DISCUSSION

The developments in cloning the basement membrane protein genes have allowed the identification of the causative genes / proteins harboring the mutations responsible for this group of EB diseases (Uitto *et al*, 2001). We can now make good estimations about the prognosis and severity of these diseases with profound beneficial effects on genetic counseling and DNA-based prenatal diagnosis. However, patients most frequently desire an appropriate therapy for EB. Corrective transfer of the *COL7A1* gene back into the skin cells is a promising treatment of DEB.

The previous northern hybridization study revealed a high level of *COL7A1* mRNA expression in cultured epidermal keratinocytes while the expression was lower in cultured dermal fibroblasts (Ryynanen *et al,* 1992). These results indicate that epidermal keratinocytes and dermal fibroblasts express the collagen VII, but also suggest that epidermal keratinocytes are the primary source of collagen VII in developing human skin. Many investigators have utilized keratinocytes as the target cells of DEB gene therapy. Several methods including viral- (Ghazizadeh and Taichman, 2000) and nonviral- (Vogel, 2000) mediated transduction have been reported for in vivo and *ex vivo* gene transfer into keratinocytes. *COL7A1* cDNA was recently transferred into cultured DEB keratinocytes using some methods including lentivirus- (Chen *et al.* 2002), retrovirus- (Baldeschi *et al,* 2003) and ϕC31 integrase-based approaches (Ortiz-Urda *et al,* 2002). The corrected DEB keratinocytes expressed the recombinant collagen VII and restored the in vivo synthesis of anchoring fibrils after implantation, demonstrating the feasibility of gene transfer using DEB keratinocytes. We also succeeded in transferring the *COL7A1* into *in vivo* keratinocytes using the naked DNA method (Sawamura *et al,* 2002) although the *COL7A1* transfer efficacy was lower than the above *ex vivo* method.

Many gene therapy protocols have already utilized retroviral vectors for clinical practices. In this study we also showed a retroviral vector could transfer the 9 kb *COL7A1* cDNA into DEB keratinocytes. Another group has succeeded in transducing *COL7A1* gene to keratinocytes using a retroviral plasmid containing the Neo selection gene (Baldeschi *et al,* 2003). They showed that transduction efficacies to primary keratinocytes were 40% and 83-93% by retroviral vectors pLSRS-Ires-zero and pMSCV, respectively. However, the use of similar plasmids is not possible to efficiently introduce this gene into keratinocytes in our experiments. In our system, the efficacy was about 30 %, which was lower than those in previous report. This study as far as we know, has been the first to try a retronectin retroviral targeting system for keratinocytes.

Retronectin is a recombinant peptide which consists of three functional fibronectin domains and significantly enhances retrovirus-mediated gene transduction into mammalian cells. Our data showed that addition of retronectin increased transfer efficacy in keratinocytes by 3 fold (data not shown), indicating that retronectin is indeed efficient in this keratinocyte / retroviral system.

Some groups have succeeded in transferring this gene into keratinocytes as mentioned above. On the other hand, cutaneous injection of the DEB fibroblasts transduced using ϕC31 integrase-based approach also restores collagen VII deposition along the dermal-epidermal junction (Ortiz-Urda *et al,* 2003). Also, gene-corrected DEB fibroblasts and normal human fibroblasts alone could supply type VII collagen deposition at the BMZ *in vivo* (Woodley *et al.* 2003) and this implies a possibility that normal cultured human dermal fibroblasts are injected intradermally into RDEB patients' skin. Moreover, intradermal injection of lentiviral vector with *COL7A1* increased collagen VII expression in fibroblasts and endothelial cells, resulting in stronger deposition of collagen VII along the basement membrane zone anchoring fibrils as seen by electron microscopy (Woodley *et al,* 2004). This study also introduced the *COL7A1* gene into DEB fibroblasts using the retroviral method and the consequent collagen VII assembly beneath the basement membrane of the fibroblast containing graft.

In this study, we compared dermal fibroblasts and epidermal keratinocytes as efficient target recipient cells for the collagen VII transgene product. After retroviral introduction of *COL7A1*, the transfer efficacy and the amount of collagen VII in the cultured keratinocytes media supernatant are almost the same as those of fibroblasts. Interestingly, a series of skin graft experiments first demonstrated that gene-transfected fibroblasts more efficiently assembled collagen VII into the dermal-epidermal junction than the gene-transferred keratinocytes. Previous northern blotting analysis revealed higher level of *COL7A1* mRNA expression in cultured epidermal keratinocytes than fibroblasts (Ryynanen *et al,* 1992). This study utilized real time PCR technique and confirmed that the epidermis produced much more collagen VII than the dermis *in vivo*. If gene-transferred fibroblasts and keratinocytes express similar amounts of type VII collagen also *in vivo*, the fibroblasts may have a better ability to supply type VII collagen to the basement membrane than the keratinocytes.

It is evident that expression of recombinant collagen VII is driven by heterologous promoters, which escape the regulatory mechanisms that govern expression of endogenous collagen VII in the different cell types. Also keratinocytes have been

preferred because of the possibility they offer of targeting stem cells and compared to keratinocytes, fibroblasts rapidly senesce in vivo (Krueger et al. 2000). However, fibroblasts are more robust and less susceptible to growth arrest and differentiation than epidermal keratinocytes (Ortiz-Urda *et al,* 2003). Furthermore, genetically engineered fibroblasts have had their use explored for therapeutic applications including visceral and cutaneous implantation to supply gene products to circulation (Roth *et al,* 2001). Given the above combined factors, it is proposed that fibroblasts may be potentially more feasible and a better target of DEB gene therapy than keratinocytes.

