vivo, and therefore unsuitable for preclinical assays in immunocompetent hosts, the dog collagen type VII cDNA was cloned into the pMSCV vector to produce plasmid MSCV-Col7 (Fig. 2C). The expression of receptors for the retrovirus envelope proteins being not clearly assessed, the retroviral vector MSCV-Col7 was pseudotyped by three different envelop proteins (MLV amphotropic, GAL V or VSV-G) using a transient transfection protocol (see Materials and Methods) to define the optimal conditions for ex vivo transduction of the RDEB keratinocytes. Retroviral vectors were first used to transduce human collagen type VII-null RDEB keratinocytes, which permits a precise evaluation of the expression level of the recombinant dog collagen type VII in the absence of the endogenous counterpart. Expression of the transferred cDNA was monitored by immunofluorescence using mAb LH7.2, which revealed efficient transduction of the infected cell cultures, because 65% of immortalized and 40% of primary keratinocytes transduced by LZRS-Col7 were immune reactive (Table 1). After 7 day selection in the presence of zeocin, 100% of the cells were immune reactive (Fig. 3A). Infection with the vectors MSCV-Col7 yielded a range of 83–93% positive cells; the highest efficiencies were obtained with the retrovirus pseudotyped with the MLV amphotropic envelope (Fig. 3D–F).

Figure 1. Identification of mutation G1906S in the RDEB dog collagen VII. (A) Immunofluorescence analysis of wild-type (a, c) and RDEB (b, d) dog keratinocytes isolated from skin (a, b) and oral mucosa (c, d) using mAb LH7.2 shows an enhanced cytoplasmic staining in the RDEB keratinocytes. (B) Immunoblot analysis of cell lysates and spent medium of wild-type (1, 3) and RDEB (2, 4) keratinocytes. The protein extracts were fractionated on a 5% SDS–PAGE under reducing conditions and subjected to immunoblotting with pAb NC2-10. The intensity of the hybridization signal in DEB cell lysates confirms the cytoplasmatic retention of collagen type VII in the skin (1, 2) and oral mucosa (3, 4). Immunoblot with anti-tubulin antibodies was included as an internal control of protein loading for the cell extracts. (C) Direct sequencing of collagen type VII cDNA detected a homozygous 5716G-to-A substitution in RDEB dogs resulting in nonsense mutation G1906S. (D) Mendelian inheritance of mutation G1906S. Transition G-to-A abolishes a HaeIII restriction site in exon 68 (127 bp) of the morbid COL7A1 allele. Digestion of the PCR-amplified 127 bp genomic DNA fragment generates a 95 bp DNA fragment in healthy unrelated (lane c) and non-carrier dogs (lanes 11 and 13), an undigested 127 bp fragment in the RDEB dogs (lanes 6, 9 and 10) and additional 95 bp fragment in the heterozygous carriers (lanes 1–5, 7, 8 and 12). The pedigree highlights the carrier (half-filled) and homozygous RDEB dogs (black).
However, after five passages in culture, 100% of the cells were immunoreactive to the anti-collagen type VII mAb, with a strong cytoplasmic labeling suggesting a sustained synthesis of the dog recombinant r-collagen type VII. These results demonstrate efficient transduction of the primary keratinocyte cell cultures, which are less proliferative, therefore less prone to transduction by retroviral vectors, than the immortalized cells. The average copy number per cell of the r-collagen type VII cDNA evaluated by Southern blot analysis of genomic DNA extracted from the transduced cells indicated integration of two copies for both LZRS-Col7 and MSCV-Col7 (Table 1). No rearrangement of the integrated proviruses was detected (data not shown).

Immunoblot analysis of the cell lysates and spent medium of the transduced cell cultures using pAb NC2-10 to the P1 and NC2 domains of the dog recombinant r-collagen type VII detected a unique migration band with an apparent molecular mass of 320 kDa both with LZRS-Col7 (Fig. 3B) or MSCV-Col7 (data not shown). These results imply that the full-length dog r-collagen type VII is actively expressed and secreted by the transduced human RDEB cells. Assembly of the r-collagen type VII into homotrimers was verified by limited proteolysis of spent medium collected from the RDEB cells transduced with LZRS-Col7 (Fig. 3C and D) or MSCV-Col7 (data not shown) (12). Treatment with collagenase, which digests the central triple-helical domain of collagen type VII heterotrimers and releases the NC-1 and the NC-2 domains, and immunoblot analysis using pAb NC1-F3 to the NC1 domain detected the NC-1 fragment (145 kDa) and the undigested procollagen type VII (320 kDa; Fig. 3C). After treatment with pepsin, which degrades the globular NC-1 and NC-2 domains and releases the collagenous C-terminal (P1) and N-terminal (P2) fragments of the triple helical (TH) domain, immunoblot analysis using pAb NC2-10 detected the 115 kDa P1 polypeptide together with the undigested collagen type VII (Fig. 3D). These observations demonstrated that the dog r-collagen type VII

### Table 1. Transduction efficiency of r-collagen VII in human keratinocytes and number of proviral integrants

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<tr>
<th>Vectors</th>
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nd, not determined.
expressed by the transduced human RDEB cells correctly assembled into homotrimers that display a correct triple helical conformation. No truncated molecules indicative of rearrangements of the transduced cDNA were seen.

