H　知的財産の出願・登録状況

特になし。

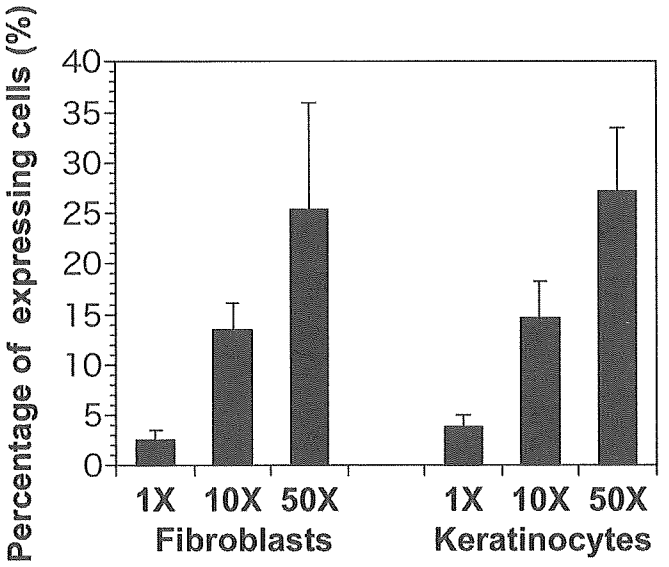

図１　Hallopeau-Siemens 劣性優性栄養障害型患者の線維芽細胞と表皮細胞へのVII型コラーゲン遺伝子の導入。ウイルス粒子を種々の濃度に濃縮して細胞に添加してある。パーセンテージはVII型コラーゲン発現細胞の割合。

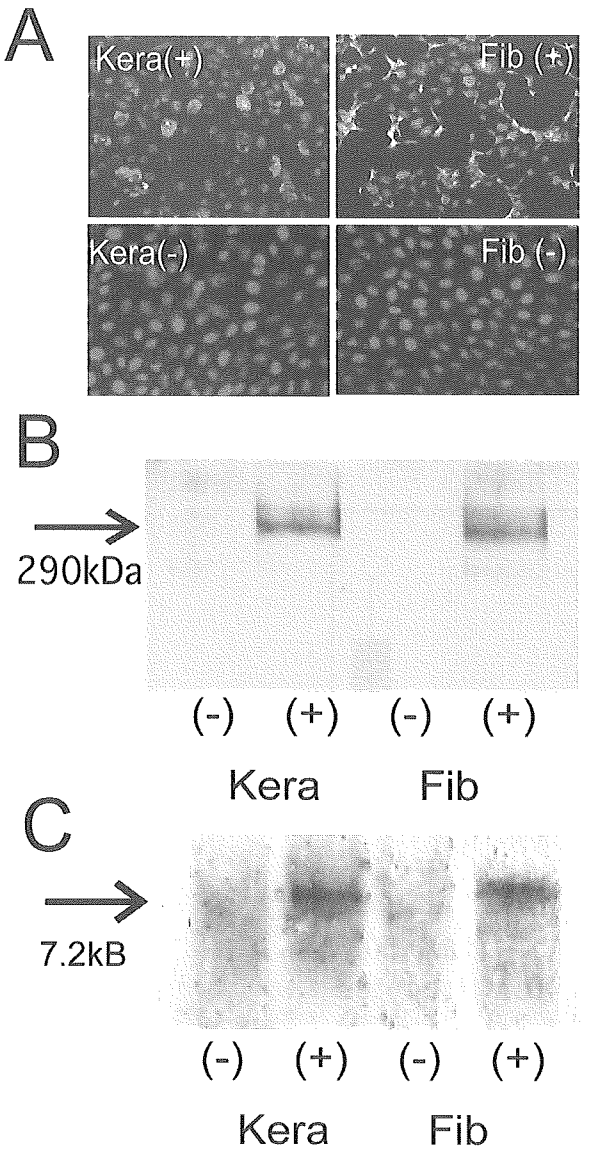

図２　Hallopeau-Siemens 劣性優性栄養障害型患者の線維芽細胞と表皮細胞へのVII型コラーゲン遺伝子の導入。A）培養細胞をVII型コラーゲンの抗体にて染色。B）培養上清のイムノブロット。遺伝子導入表皮細胞と線維芽細胞で等量のVII型コラーゲンが発現されている。C）サザンブロット所見。遺伝子導入表皮細胞と線維芽細胞で等量のVII型コラーゲン遺伝子が挿入されている。