Materials and Methods

Cells culture

Primary keratinocytes were isolated and grown in the presence of an irradiated 3T3 feeder layer (Rheinwald and Green, 1975). Briefly, keratinocytes, which were obtained from skin biopsy of a dystrophic epidermolysis bullosa (DEB) patient and healthy controls, were cultured on feeder layers of mitomycin C-treated mouse 3T3 fibroblasts in Dulbecco's modified Eagles' medium (DMEM): Ham's F-12 (3:1) supplemented with 10% FCS fetal calf serum (FCS). 5 μg/ml insulin, 10 ng/ml epidermal growth factor, 0.4 μg/ml hydrocortisone, and 8 ng/ml cholera toxin. The DEB patient was diagnosed as the most severe subtype, Hallopeau-Siemens type, showed no *COL7A1* expression in the skin and harbored heterozygous premature stop codon mutations 1474del8 and 5818delC. Fibroblasts were also obtained from skin biopsy from the DEB patient and healthy controls, and were cultured in DMEM with 10% FCS. Packaging cells amphopack-293 and GP2-293 (Clontech, Palo Alto, CA) were maintained in DMEM with 10% FCS, 2 mM glutamine, and 2mM sodium pyruvate.

Intrinsic expression of collagen VII in control keratinocytes and fibroblasts

Human skin was obtained from normal volunteers, and treated with 10 mg/ml dispase for 3 h at 37° C to separate the epidermis from the dermis. The epidermal and dermal sheets were minced and total RNA was extracted using an RNeasy RNA extraction kit (QIAGEN, Germany). First strand cDNA was synthesized with reverse transcriptase (Life Sciences Inc., St. Petersburg, FL) using an oligo-dT primer. Assays-on-Demand™ Products for *COL7A1* and *GAPDH* were purchased from Applied Biosystems (Foster City, CA). The 50 μL reaction in each well contained 1 μL of total cDNA, 300 nM sequence-specific primers and 200 nM dual-labeled fluorogenic probe

with 1 unit of Taqman Universal PCR master mix (Applied Biosystems). A negative control PCR without template and a positive PCR control with a template of known amplification were included in each assay. The samples underwent following stages: stage 1, 50°C for 2 min; stage 2, 95°C for 10 min; and stage 3, 95°C for 15 s followed by 60°C for 1 min. Stage 3 was repeated 45 times. Gene-specific products were measured by means of an ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, CA) continuously for 45 cycles. The *COL7A1* specific signal was normalized by constitutively expressed *GAPDH* and expressed as arbitrary scale.

Construction of retroviral COL7A1 expression vectors and transfection

Human full length *COL7A1* cDNA was constructed from several overlapping cDNA clones (Sawamura *et al,* 2002). We employed two retroviral vectors, pLIXN (Clontech) and pDON-AI (TAKARA, Japan), and full-length *COL7A1* cDNA was inserted into the retroviral vectors to generate plasmids termed pLI-COL and pDON-COL, respectively (Fig. 1). Also we created pDON(Δ) by removing the SV-40 promoter and *Neo* gene from pDON-AI and constructed a retroviral vector with *COL7A1* cDNA pDON(Δ)-COL (Fig. 1). The recombinant retroviruses were produced by transfecting the retroviral plasmids into the amphotropic amphopack-293 packaging cells (Clontech) using calcium-phosphate co-precipitation. In addition, we tried vesicular stomatitis virus G protein (VSV-G) pseudotyped retrovirus vectors. The retroviral plasmids and plasmid pVSV-G were cotransfected into pantropic GP2-293 packaging cells (Clontech). The viral particles were recovered from the cell culture medium 48 h later and applied to keratinocyte or fibroblast cultures. To increase transfer efficacy, ultracentrifugation was performed to concentrate the VSV-G virus particles. The titer of the viral supernatant was determined by real time quantitative PCR (Towers *et al,* 1999).

Cells infection with retrovirus

Keratinocytes and fibroblasts were cultured to up to 60 % of confluency and then infected with the viral suspensions in 5 μg/ml polybrene. To increase the virus-cell interactions, we coated the surface of the culture plates with 10 ng/ml retronectin (TAKARA: fibronectin fragment CH-296). After incubation for 24 h at 32° C, we maintained the treated cells under fresh medium for another 24 h until the transduction efficiency was assessed by immunofluorescence examination of the infected cells.

Immunostaining and immunoblot

Transfected cultured keratinocytes and fibroblasts were fixed with 2 %

parafromaldehye in PBS, and were then incubated with the monoclonal antibody LH7.2 (1:100) against the NC1 domain of collagen VII (Chemicon, Temecula, CA) for 18 h at 4° C. They were treated with secondary goat anti-mouse IgG antibodies conjugated with FITC (1:50) for 1 h at 37° C, and preparations were examined under a fluorescence microscope. Nuclei were counterstained with propidium iodide (Dojindo Laboratories, Japan). Subconfluent cell cultures were fed for 48 h with serum-free medium supplemented with 50 μg/ml ascorbic acid. For SDS-PAGE analysis, the culture medium was treated with Amicon Ultra-100,000 Centrifugal Filter Devices (Millipore, Bedford, MA) for concentration and desalting. The samples were separated on a 5% polyacrylamide gel under reducing conditions. Immunoblotting analysis was performed by treating with the LH7.2 monoclonal antibody (1:1000) for 18 h at 4° C and then secondary goat anti-mouse IgG antibodies conjugated with peroxidase (1:2000) for 1 h at 37° C . The resultant complexes were processed for Phototope HRP Western Blot Detection System (Cell Signaling, Beverly, MA) according to the manufacturer's protocol.

Southern blot analysis

The average copy number per cell of the *COL7A1* cDNA evaluated by Southern blot analysis (Baldeschi *et al*, 2003). Briefly, genomic DNA was extracted from subconfluent cell cultures and digested with *Bgl II* and *Hind III*. Plasmid pDON(*Δ*)-COL was serially diluted with yeast genomic DNA at the final concentration ranging from 0.5 to 20 copies/cell. The digested DNA was electrophoresed on a 0.8 % agarose gel and transferred to Zeta-Probe membrane (Bio-Rad, Hercules, CA). The 375pb cDNA extending from Exons 58 to 64 was generated by PCR amplification of *COL7A1* cDNA as a template. This fragment was designed to recogniezed a 7.2kb band from the integrated cDNA, and also 1.0 and 0.3 kb bands from the intrinsic *COL7A1* gene. The membranes were hybridized with the 375pb cDNA probe was labeled by random primed incorporation of digoxigenin-labeled 2'-deoxyuridine 5'-triphosphate (DIG-labeled DNA) using the DIG DNA Labeling Kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. After high stringency washes, blots were visualized using an enhanced chemiluminescence system.