Retroviral transduction of dog RDEB keratinocytes

Because expression of the r-collagen type VII had been validated in human collagen type VII-null cells, the retroviral vectors were subsequently used to transduce primary dog keratinocytes that express collagen type VII molecules carrying mutation G1906S. As shown in Figure 4A, immunofluorescence analysis of the transduced cells using mAb LH7.2 showed an enhanced intracellular fluorescent staining compared with the dog RDEB keratinocytes infected with the empty vector. After zeocin selection of the cell cultures transduced with vector LZRS-Col7, all the keratinocytes were immunoreactive to the antibody directed against the zeocin gene product Sh-ble, confirming the efficient expression of the recombinant bicistronic transgene. Western blot analysis of cell lysates and medium spent by the transduced keratinocyte cultures detected a unique 320 kDa band with pAb NC2-10, which corresponds to the apparent molecular mass of the endogenous mutated collagen type VII and the recombinant wild-type counterpart (Fig. 4B). Collagenase and pepsin treatment of the spent culture medium of the RDEB keratinocytes before and after transduction released the P1 and NC1 fragments of collagen type VII, respectively (Fig. 4C and D). These results indicated that the r-collagen type VII secreted by the transduced dog keratinocytes incorporated in homotrimers with a triple helical conformation indistinguishable from that of the endogenous counterpart.

Expression of r-collagen type VII by artificial skin

As an attempt to evaluate the capacity of the transduced cells to deposit the r-collagen type VII at the epithelial dermal–epidermal junction, skin equivalents (SE) were first made with human keratinocytes seeded onto a synthetic matrix embedded with human collagen type VII-null fibroblasts and analyzed by immunofluorescence using anti-collagen type VII mAb (Fig. 5). SE made with human RDEB keratinocytes was not immunoreactive to anti-collagen type VII mAb, while SE obtained with transduced RDEB keratinocytes analyzed 15 days after emergence at the air–liquid interface displayed a bright linear staining at the dermal–epidermal junction (data not shown). Interestingly, labeling of collagen type VII in the artificial skin was enhanced with time. At 30 days, the intensity of the fluorescence signal was comparable to that observed in SE made with wild-type keratinocytes, which indicated efficient expression of the r-collagen type VII (Fig. 5A). Deposition of the r-collagen type VII at the dermal–epidermal junction of artificial skin was then assessed by seeding the transduced dog RDEB keratinocytes on sponges embedded with human RDEB–collagen type VII-null fibroblasts (Fig. 5B). Reflecting the results of the epitope mapping performed in vivo (11), collagen type VII staining was less intense in SE constructed with RDEB dog keratinocytes than in SE made with wild-type keratinocytes (Fig. 5B). In contrast, the staining was very strong in SE constructed with the transduced RDEB keratinocytes. This

Figure 4. Expression of r-collagen type VII in transduced RDEB dog keratinocytes. (A) Immunoreactivity to mAb LH7.2 in skin (a) and oral mucosa (c) keratinocytes is enhanced after transduction with retrovirus LZRS-Col7 (b, d). All the keratinocytes are labeled by the antibody to the zeocin gene product (f); the original RDEB keratinocytes are not immunoreactive (e). (B) Immunoblot analysis of cell lysates and spent medium of skin (lanes 1 and 2) and oral mucosa (lanes 3 and 4) dog keratinocytes demonstrates a stronger immunoreactive 320 kDa collagen type VII band in cells transduced with vector LZRS-Col7 (lanes 2 and 4) than in cells infected with LZRS-Zeo (lanes 1 and 3). Immunoblot with anti-tubulin antibodies was included as an internal control of protein loading for the cell extracts. (C) Collagenase digestion of spent medium of wild-type (lane 1), RDEB (lane 2) and reverted RDEB (lane 3) dog keratinocytes generated the NC-1 domain recognized by pAb NC1-F3. (D) Pepsin digestion of spent medium of wild-type (lane 1), RDEB (lane 2) and transduced RDEB (lane 3) dog keratinocytes generates the NC-1 domain recognized by pAb NC1-F3. (–) Not-digested; (+) digested.
Expression of r-collagen type VII reduces cell motility