厚生労働科学研究費補助金
（難治性疾患克服研究事業）
分担研究報告書

造血幹細胞由来表皮細胞の遊走機序に関する研究

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研究要旨

これまで再生医学領域の研究により、種々の体細胞成分が骨髄幹細胞から分化可能であることが明らかとなった。骨髄由来幹細胞が表皮細胞に分化しうることも予想されている。よって、皮膚基底膜構造タンパク欠損疾患である表皮水疱症に対して、同種骨髄移植によりドナー骨髄由来表皮細胞を分化させ、欠損構造タンパクを産生させることで根治させることを目的として検討を行った。本年度の研究で、これまでの検討で確認した骨髄由来表皮細胞の割合を増加させるために、骨髄由来表皮細胞特異的遊走因子を同定した。さらに実際に、この因子が、生体内における骨髄由来表皮細胞の割合を増加させることも明らかにした。加えて、骨髄由来表皮細胞増加の創傷治癒への影響も検討した。

A　目的

これまで再生医学領域の研究により、種々の体細胞成分が骨髄幹細胞から分化可能であることが明らかとなった。骨髄幹細胞から表皮細胞への分化の可能性も示唆されているが、なお確定はえられていない。一方先天的酵素欠損症（Hunter 症候群など）は同種骨髄移植によりドナー由来細胞から欠損酵素を供給する治療が試みられ臨床的にも有用であることが証明されている。しかしながら同種骨髄移植を用いた、構造タンパク欠損症に対する治療法の研究はなされておらず、定説もない。そこで、国の稀少難治性疾患に指定されている皮膚基底膜構造タンパク欠損疾患（基底膜構造タンパク欠損により、微細な外力でびらん、潰瘍をきたす重症難知性疾患）である表皮水疱症に対して、同種骨髄移植により構造タンパク欠損症を根治させることを目的として、検討を行った。

B　研究方法

①骨髄由来表皮細胞特異的遊走因子の同定：

皮膚再生の現象の場である、創傷治癒過程の皮膚における細胞遊走因子のスクリーニングを行い、候補となる遊走因子を数種同定した。同定したそれぞれの遊走因子において、骨髄幹細胞への遊走惹起能を in vitro で検討した。さらに皮膚創傷部位への骨髄由来表皮細胞遊走への、それぞれの遊走因子の影響を検討した。

②骨髄由来表皮細胞増加の創傷治癒への影響の検討：

骨髄由来表皮細胞増加の、皮膚再生過程に対する寄与を解析するため、上記

同定遊走因子を皮膚創傷部位に投与することによる、創傷治癒への影響を検討した。

C　研究結果

①骨髄由来表皮細胞特異的遊走因子の同定：

現在までの検討で、皮膚創傷部位に特異的に発現し、かつ骨髄幹細胞の遊走を特異的に誘導する、骨髄由来表皮細胞特異的遊走因子を同定した。この同定因子は生体内においても骨髄由来表皮細胞の数を増加させた。

②骨髄由来表皮細胞増加の創傷治癒への影響の検討：

上記同定遊走因子を皮膚創傷部位に投与することにより、創傷治癒が有意に促進した。

D　考察

本年度の研究で、骨髄由来表皮細胞の割合を増加させるために、骨髄由来表皮細胞特異的遊走因子を同定した。さらに実際に、この因子が、生体内における骨髄由来表皮細胞の割合を増加させることも明らかにした。加えて、骨髄由来表皮細胞増加の創傷治癒への影響も検討し、創処治癒を促進させることを明らかにした。