Grafting of gene-transferred DEB cells

Gene-transferred and untreated DEB keratinocytes and DEB fibroblasts were cultured using the above methods. 10^6 fibroblasts were seeded into a collagen sponge scaffold and maintained in DMEM with 10% FCS for 3 days. In nude rats (F344/N

Jcl-rnu), the sites for transplantation were prepared by excising a 2 cm^2 area of dorsal epidermis and dermis, and then the collagen sponge (3 cm^2) containing the fibroblasts was placed into the skin wound. The confluent cultures of 10^6 keratinocytes were treated with dispase (1nU/ml: Godo Shusei, Japan), and the floating epidermal sheet placed on the collagen sponge. Preliminary experiment showed that the number of fibroblasts was almost equal to that of keratinocytes when we applied the graft to the animal. An occlusive dressing was quickly placed over the graft to hold it in position and to prevent it from drying out and then the dressing was removed after 7 days. We prepared combinations of gene-transferred keratinocytes and untreated fibroblasts, of untreated keratinocytes and gene-transferred fibroblasts, and of untreated keratinocytes and fibroblasts as control. Skin biopsies were taken from the grafted skin at various time points and subjected to routine immunohistochemical staining using the LH7.2 monoclonal antibody and ultrastructural analysis. To semiquantify *COL7A1* accumulation in basement membrane zone, we converted color images to grayscale images, and measured fluorescence intensity (arbitrary scale) in basement membrane zone at ten areas using NIH Image software. The *COL7A1* deposition index was expressed as the mean ± SD from the ten values.

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FIGURE LEGEND

Fig.1 Schematic representation of retroviral *COL7A1* expression vectors. We employed two retroviral vectors, pLIXN (Clontech) and pDON-AI (TAKARA, Japan), and full-length *COL7A1* cDNA was inserted into the retroviral vectors to generate plasmids pLI-COL and pDON-COL, respectively. A pDON (Δ) vector was created by removing the SV-40 promoter and *Neo* gene from pDON-AI and *COL7A1*cDNA constructed retroviral vector made termed pDON(Δ)-COL. These vectors harbor long terminal repeat (LTR) derived from mouse moloney leukemia virus (MMLV) and human cytomegalovirus (HCMV). The internal ribosome entry site (IRES) enables expression of two unrelated reading frames from a single transcription unit. ψ: packaging signal.

Fig. 2 Successful gene transfer of *COL7A1* into DEB fibroblasts and keratinocytes using retroviral systems. *COL7A1* was transferred into DEB fibroblasts and keratinocytes using the retroviral systems. Immunostaining revealed the transfer rates in DEB keratinocytes and fibroblasts were almost equal. The concentration of virus particles (by 10 or 50 times) using the VSV-G system improves the transfer rate. The values were represented the mean $\pm$ SD of six individual samples.

Fig.3 Corrective gene transfer of the *COL7A1* into DEB fibroblasts and keratinocytes. **(A)** Immnostaining (A) showed that the gene transfected DEB keratinocytes Kera(+) and fibroblasts Fib (+) expressed collagen VII while no expression was found in either the untreated keratinocytes Kera(-) or fibroblasts Fib(-). Nuclei were counterstained with propidium iodide. **(B)** Western blot analysis demonstrated that the amount of transgene product in culture medium was almost the same between keratinocytes and fibroblasts. (C) Southern blot analysis of genomic DNA extracted from the transduced cells showed the *COL7A1* cDNA integration copies for keratinocytes and fibroblasts were almost equal.

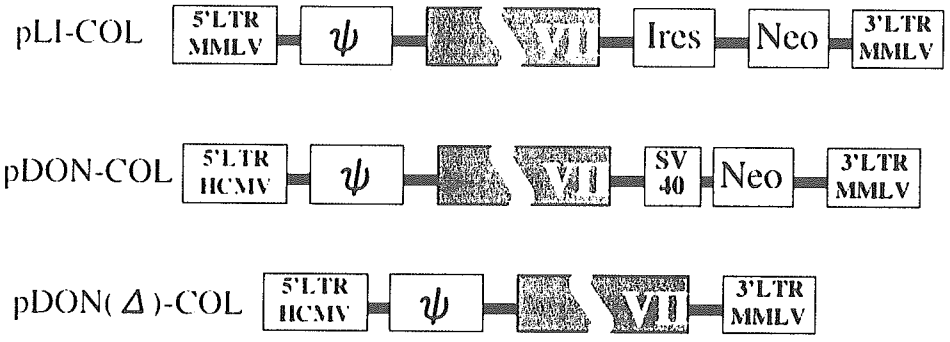
Fig 4 *In vivo COL7A1* expression in epidermis is higher than that in dermis. We measured *COL7A1* mRNA levels in the epidermis (Epi) and dermis (Derm) *in vivo*. Real time PCR demonstrated that the *COL7A1* specific signal (per RNA) of the epidermis was higher than that of the dermis. Comparison of the total RNA amounts from the epidermis and dermis in the same area of the excised normal skin showed that the amount from the dermis was higher than that from the epidermis. The *COL7A1* mRNA expression and total RNA amounts were expressed as an arbitrary scale. The values

were represented the mean + SD from 3 separate samples. *P< 0.01: significant difference.