Compared with wild-type keratinocytes, RDEB keratinocytes seeded in Petri dishes coated with collagen type I display hypermotility (10). To determine whether expression of dog r-collagen type VII reverts the hyper-motile phenotype, the migration of the transduced RDEB cells was evaluated using a haptotactic assay in Boyden chambers in the presence of either BSA or ECM (Fig. 6). On ECM, both the human and dog RDEB keratinocytes, before and after infection with the empty LZRS-Zeo vector, were hyper-motile. In contrast, migration of the transduced human RDEB keratinocytes was reduced and comparable to that of the wild-type human keratinocytes, which suggests that expression of the dog r-collagen type VII fully reverts the hyper-motile phenotype. Conversely, migration of the transduced dog RDEB keratinocytes appeared reduced, but not to the extent observed with the wild-type dog keratinocytes and the reverted human RDEB collagen type VII-null cells, possibly because the endogenous mutated collagen type VII partially counteracts the curative effect of the r-collagen type VII.

DISCUSSION

Our studies have disclosed the genetic basis of RDEB in a strain of dogs that represents the unique animal model for preclinical gene therapy trials of skin blistering genodermatosis. Dog RDEB is caused by a glycine substitution in the collagenous P2 domain of collagen type VII that is predicted to interfere with the assembly of the collagen type VII homotrimers and formation of AF. Our studies also provide evidence that retroviral vectors efficiently shuttle large DNA inserts (>12 kb) into eucaryotic cell cultures. By using recombinant MMLV and MSCV retroviruses to deliver the 8.8 kb cDNA for dog collagen type VII, we have achieved reproducible and persistent correction of the RDEB phenotype both in human collagen type VII-null cells and the dog keratinocytes. This correction was realized on primary cell cultures and was also possible without preselection of the transduced cells. Artificial skin made with the genetically modified keratinocytes permanently expressed high levels of recombinant collagen type VII at the dermal–epidermal junction of the reconstructed tissue. According to recent observations that lengthy retroviral genomes can be efficiently replicated and encapsidated by producer cell lines after a single replication cycle (13), the retroviral vectors produced infectious viral preparations with titers high enough to achieve efficient infection of the RDEB cells without any evidence of viral instability through insertion of rearranged proviral sequences and of synthesis of modified recombinant collagen type VII polypeptides. The transduction efficiencies were higher with the MSCV-based vectors, which reflects the higher multiplicity of infection (m.o.i.) achievable with these constructs due to the relatively reduced size of their genome that results in a more efficient encapsidation (13). The finding that in cell cultures infected with the MSCV-based
vectors the number of transduced keratinocytes rapidly increased along with passages to 100% of reversion indicates efficient targeting of the epithelial progenitor cells or of the highly proliferative keratinocytes that in vivo assure the self-renewal of the epithelium (14). This interpretation is in agreement with the broad host-range of MMLV vectors, and with their capacity to efficiently transduce stem cells in vitro (15). However, since expression of collagen type VII enhances cell adhesion and growth potential (10), the progressive enrichment of cell cultures in reverted RDEB cells may also reflect a selective advantage acquired by the infected keratinocytes on the minority of untransduced cells (15). These results constitute a significant advance over previous results obtained by using either a non-viral gene delivery system in primary RDEB keratinocytes, which yielded low gene transfer efficiencies (9), or a lentiviral construct in infection of immortalized RDEB keratinocyte cell lines, which are much more easily transduced than transplantable primary keratinocyte cultures but unsuitable for construction of transplantable epithelia (10).

Clinically useful molecular therapy requires efficient and sustained expression of the therapeutic gene. This is particularly important when the pathological phenotype results from the expression of a mutated protein, as in the RDEB dogs and the majority of DEB patients, and when the dominant negative interference may be reduced by a high ratio of normal vs mutated molecules during formation of supramolecular aggregates, such as polymerization of anchoring fibrils. Our protein chemical analyses indicate that in canine RDEB cells, r-collagen type VII may associate with the endogenous mutated counterpart to form hybrid molecules, which are indistinguishable from normal controls. An analogous situation has been reported in a inducible mouse model for EB simplex, where a ratio 3:1 of normal:mutated keratin 5 was functionally well tolerated, but lower ratios lead to abnormalities of keratin filaments and to epidermal fragility (16).