今後は現在までの成果を、臨床応用に近づけるべく、表皮構造タンパク欠損マウス（VII 型コラーゲンノックアウトマウス）への正常マウスから骨髄移植療法を行う。

E　結論

今回の研究で、骨髄由来表皮細胞特異的遊走因子を同定した。加えて、骨髄由来表皮細胞増加の創傷治癒への影響も検討し、創処治癒を促進させることを明らかにした。

F　健康危険情報

特になし。

G　研究発表

特になし。

H　知的財産の出願・登録状況

出願番号：JP2004/369272、「骨髄幹細胞移植治療用医薬」
発明者：清水　宏、阿部理一郎、猪熊大輔
出願人：北海道大学

厚生労働科学研究費補助金
（難治性疾患克服研究事業）
分担研究報告書

表皮の幹細胞と RNA 結合蛋白 Musashi
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研究要旨

Musashi family の RNA-binding protein は、神経幹細胞の維持と神経の幹細胞から神経細胞へ分化する際の非対称性分裂に関連していることが知られている。今回、RT-PCR と免疫ブロットによって、Musashi1 と Musashi2 の mRNA と蛋白は培養マウス表皮細胞に発現していることが確認されたが、ヒト表皮細胞では、Musashi1 のみ、mRNA と蛋白が認められる。免疫組織学的に、Musashi1、Musashi2 は、胎生 14.5 日から成体までのマウス表皮と毛包に発現していた。マウス毛周期においては、成長期早期では、Musashi１、Musashi2 はバルジと２次毛芽に発現しており、成長期後期では、内毛根鞘、とりわけ、毛包中位の内毛根鞘の細胞に限局存在していた。ヒト皮膚では、Musashi1 は胎生期毛包の細胞に発現していたが、成人の毛包では、発現は認められなかった。これらのデータより Musashi は毛包形成においては、幹細胞からの早期の非対称性分裂に関与するのみでなく、毛包の発生と毛周期の成長期において内毛根鞘の分化に関与していると考えられる。

A　目的

表皮水疱症の治療として、上皮細胞の幹細胞への遺伝子導入が期待されている。上皮細胞の幹細胞は毛包のバルジ領域に存在し、皮膚を構成する種々の上皮細胞へ分化する能力を有している。系統発生学上、比較的保たれている Musashi family の RNA-binding protein は、神経幹細胞の維持と神経の幹細胞から神経細胞へ分化する際の非対称性分裂に関連していることが知られている。哺乳類の Musashi 蛋白は、消化管や乳腺をはじめとして上皮系の種々の幹細胞および幹細胞からの早期由来細胞に発現している。これらの事実から、Musashi は毛包上皮の幹細胞および幹細胞に由来する未分化な細胞にも発現していると考えられた。
そこで今回の研究では、（１）マウスおよびヒトにおいて、胎生期の表皮、毛包上皮の発生段階で Musashi がどのように働いているか、（２）マウスおよびヒト皮膚の毛周期の形成に Musashi がどのように関っているか、を明らかにすることを目的とした。

B　研究方法

（１）培養表皮細胞での Musashi の発現の検討
RT-PCR と免疫ブロット法を用いて、Musashi1 と Musashi2 の mRNA と蛋白の発現を検討した。対象とする細胞は、培養マウス表皮細胞（C57BL/6J 由来）と培

養ヒト表皮細胞　(Cambrex Bio Science, Walkersville, MD, USA) であった。

（２）胎生期皮膚における Musashi の分布の検討

抗 Musashi-1 抗体 (Msi-1)、および、抗 Musashi-2 抗体 (Msi-2) を用いて免疫染色を行なった。対象組織としては、C57BL/6J マウス (Clea, Japan Inc., Tokyo, Japan) 胎児皮膚、および、ヒト胎児皮膚 (胎生４９日―１６３日) を用いた。

（３）毛周期における Musashi の局在の検討

抗 Musashi-1 抗体 (Msi-1)、および、抗 Musashi-2 抗体 (Msi-2) を用いて新生児マウスの皮膚の免疫染色を行った。対象とした組織は、C57BL/6J マウス (Clea, Japan Inc., Tokyo, Japan)新生児皮膚を用いた（１日齢、４日齢、８日齢、１１日齢、１８日齢、２１日齢)。