Fig. 5. Gene-transferred fibroblasts can supply more collagen VII to the sub-basement membrane zone than gene-transferred keratinocytes. We transplanted gene-transferred DEB keratinocytes and DEB fibroblasts to nude rats wounded back skin, and observed *COL7A1* deposition by immunohistochemistry at 3, 6 and 9 weeks after transplantation. The skin graft with gene-transferred keratinocytes and untreated fibroblasts (Kera3W, Kera6W, Kera9W) started dermal-epidermal junction collagen VII deposition at 3 week and maintained it until 9 weeks. A greater accumulation of collagen VII in dermal-epidermal junction of the grafts using untreated keratinocytes and gene-transferred fibroblasts was found 3 weeks after transplantation (Fib3W). The DEB fibroblasts transfected with *COL7A1* demonstrated more dermal epidermal junction collagen VII staining than *COL7A1* transfected DEB keratinocytes / untreated fibroblast from 6 to 9 weeks (Fib6W, Fib9W). The controls DEB keratinocyte and fibroblast cell grafts (Cont3W, Cont6W, Cont9W) demonstrated no deposition. Arrowheads define the limit between dermis and epidermis.

Fig. 6 Semiquantification of COL7A1 deposition in basement membrane zone. We transplanted gene-transferred DEB keratinocytes and DEB fibroblasts to nude rats wounded back skin, and observed *COL7A1* deposition by immunohistochemistry at 3, 6 and 9 weeks after transplantation. To semiquantify *COL7A1* deposition in basement membrane zone, we measured fluorescence intensity (arbitrary scale) in basement membrane zone at ten areas at each point and the *COL7A1* deposition index was expressed as the mean ± SD from the ten values. K: the skin graft with gene-transferred keratinocytes and untreated fibroblasts, F: the graft with untreated keratinocytes and gene-transferred fibroblasts grafts, C: the graft with untreated keratinocyte and untreated fibroblasts. *P< 0.01: significant difference.

Fig. 7 Collagen VII released from gene-transferred cells forms anchoring fibrils similar to normal skin. We examined the ultrastructural formation of anchoring fibrils in the grafts. The grafts with the gene-transferred keratinocytes (Kera) or the gene-transferred fibroblasts (Fib) demonstrated cross banded, filamentous structures sometimes forming semicircular loops immediately beneath the lamina densa, corresponding to anchoring fibrils whereas we failed to identify these filamentous structures in control without *COL7A1* transfection (Cont).



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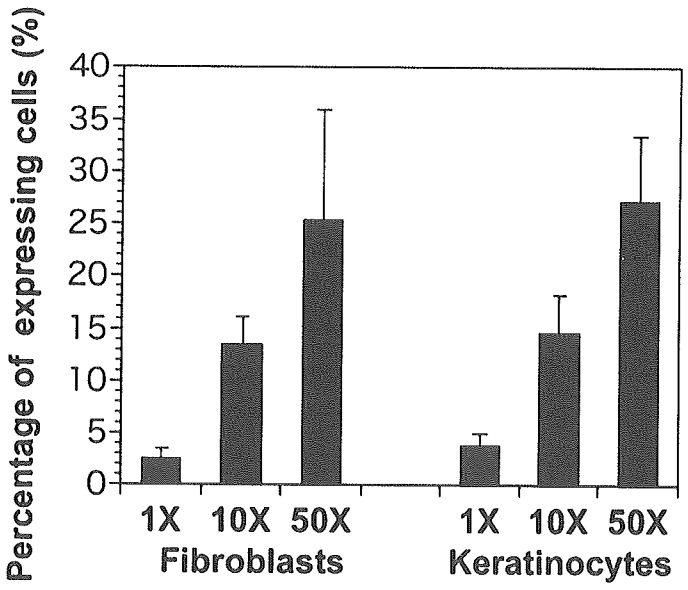

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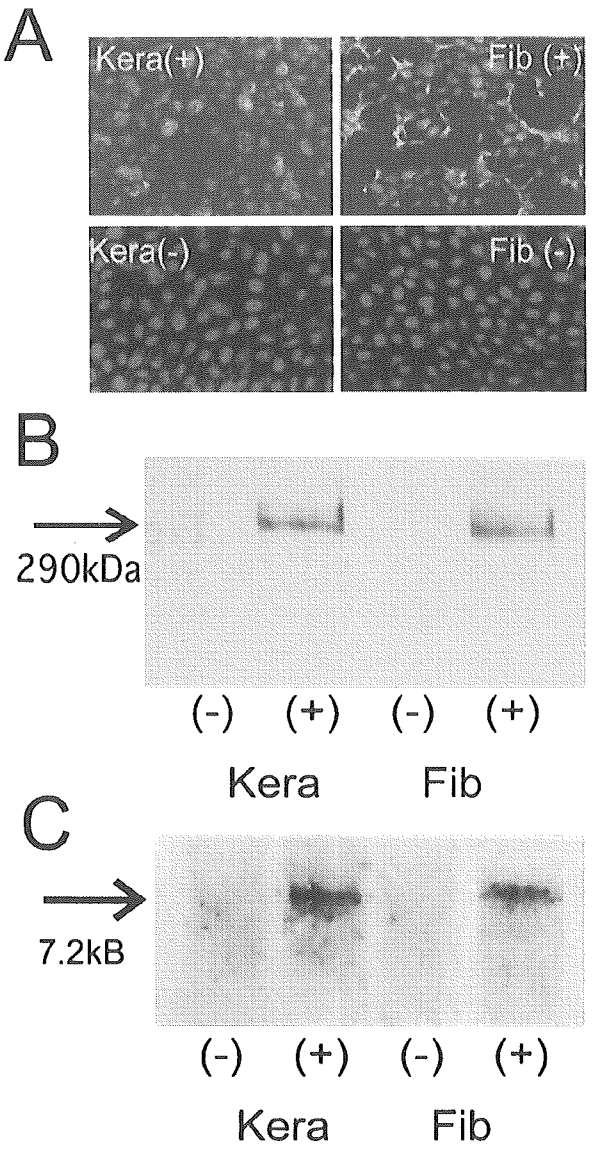