Assuming that the constitutive promoters of the viral vectors sustain comparable expression rates in human and dog cells, hypermotility was fully reverted in human RDEB keratinocytes and significantly, but not completely, in the dog cell cultures. This observation further stresses that not only infection efficiency but also high expression levels may be required to ensure therapeutic efficacy in the presence of mutated gene products. Gene-transfer directed overexpression of basement membrane proteins that undergo posttranslational regulation supports the appropriate localization of the recombinant proteins in vivo (5,17,18). Accordingly, recombinant dog collagen type VII was progressively deposited to the basement membrane of the in vitro reconstructed skin at amounts comparable to normal. Presence of immunoreactive collagen type VII in the suprabasal layers of the stratified epithelium consequent to overexpression of the recombinant protein did not alter the tissue differentiation process and may reflect less efficient posttranslational clearing by the homeostatic mechanisms that in vivo regulate the focal distribution of proteins (19).

In conclusion, we have set the basis for preclinical assays of gene therapy of an inherited skin disease in an immune competent large animal model. By showing the suitability of retroviral vectors to efficiently shuttle the collagen type VII cDNA into eucaryotic cells, our results broaden the spectrum of applications of these vectors in the transfer of large recombinant genes in epithelial cells.

**MATERIALS AND METHODS**

**Cell cultures**

Keratinocytes and fibroblasts were obtained from skin biopsies of collagen type VII-null RDEB patients and healthy controls, and from skin and oral mucosa biopsies of RDEB and healthy dogs (11). Keratinocytes were cultured on feeder layers of lethally irradiated mouse J2-3T3 fibroblasts in DMEM: Ham's F-12 (3:1; Hyclone, Perbio Sciences, Bezons, France) (20). Fibroblasts were grown in DMEM, 10% FCS, 2 mM glutamine, 10 ng/ml EGF. Packaging cells were grown in DMEM, 10% inactivated FCS, 2 mM glutamine, and 2 mM sodium pyruvate (21). Primary keratinocytes were immortalized by infection with purified SV40 virions as described (22).

**Isolation of dog collagen type VII cDNA and identification of mutation 5716G→A**

Overlapping cDNA fragments spanning the open reading frame of collagen type VII were obtained by RT–PCR amplification of total RNA purified from wild-type and RDEB dog keratinocytes using 17 pairs of oligonucleotide primers (Supplementary Table 1). The cDNA amplicons were analyzed in 1% agarose gels and submitted to direct sequencing using an ABI PRISM Dye Terminator Sequencing kit (Perkin Elmer, Foster City, CA, USA). Sequence analyses, alignments and amino acid translation were performed using the Lalign software (http://vega.igh.cnrs.fr) and cDNA nucleotide sequences were compared using the software program Sequencer (Gene Codes Corp, Ann Arbor, MI, USA). Identification of mutation 5716G→A at the genomic level was verified by PCR amplification of exon 68 of dog COL7A1 gene using genomic DNA as template and the intronic primers: (L) 5'-TCCACCACTACCCCTGGG-3' and (R) 5'-CTCTATCG-ACCACACACTG-3'. The amplification products (127 bp) were digested using the endonuclease HaeIII and fractionated on a 2.5% agarose gel.