C　研究結果

RT-PCR と免疫ブロットによって、Musashi1 と Musashi2 の mRNA と蛋白は培養マウス表皮細胞に発現していることが確認されたが、ヒト表皮細胞では、Musashi1 のみ、mRNA と蛋白が認められる。マウスでは、免疫組織学的に、Musashi1、Musashi2 は、胎生 14.5 日から成体までの表皮と毛包に発現していた。Musashi と毛周期の関連について調べると、成長期早期では、Musashi、Musashi2 はバルジと２次毛芽に発現しており、成長期後期では、内毛根鞘、とりわけ、毛包中位の内毛根鞘の細胞に限局存在していた。ヒト皮膚では、Musashi1 は胎生期毛包の細胞に発現していたが、成人の毛包では、発現は認められなかった。

D　考察

RNA-binding protein である Musashi family は、神経幹細胞の維持と神経の幹細胞から神経細胞へ分化する際の非対称性分裂に関連している。ヒトを含めて哺乳類の Musashi 蛋白は、消化管や乳腺等の種々の上皮組織の幹細胞および幹細胞からの早期由来細胞に発現している。今回の我々の研究の結果から、マウス表皮細胞は Musashi1 と Musashi2 発現しているが、ヒト表皮細胞は Musashi1 のみを発現していることが確認された。さらに、胎生期マウスでは、Musashi1、Musashi2 は、胎生 14.5 日から成体までの表皮と毛包に発現していた。毛周期の各時期の Musashi の発現については、成長期早期では、Musashi１、Musashi2 はバルジと２次毛芽に発現しており、成長期後期では、内毛根鞘、とりわけ、毛包中位の内毛根鞘の細胞に限局して発現していた。他の組織における Musashi の発現様式から、表皮、毛包上皮においても、Musashi は幹細胞および幹細胞に由来する未分化な細胞に発現していると予想されたが、今回の結果は、Musashi は毛包中位の内毛根鞘の細胞にも特異的に発現し、機能していることを示していた。

E　結論

Musashi は毛包形成においては、幹細胞からの早期の非対称性分裂に関与するのみでなく、毛包の発生と毛周期の成長期において内毛根鞘の分化に関与していると考えられる。

F　健康危険情報

特になし

G　研究発表

論文１）Sugiyama-Nakagiri Y, Akiyama M, Shibata S, Okano H, Shimizu H. Expression of RNA-binding protein Musashi in hair follicle development and hair cycle progression. Am J Pathol (in press).

H　知的財産の出願・登録状況

特になし。

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表（雑誌）

発表者氏名	論文タイトル名	発表雑誌	巻号	ページ	出版年
Sawamura D, Niizeki H, Miyagawa S, Shinkuma S, Shimizu H	A novel indel *COL7A1* mutation 8068del17ins-GA causes dominant dystrophic epidermolysis bullosa.	**Br J Dermatol**			in press
Goto M, Sawamura D, Ito K, Abe M, Nishie W, Sakai K, Shibaki A, Akiyama M, Shimizu H	Fibroblasts are more potential target cells than keratinocytes for *COL7A1* gene therapy of dystrophic epidermolysis bullosa.	**J Invest Dermatol**			in press
Ota M, Sawamura D, Yokota Y, Ueda M, Horiguchi Y, Kodama K, Goto M, Shimizu H	A unique monoclonal antibody 29A staining the cytoplasm of amniotic epithelial in the placenta and cutaneous basement membrane zone.	**J Dermatol Sci**			in press
Onozuka T, Sawamura D, Goto M, Yokota K, Shimizu H	Possible role of endoplasmic reticulum stress in the pathogenesis of Darier's disease.	**J Dermatol Sci**			in press
Sawamura D, Sato-Matsumura K, Shibata S, Tashiro A, Frue M, Goto M, Akiyama M, Nakamura H, Shimizu H	*COL7A1* mutation G2037E causes epidermal retention of type VII collagen.	**J Hum Genet**			in press
Yasukawa K, Sawamura D, Goto M, Nakamura H, Jung SY, Kim SC, Shimizu H	Epidermolysis bullosa simplex in orientals: genetic studies in 19 cases.	**Br J Dermatol**			in press
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Suzuki N, Suzuki T, Inagaki K, Ito S, Kono M, Fukai K, Takama H, Sato K, Ishikawa O, Abe M, Shimizu H, Kawai M, Horikawa T, Yoshida K, Matsumoto K, Terui T, Tsujioka K, Tomita Y	Mutation analysis of the ADAR1 gene in dyschromatosis symmetrica hereditaria and genetic differentiation from both dyschromatosis universalis hereditaria and acropigmentatio reticularis.	**J Invest Dermatol**	124	1186-1192	2005
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Zhao Y, Shimizu T, Nishihira J, Koyama Y, Kushibiki T, Honda A, Watanabe H, Abe R, Tabata Y, Shimizu H	Tissue regeneration using macrophage migration inhibitory factor-impregnated gelatin microbeads in cutaneous wounds.	**Am J Pathol**	167	1519-1529	2005