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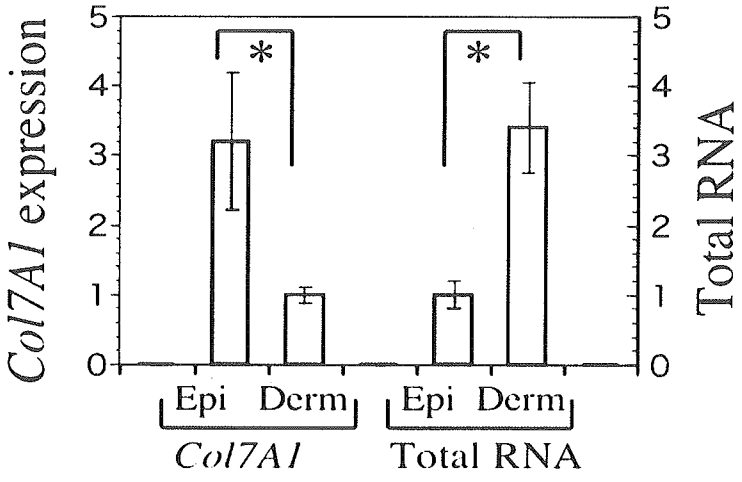

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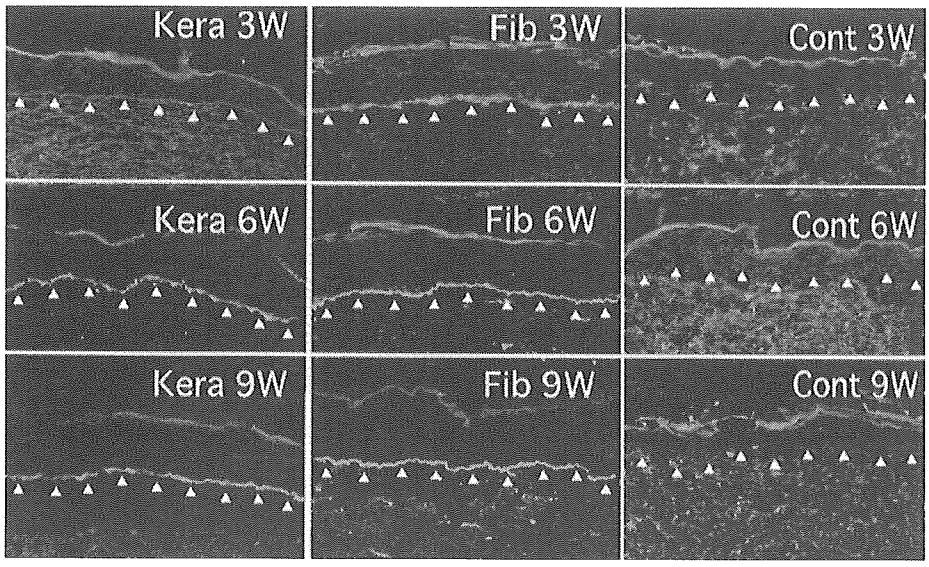

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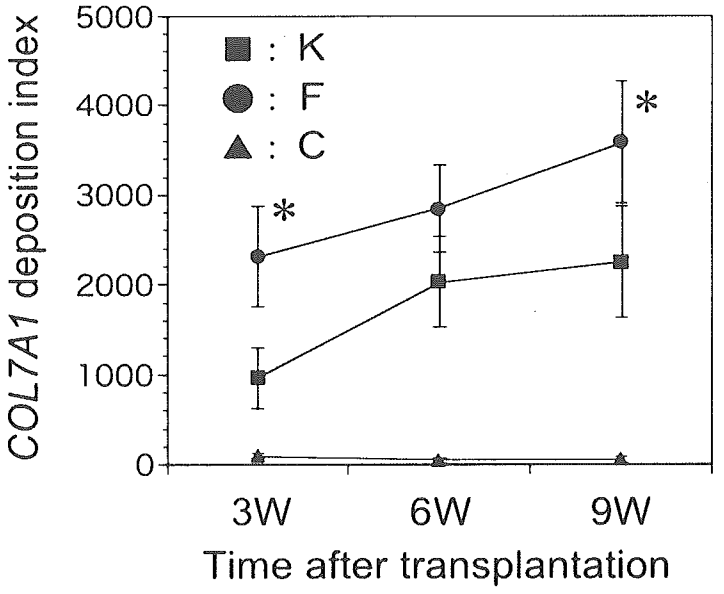

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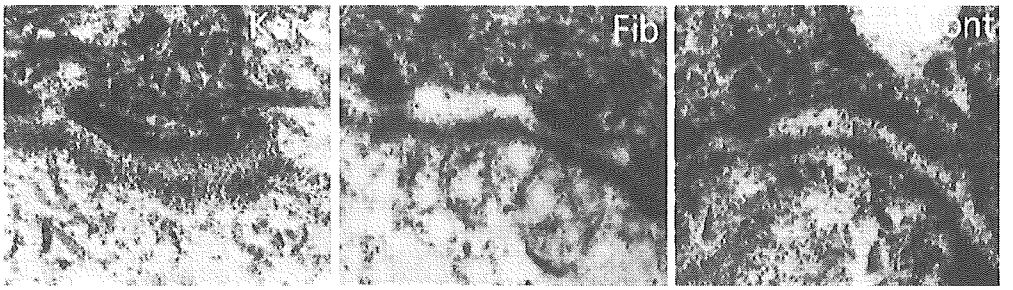

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A unique monoclonal antibody 29A stains the cytoplasm of amniotic epithelia and cutaneous basement membrane

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KEYWORDS
Epithelia;
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Summary

Background: The basic function of epithelia is to provide a boundary between tissue and its external environment, and is achieved by a wide variety of components including extracellular molecules. Multiple monoclonal antibodies raised against epithelial antigens have helped identify a range of distinct, novel protein epitopes.
Object: In this study, we raised a monoclonal antibody to detect a novel epithelial molecular component.
Methods: We have produced a mouse monoclonal antibody using normal human amniotic tissue as an immunogen. The monoclonal antibody was subsequently immunohistochemically screened, and the target antigen was cloned using an immunoscreening method.
Result: In the course of the screening, we identified unique antibody staining patterns within the cytoplasm of a subset of amniotic cells at intervals within the normal placental epithelia. By immunoscreening, we identified this candidate gene as

Abbreviations: BM, basement membrane; FITC, fluorescein isothiocyanate; IF, immunofluorescence; LR, laminin receptor; PBS, phosphate buffered saline; TBS, tris-buffered saline; TRITC, tetramethylrhodamine isothiocyanate

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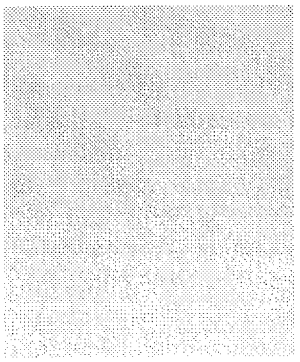

laminin receptor (LR). By dot blot analysis, this antibody reacted with recombinant LR. The same localization of the antigen and LR was proved by a double staining immunofluorescence test in the placenta. This monoclonal antibody unexpectedly demonstrated linear staining within the dermal–epidermal junction of normal human skin but failed to react within the keratinocyte cytoplasm.