**Retroviral expression vectors**

Three overlapping cDNA fragments (3770, 3970 and 2699 bp) were amplified by RT–PCR of total RNA using the Pfu Turbo polymerase (Stratagene, Amsterdam, The Netherlands) and primers pairs: L1 5'-GGATGAGGCTGGCCGCC-3', R1 5'-TTGGACACATGCACCTGTGAT-3'; L2 5'-GGGCTA-GACGCTGGCAAC-3', R2 5'-GGGAAACCAAGCCAACACC-AGG-3', and L3 5'-AGCGTTGGAAAGCCTGGTATT-3', R3 GCACGTCCACGTGCAC-3', respectively. The amplification products were cloned into the Zero Blunt Topo vector (Invitrogen, Cergy Pontoise, France), submitted to nucleotide sequencing, and assembled in the retroviral vectors pLZRS-Ires-Zeo (gift of G.P. Nolan, Stanford University, CA, USA) and murine stem cell virus (MSCV; Clontech, Palo Alto, CA, USA) to generate plasmids LZRS-Col7 and MSCV-Col7. The recombinant retroviruses were produced by transfecting plasmid LZRS-Col7 into the amphotropic Phoenix packaging...
The viral particles were recovered from the cell culture medium 48 h later and the titer (2.5 × 10⁶ cfu/ml) of the viral supernatant was determined by transduction of NIH-3T3 cells in the presence of 5 µg/ml polybrene and zeocin selection (200 µg/ml; Invitrogen). The MSCV-Col7 recombinant viruses were obtained from cell culture medium 48 h after transient expression in 293T cells transfected by calcium phosphate co-precipitation of a plasmid encoding an envelope glycoprotein (pBA-Ampho, pBA-GALV and pMDG, encoding MLV Amphotropic, GALV and VSVG envelope glycoproteins respectively), a plasmid coding the MLV Gag and Pol proteins (pMN gag-pol) and the vector plasmid (MSCV-Col7) in a proportion of 4, 6 and 10 µg of DNA, respectively. Titers (1.9 × 10⁷ GI/ml for MSCV-Col7 GALV, 4.4 × 10⁷ GI/ml for MSCV-Col7 VSVG, 2.1 × 10⁷ GI/ml for MSCV-Col7 Amphi) were determined by real time quantitative PCR (23). Cell cultures were transduced with the viral suspensions in 5 µg/ml polybrene for 24 h at 32°C in 5% CO₂ then fed with fresh medium containing 200 µg/ml zeocin (Invitrogen) when specified. The transduction efficiency was assessed by immunofluorescence examination of the infected cells. For Southern blot analysis, genomic DNA was extracted from subconfluent cell cultures using QIAamp DNA blood kit (Qiagen) and digested with EcoRI to release the full-length collagen type VII proviral fragment (9 kb). Plasmid pLZRS-Col7 was serially diluted with yeast genomic DNA at the final concentration range corresponding to 1–20 copies/cell. The restricted DNA was electrophoresed on a 0.8% agarose gel and transferred to a nylon membrane (Amersham Pharmacia Biotech, Saclay, France). The cDNA probe (1177 bp) was generated by PCR amplification of dog collagen type VII cDNA as a template using primers 4L and 5R (Supplementary Table 1) and ³²P-labeled by random priming (Stratagene). Hybridization and detection were performed according to manufacturer’s recommendations (Amersham). The intensity of the hybridization bands was quantified by densitometry using a Molecular Dynamics Storm Phosphoimager.

Skin equivalents were generated as detailed elsewhere (24). RDEB fibroblasts (2 × 10⁶ cells/cm²) were seeded on collagen–chitosan–glycosaminoglycan sponges (Genevrier, Sophia Antipolis, France) and maintained for 15 days in DMEM medium supplemented with 50 µg/ml ascorbic acid. Keratinocytes (2 × 10⁶ cells/cm²) were seeded on the fibroblast-embedded sponges, incubated for 4 days in DMEM/F-12 medium containing ascorbic acid, and raised at the air–liquid interface for either 15 or 30 days.

Immunohistochemistry

Immunolabeling was performed on 4µm cryosections of reconstructed skin and on keratinocyte cultures grown on glass coverslips and fixed as previously described (25). Antibodies were mAb LH-7.2 to the NC1 domain of collagen type VII (Chemicon, Temecula, CA) and a pAb directed against Sh-ble (Cayla, Toulouse, France). Secondary antibodies were FITC-conjugated goat anti-mouse or anti-rabbit Ig (Dako SA, Trappes, France). Preparations were examined under a Zeiss Axioshot microscope (Carl Zeiss Inc., Thornwood, NY, USA).

Immunochrometry

Subconfluent keratinocyte cultures were fed for 48 h with serum-free medium supplemented with 50 µg/ml ascorbic acid. Collagenase and pepsin digestion of crude cell extracts and spent culture medium was performed as described (12). For SDS–PAGE analysis, proteins were precipitated with trichloroacetic acid in the presence of deoxycholic acid (26) and separated on a 5% polyacrylamide gel under reducing conditions (27). Immunoblotting analysis was performed using pAb NC2-10 to the C-terminus (P1) of the collagenous triple helix, the NC2 domain of collagen type VII or pAb NC1-F3 to the NC1 domain (28,29) and mAb TUB 2.1 directed against tubulin (Sigma-Aldrich, Saint Quentin, France).

Migration assays

The lower side of transwell filters (Costar, Cambridge, MA, USA) was coated with 500 µg/ml heat-denatured BSA or 2.5 µg/ml of Engelbreth Holm–Swarm Mouse Sarcoma–extracellular matrix (EHS-ECM; Sigma-Aldrich) diluted in serum-free DMEM/F-12 medium for 4 h at 37°C. The upper chamber was saturated with 500 µg/ml BSA. Keratinocytes (2.5 × 10⁴ cells/cm²) in serum-free DMEM:F-12 medium were seeded in the upper chamber and allowed to migrate for 18 h at 37°C, 5% CO₂. The cells found on the lower side of the filter were fixed with 3% paraformaldehyde, stained with DAPI and counted as described (30).

Supplementary Material

Supplementary Material is available at HMG Online.

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References