IV. 研究成果の刊行物・別刷

DOI 10.1111/j.1365-2133.2006.07148.x

A novel indel *COL7A1* mutation 8068del17insGA causes dominant dystrophic epidermolysis bullosa

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Key words
COL7A1, epidermolysis bullosa pruriginosa, pruritus, type VII collagen

Conflicts of interest
None declared.

Summary

We report a 21-year-old Japanese man with skin fragility and severe itching, who showed multiple erosions, blisters, scarring, pruriginous papules and lichenoid plaques. Blister formation was demonstrated beneath the lamina densa corresponding to dystrophic epidermolysis bullosa (EB), and there were multiple affected family members, indicating a dominant type of inheritance. Mutation analysis detected a novel indel (insertion/deletion) *COL7A1* mutation 8068del17insGA. This is the first indel mutation identified as a cause of the dominant dystrophic EB (DDEB) phenotype. Nine other family members were affected, but they did not show severe pruritus or prurigo. The proband had elevated IgE, and we diagnosed him as having DDEB with atopic dermatitis. This case highlights the difficulty in differential diagnosis of EB pruriginosa and EB associated with atopic dermatitis.

Dystrophic epidermolysis bullosa (DEB) is characterized clinically by mucocutaneous blistering in response to minor trauma, followed by scarring and nail dystrophy, and shows blister formation beneath the epidermal lamina densa at the level of the anchoring fibrils. It occurs in autosomal dominant (DDEB) and recessive (RDEB) forms, each form comprising different subtypes with variable clinical presentation and severity.[1] Based on the genetic background, both DDEB and RDEB are caused by mutations in the *COL7A1* gene encoding type VII collagen, the major component of anchoring fibrils.[1] Examination of the *COL7A1* mutations in DEB has so far disclosed certain genotype–phenotype correlations.[2] Patients with DDEB usually harbour glycine substitution mutations within the collagenous domain on one *COL7A1* allele, leading to disruptions in anchoring fibril assembly and relatively mild clinical features. This study describes the first indel (insertion/deletion) mutation[3] in the *COL7A1* gene that causes a clinical DDEB phenotype.

Case and methods

The proband was a 21-year-old Japanese man with blistering mainly on his knees and elbows after minor trauma since infancy. The healing lesions left scars and milia. Although the frequency of blister formation gradually diminished with age, severe pruritus started at around the age of 13 years. Afterwards, excoriations and skin lesions appeared over the whole body. There were nine other affected family members with skin fragility, but they failed to show any severe pruritus (Fig. 1a). Examination revealed multiple erosions, scarring papules and lichenoid plaques on the proband's trunk and extremities, and some tense blisters on his legs (Fig. 1b,c). Laboratory tests showed IgE levels elevated to 149 667 U mL^{-1}. Skin biopsies from the proband revealed subepidermal blister formation. Further immunostaining with the monoclonal antibody LH7:2 recognizing type VII collagen demonstrated bright linear staining along the basement membrane zone and on the roof of the blister (Fig. 2a), and electron microscopy showed tissue separation beneath the lamina densa within the dermal sublamina densa region (Fig. 2b). The combination of topical betamethasone and oral antihistamines was moderately effective for the pruritus and prurigo.