Conclusion: We have produced and characterized a novel monoclonal antibody 29A that recognizes an LR-related molecule, which demonstrated a unique staining pattern. This monoclonal antibody might be a useful tool for further investigations into the epithelial tissues and the cutaneous basement membrane (BM).

1. Introduction

The evolution of novel developmental mechanisms in epithelia allows animals to live in diverse ecological environments. This enables epithelia to have many functions as a protective barrier, play roles in the immune or endocrine systems. A wide variety of molecules participate in this maintenance and protection of epithelia. Many organs including the placenta and skin are thought to be related and possess similar epithelial structures, and it is known that organs can share multiple common molecules. Advances in immunology and molecular biologic methods have enabled the production of novel monoclonal antibodies and allowed us to clone the associated genes using these antibodies. Previous studies have identified several novel BM proteins which are expressed in both the placenta and skin [1–3].

The basement membrane (BM) of many epithelia is a highly specialized extracellular matrix that serves many functions, of which the most obvious is the attachment of the epithelia to underlying mesenchyme. It also influences such tissue behavior such as the growth, migration, and differentiation [4,5] of nearby cells. Moreover, degradation of this matrix is associated with tumor cell invasion and cancer progression [6]. Previous studies have showed that extracellular proteins such as type IV collagen, laminins, fibronectin, nidogen, and heparin sulfate proteoglycans appear to be BM components including skin and placenta BM [1–3,7,8].

Novel as yet unidentified molecules which play a crucial role in normal and abnormal conditions are though to be expressed in the epithelial and cutaneous BMs, and identification of such molecules may lead to significant progress in epithelial biology and furthermore in dermatological research. In this report, we have produced a monoclonal antibody using normal human amniotic epithelia as an immunogen. Interestingly, the resulting unique monoclonal antibody 29A stained the cytoplasm of amniotic epithelial in the placenta but was restricted to the basement membrane zone of the skin.

2. Materials and methods

2.1. Monoclonal antibody production

A monoclonal antibody was produced against normal human placental amnion using a technique previously described with some modifications [9]. Amnion was separated from the underlying connective tissue, and cut using scissors. Ten grams of the tissue was homogenized with a Polytron (Kinematica AG) in 20 ml of 40 mM phosphate buffer (pH 7.3) containing 150 mM NaCl and protease inhibitors, 1 mM *p*-amidinophenyl methanesulfonyl fluoride hydrochloride (Wako Pure Chemicals), 10 μg/ml leupeptin and pepstatin (Peptide Institute Inc.). After centrifugation at 10,000 × *g* for 20 min, the supernatant was again centrifuged at 100,000 × *g* for 60 min. The whole process was performed at 4 °C. The precipitate was suspended in 1 ml of the above buffer and immediately used for mouse immunization, and the rest stored as stock at −80 °C for further immunizations.

A BALB/c mouse was injected subcutaneously with 100 μg protein after mixing with adjuvant (TiterMax Gold, Cytrx Corp.) once a week for 3 weeks. On the third day after the last intraperitoneal immunization with 100 μg protein in buffer, spleen cells of the immunized mouse were fused with X63Ag8.653 mouse myeloma cells, using 50% polyethylene glycol 1500 (BDH Chemicals). The fused cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 15% fetal calf serum and 10% BM-condimed H1 (Boehringer) including HAT (Flow Co.) in 96-well plates. An indirect immunofluorescence (IF) method using frozen sections of normal human placental amnion was used for screening the supernatants from the growing cells. Every 3 weeks, the hybrid cell line of interest was cloned twice and injected to mice intraperitoneally previously treated with pristane (2,6,10,14-tetramethylpentadecane, Tokyo Kasei Co.).

2.2. Antibodies used in this study

The monoclonal antibody of interest, termed 29A, was purified from ascites fluids of the SCID mice by HiTrap IgM Purification HP (Amersham) according to the manufacturer's instructions, as the 29A antibody was of the IgM subclass. A monoclonal antibody MLuC5 against laminin receptor (LR) was from Quartett (Berlin, Germany). The polyclonal anti-LR antibody FD4818 derived from rabbit raised against the polypeptide corresponding to amino acids 3–15 of LR [10] was kindly provided by Dr. Seiji Takashima (Osaka University Graduate School of Medicine, Osaka, Japan). Another polyclonal antibody against the polypeptide corresponding to amino acids 279–295 of LR was produced by rabbit immunizations. The following commercial secondary antibodies were obtained: fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM antibody from Biosource International, tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-rabbit IgG antibody from Dako, and peroxidase-conjugated second antibodies from Jackson ImmunoResearch Laboratories Inc.

2.3. Immunofluorescence staining

Immunofluorescence staining of 5 μm cryosections of normal human placental amnion and normal human skin were carried out using standard methods. Briefly, after being washed with a PBS/BSA mixture, the tissues were incubated with the first antibodies overnight at 4 °C, and then incubated with FITC- or TRITC-conjugated second antibodies for 1 h at room temperature. Following extensive washing, fluorescence was observed with confocal laser scanning microscope (Olympus Fluoview FV300, Tokyo, Japan).