Total genomic DNA from the proband was examined to detect the precise *COL7A1* mutational defects. The *COL7A1* DNA segments including all 118 exons, all intron–exon borders and the promoter region were amplified by polymerase chain reaction (PCR) using pairs of oligonucleotide primers synthesized on the basis of intronic sequences according to Christiano et al.[4] The PCR products were subjected to automated nucleotide sequencing using an ABI 3100 Genetic Analyzer (Perkin Elmer, Warrington, U.K.).

This study was approved by the Ethical Committees at Hokkaido University Graduate School of Medicine and Nara Medical University School of Medicine. Informed consent was obtained from the proband.

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COLOUR FIG.

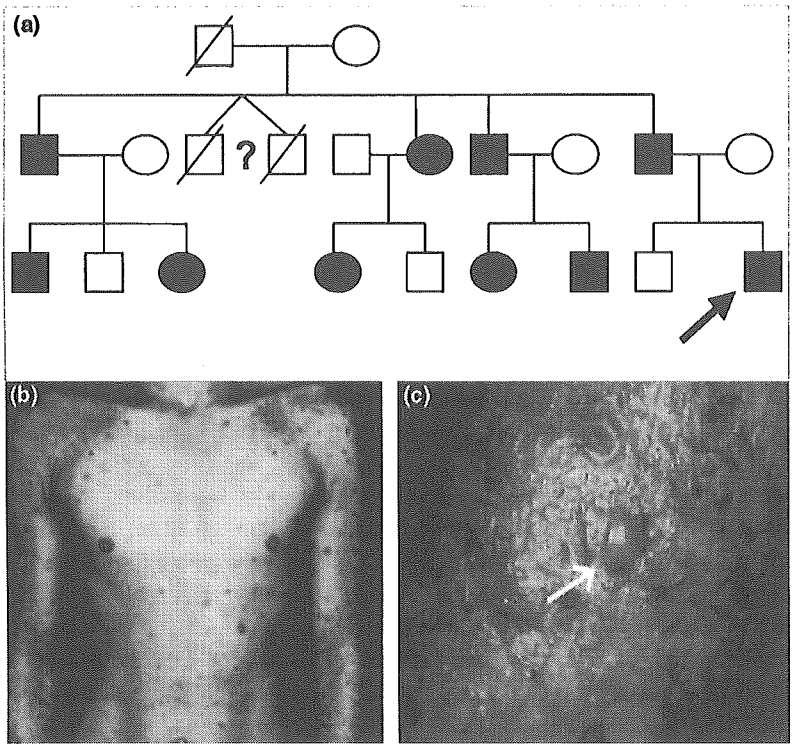

Fig 1. (a) Family pedigree. The proband is indicated with an arrow. The other nine family members affected with skin fragility failed to show severe pruritus. (b) Multiple erosions, scarring pruriginous papules and lichenoid plaques on the trunk and arms. (c) Tense blisters on the leg (white arrow).

COLOUR FIG.

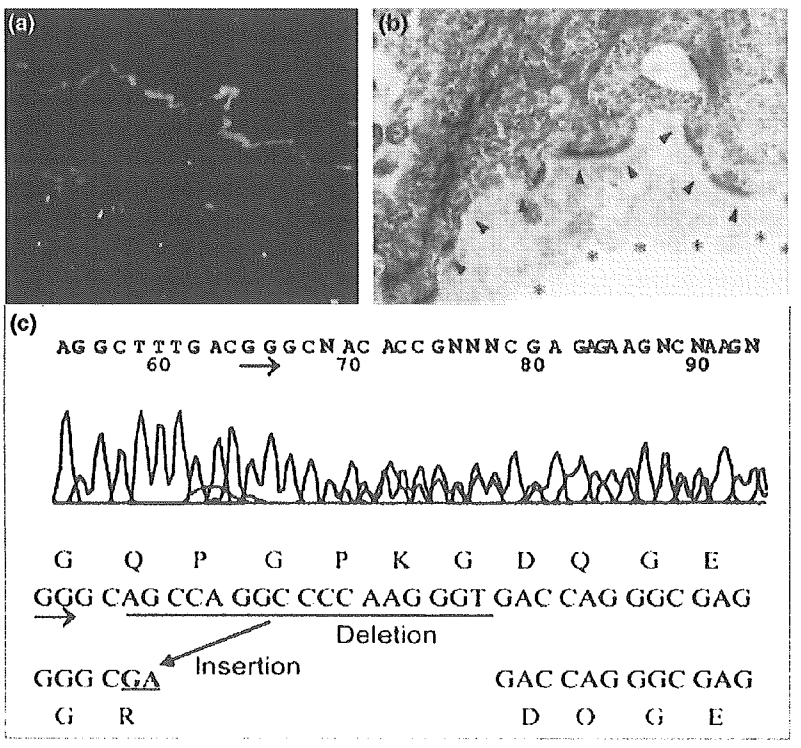

Fig 2. Immunohistochemical, ultrastructural and mutation analyses of the proband. (a) Immunostaining with monoclonal antibody LH7:2 to type VII collagen demonstrated positive, linear staining along the basement membrane zone and on the roof of the blister. (b) Electron microscopy showed tissue separation in the dermal side of the basement membrane: arrowheads, lamina densa; asterisks, blister site. (c) Direct DNA sequencing detected a heterozygous indel *COL7A1* mutation 8068del17insGA in exon 109. The 17-nucleotide deletion (green underlined area) from 8068 to 8084 with a GA insertion resulted in a 15-nucleotide deletion within the collagenous domain, which did not change the open reading frame of *COL7A1*, but interfered with the collagen triple helix (Gly–X–Y repeat). Red arrows start from G, nucleotide 8064.

Results and discussion

Direct DNA sequencing detected a heterozygous indel *COL7A1* mutation 8068del17insGA in exon 109. The 17-nucleotide deletion from 8068 to 8084 with a GA two-base insertion resulted in a 15-nucleotide deletion within the collagenous domain, which failed to change the downstream *COL7A1* open reading frame (Fig. 2c). The other affected and unaffected family members refused DNA analysis.

This study examined a 21-year-old Japanese man with skin fragility. The blister formation beneath the lamina densa and the presence of multiple affected members in his family suggested DDEB, the diagnosis of which was confirmed by the identification by mutational analysis of a *COL7A1* mutation in one allele. The characteristic clinical feature of this patient was his severe itching, resulting in scratching and prurigo formation, similar to the EB pruriginosa phenotype.[5,6]

EB pruriginosa is a form of DEB characterized by prurigo-like or lichenoid lesions associated with scarring. Blistering is usually confined to the shins and forearms. The mode of inheritance is variable. McGrath *et al.*[5] described eight unrelated families with EB pruriginosa, of which five were sporadic, two were autosomal dominant and one was autosomal recessive. In the present family, nine affected family members, unlike the proband, showed no severe pruritus or prurigo formation. Therefore, considering the elevated IgE levels, we made the diagnosis of DDEB with atopic dermatitis, not EB pruriginosa. As atopic dermatitis may show familial aggregation, we highlight here the difficulty in differential diagnosis of EB pruriginosa and EB associated with atopic dermatitis. In this case, instability in the epidermal basement membrane zone caused by anchoring fibril dysfunction might have accelerated the tendency for prurigo formation.

The mutation 8068del17insGA is a novel DEB mutation. An indel *COL7A1* mutation is extremely rare, as only two indel mutations have been reported in RDEB patients among approximately 250 previously reported *COL7A1* mutations according to the Human Gene Mutation Database (http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html). The 17-nucleotide deletion with a GA two-base insertion resulted in a 15-nucleotide deletion within the collagenous domain, which failed to change the downstream *COL7A1* open reading frame. Consequently, the deletion of 15 nucleotides (five amino acids) interfered with the collagen triple helix (Gly–X–Y repeat) and caused this DDEB phenotype probably in a dominant negative fashion. If the deleted nucleotide number had not been a multiple of 3, then a premature stop codon would have appeared downstream, resulting in a RDEB pattern mutation; therefore, this family would not have been affected.

Patients with DDEB usually harbour glycine substitution mutations within the collagenous region of collagen VII. As far as we know, only six dominant mutations other than glycine substitution mutations have been reported in the literature. Of these, three mutations, 4084–1G → C,[7] 6899A → G[8] and 8045Ae → G,[9] were one-nucleotide substitutions and the others were gross deletion mutations, 6847del27,[10] 6863del16[6] and 6081del28.[10] Thus, we have demonstrated for the first time that indel mutations may result in the DDEB phenotype and have therefore further extended the body of evidence implicating a full range of *COL7A1* gene mutations in DEB.