2.4. Western blotting

Normal human amnion was extracted with Laemmli sample buffers with or without 2-mercaptoethanol. Protein samples were separated on SDS-PAGE with polyacrylamide gels, and then transferred to nitorocellulose membranes. After the membranes were incubated for 1 h in a blocking buffer of 2% fat-free milk in 50 mM Tris–HCl, pH 7.4, 150 mM NaCl (tris-buffered saline; TBS). The first antibody was applied overnight at room temperature. After washes with 0.05% Tween-20 in TBS, the membranes were incubated for 2 h with peroxidase-conjugated second antibody diluted 1:1000 in blocking buffer. After the membranes were washed, the signals were detected with chemiluminescence (Amersham Inc., Amersham, UK).

2.5. Immunoscreening

Normal human keratinocyte cDNA library (Clontech) was screened with the 29A antibody. Briefly, single *E. coli* Y1090 colony was grown at 37 °C in 10 ml LB medium (10 g/l trypton, 5 g/l bacto-yeast extract, 10 g/l NaCl, and 1 mM NaOH) supplemented with 10 mM $MgSO_4$, 0.4% maltose, and 50 μg/ml ampicillin until the OD_{660} reached 0.3. The cells were harvested by centrifugation and resuspended in 10 mM $MgSO_4$. Phage solution containing approximately 10^4 pfu phages in SM buffer (0.1 M NaCl, 50 mM Tris–HCl, pH 7.5, 10 mM $MgSO_4$, 0.01% gelatin) was incubated with 200 μl of Y1090 cell suspension at 37 °C for 20 min, and then plated on the 100 mm LB–agar plates containing 50 μg/ml ampicillin with an LB soft top agarose. After the plates were incubated at 42 °C for 4 h, nitrocellulose filters presoaked in 10 mM isopropyl-B-thiogalactopyranoside were overlaid on the plates and incubated for 4 h at 37 °C. After being washed and blocked with milk proteins, the filters were incubated with the 29A antibody. The positive signal was detected using diaminobenzidine. Positive plaques were picked up on the original plate, and eluted in SM buffer containing chloroform. After repeated isolation and screening of these clones, candidate clones were obtained. As a control, normal mouse IgM (Sigma) was used instead of the 29A antibody.

2.6. Sequence analysis

The insert cDNA of positive phage clones were sequenced on a Perkin-Elmer 3100 sequencer using the forward primer; 5′-GAA GGC ACA TGG CTG AAT ATC GAC GGT TTC-3′ and reverse primers; 3′-CAG CGA TGG TAA TGG TCA ACC AGA CCA CAG-5′, and the sequence results were searched on the BLAST site (http://www.ncbi.nlm.nih.gov/BLAST/).

2.7. Recombinant LR protein generated in the baculovirus

Recombinant LR was expressed in baculovirus-infected insect cells using Bac-to-Bac baculovirus expression system (Invitrogen, Tokyo, Japan). A full-length cDNA encoding the human LR was cloned by RT-PCR using mRNA isolated from normal human skin into pFastBac plasmid (Invitrogen). For construction of the donor plasmid, a pair of the gene-specific primers was designed; 5′-TAG GAA TTC TCA CAA TGT CCG GAG CCC TT-3′ (spacer and *Eco*R I site are included) and 5′-CTA CTC GAG TTA AGA CCA GTC AGT GGT TG-3′ (spacer and *Xho* I site are included). The PCR product was digested with *Eco*R I and *Xho* I (Takara Bio, Otsu, Japan), separated by agarose-gel

electrophoresis and purified from the gel with QIAquick Gel Extraction Kit (Qiagen). The purified DNA was ligated into a pFastBac plasmid predigested with *Eco*R I and *Xho* I, and then sequenced. The expression cassette of the plasmid was incorporated into the baculovirus genome (bacmid DNA) in DH10Bac competent cells (Invitrogen) by using Tn7 site-specific transposition according to the manufacturer's instruction. The resulting recombinant bacmid DNA was purified with QIAprep Spin Miniprep Kit (Qiagen) and transfected into *Spodoptera frugiperda* Sf9 cell (Invitrogen) using CellFECTIN (Invitrogen). After incubation at 27 °C for 72 h in Sf-900 II SFM medium (Invitrogen), the resultant recombinant baculovirus was harvested. A control baculovirus was prepared in the same way by using pFastBac. Viral titers were determined with a conventional viral plaque assay.

10^7 Sf9 cells were seeded in a 150 mm dish and infected with above recombinant baculoviruses with a multiplicity of infection of 10. After incubation in serum-free Sf-900 II SFM medium at 27 °C for 96 h, the generated recombinant LR protein was collected from the Sf9 cells. The cell pellets were homogenized in ice-cold 50 mM Tris–HCl buffer (pH 7.2) containing protease inhibitors and centrifuged at 15,000 × *g* for 30 min. Then the samples were concentrated with Amicon Ultra YM-30 and stored at −80 °C until use.

2.8. Dot binding assays

The extracted recombinant LR protein was eluted in TBS buffer and dotted onto nitrocellulose membranes using a vacuum manifold [11]. After blocking with 2% milk protein in TBS, the filters were incubated with the first antibody followed by peroxidase-conjugated second antibody. Bound antibodies were revealed using chemiluminescence.

3. Results

3.1. Isolation of the 29A antibody

The monoclonal antibody, which one of the single-cloned hybridoma cells secreted, the 29A antibody, intermittently stained the cytoplasm of amniotic cells using immunofluorescence staining (Fig. 1). We were interested in this unique staining pattern; therefore, we analyzed the amniotic protein extracts by western blotting. However, no positive band was shown under both reduced and non-reducing conditions (data not shown). These results indicated that the 29A antibody probably recognized a conformational epitope on a low level-expressed cytoplasmic protein in amniotic cells.

Fig. 1 Isolation of the 29A antibody. The monoclonal 29A antibody intermittently stained the cytoplasm of normal human amniotic cells using an indirect immunofluorescence staining technique. (Green): FITC-conjugated anti-mouse IgM antibody; (Red): propidium iodide, nuclear stain.

3.2. Immunoscreening using the 29A antibody

For the purpose of cloning of the 29A antigen, we used a keratinocyte cDNA expression library and immunoscreened it. Recombinant phages were plated with *E. coli* Y1090, and plaques were immunoscreened with the 29A antibody. Upon the first screening of 8×10^6 clones, we obtained three candidate clones after repeated screening and isolations. Each cDNA insertion was sequenced and found to contain one continuous open reading frame. BLAST research of these sequences revealed identical cDNA fragments of laminin receptor (Fig. 2).

3.3. The 29A antibody reacted with recombinant LR

To confirm that the 29A antibody recognizes the LR protein, full-length recombinant LR protein was expressed in insect cells. The size of the generated recombinant LR protein was 37 kDa under non-reducing conditions. The 29A antibody reacted with the

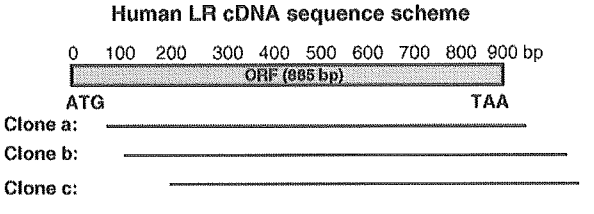

Fig. 2 The schema selecting positive clones for immunoscreening. Upon the first screening of 8×10^6 clones, finally three candidate clones (a–c) were obtained. Each cDNA insertion contained one continuous open reading frame, and their sequences revealed identical cDNA fragments encoding for the laminin receptor (LR).

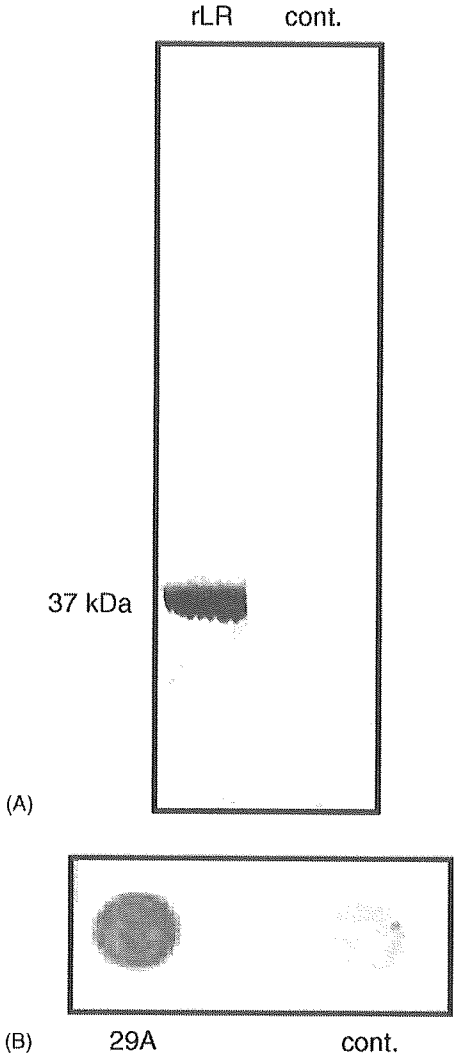

Fig. 3 Reactivity of the 29A antibody with human LR. Full-length recombinant human LR was expressed in insect cells. (A) Western blotting. Generated recombinant LR (rLR) was present in a 37 kDa form under non-reducing condition. This was confirmed using the anti-LR antibody. (B) Dot binding assay. The 29A antibody reacted with recombinant LR. Cont., control baculovirus-infected insect cell lysate.

recombinant LR by a native dot binding assay (Fig. 3).

3.4. The 29A antigen and LR co-localized in the amnion

The presence of LR in the amnion was examined using the polyclonal anti-LR antibody. It stained the partial cytoplasm of amniotic cells. Moreover, double staining with the 29A antibody and the anti-LR antibody revealed co-localization of the 29A antigen and LR in the amnion (Fig. 4).

3.5. The 29A antibody reacted to the basement membrane zone in the normal human skin

We examined human skin tissue with the 29A antibody. Unexpectedly the 29A antibody reacted in a linear fashion along the BM of normal human skin by IF staining (Fig. 5a). However, the cytoplasm of keratinocytes was not stained. Conversely, we investigated the localization of LR in the skin using MLuC5 monoclonal antibody. In normal human skin, MLuC5 partially stained keratinocyte cell membranes but only very weakly along the BM (Fig. 5b).

4. Discussion

In this study, we have produced and characterized a novel monoclonal 29A antibody against human placental amnion. The 29A antibody stained the cytoplasm of selected amniotic cells at intervals in a unique manner. However, the 29A antigen was not detected by western blot analysis, therefore it is considered that the 29A probably recognizes a conformationally sensitive epitope. We cloned this antigen using an immunoscreening method, and identified the candidate gene of interest as a laminin receptor. We confirmed that the 29A antibody actually reacted with the recombinant LR protein by dot binding assay. In addition, we proved that the 29A antigen showed the same staining pattern as LR in the placental amnion by double staining IF microscopy analysis. We therefore concluded that the 29A antibody recognized the LR-related protein. To further confirm these findings, we have repeatedly tried to isolate the 29A antigen by affinity chromatography and immunoprecipitation using amniotic extracts. However, it was impossible to isolate this specific protein by the affinity chromatography or immunoprecipitation methods using this 29A antibody (data not shown).

LR was originally identified as a 67 kDa protein that binds to laminin 1 and is expressed in a colon cancer cell line [12]. It seems that the evidence for LR being a receptor was based on the finding that it was retained in a laminin column [13], and in addition to laminin, the known ligands for LR are type IV collagen and fibronectin [14,15]. LR cDNA encodes for only 295 aa (37 kDa), and it is still unknown if cells make the 67 kDa LR through the addition of extra glycoprotein moieties or via other post-translational modifications. Castronovo et al. reported that transfection of the full-length LR cDNA into COS-7 cells induced an increase in the synthesis of the 37 kDa form but not of the 67 kDa form [16]. In