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Fibroblasts are More Potential Target Cells than Keratinocytes for *COL7A1* Gene Therapy of Dystrophic Epidermolysis Bullosa

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Running title: *COL7A1* gene transfer

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Key words: retroviral transfer, basement membrane, skin, genetic disorder

Abbreviations: DEB; dystrophic epidermolysis bullosa, DIG;digoxigenin, DMEM; Dulbecco's modified Eagles' medium, FCS; fetal calf serum, VSV-G; G protein of vesicular stomatitis virus

ABSTRACT

Dystrophic epidermolysis bullosa (DEB) is an inherited blistering skin disorder caused by mutations in the type VII collagen gene (*COL7A1*). Therapeutic introduction of *COL7A1* into skin cells holds significant promise for the treatment of DEB. The purpose of this study was to establish an efficient retroviral transfer method for *COL7A1* into DEB epidermal keratinocytes and dermal fibroblasts, and to determine which gene-transferred cells can most efficiently express collagen VII in the skin. We demonstrated that gene transfer using a combination of VSV-G pseudotyped retroviral vector and retronectin introduced *COL7A1* into keratinocytes and fibroblasts from a DEB patient with lack of *COL7A1* expression. RT-PCR analysis of the normal human skin demonstrated that quantity of *COL7A1* expression in the epidermis was significantly higher than that in the dermis. Subsequently, we have produced skin grafts with the gene-transferred or untreated DEB keratinocytes and fibroblasts, and have transplanted them into nude rats. Interestingly, the series of skin graft experiments showed that the gene-transferred fibroblasts supplied higher amount of collagen VII to the new dermal-epidermal junction than the gene-transferred keratinocytes. An ultrastructural study revealed that collagen VII from gene-transferred cells formed proper anchoring fibrils. These results suggest that fibroblasts may be a better gene therapy target of DEB treatment than keratinocytes.

INTRODUCTION

Type VII collagen, a non-fibrillar collagen, is a major component of anchoring fibril loop structures beneath the epidermal basement membrane (Uitto *et al*, 1992; Burgeson, 1993). Cloning of collagen VII cDNA demonstrated a primary sequence of 2944 amino acids and the basic organization of the functional domains (Christiano *et al*, 1994a). Subsequent genomic cloning has highlighted the structural organization of the collagen VII gene (*COL7A1*) (Christiano et al, 1994b). This cloning information has enabled genomic DNA sequence analysis of *COL7A1* and has demonstrated that mutations within *COL7A1* are associated with the dystrophic forms of epidermolysis bullosa (DEB). DEB comprises a group of mechanobullous diseases characterized by cutaneous fragility with a tendency to form sub-basal lamina densa blisters (Christiano *et al,* 1993b; Pulkkinen *et al,* 1999; Chen *et al.* 2002). In addition, targeted disruption of *COL7A1* in a mouse model demonstrated an almost identical phenotype to DEB in humans (Heinonen *et al,* 1999). These results indicate that collagen VII is of critical importance for dermal-epidermal adhesion.

Approximately 300 distinct *COL7A1* mutations have been identified in DEB patients so far. Therapeutic introduction of *COL7A1* into skin cells is a promising treatment of DEB. Despite the relatively large size of *COL7A1*, the cDNA of which is still 9 kb, makes gene transfer relatively problematic, and several methods including lentivirus- (Chen *et al.* 2002), retrovirus- (Baldeschi *et al,* 2003) and ϕC31 integrase-based approaches (Ortiz-Urda *et al,* 2002) have attempted to transfer *COL7A1* into keratinocytes. These studies used keratinocytes as target cells since collagen VII has been reported to be mainly synthesized and secreted by keratinocytes and to lesser extent by fibroblasts (Ryynanen *et al,* 1992). However, application of gene-transferred DEB fibroblasts into the skin restored collagen VII expression in the dermal-epidermal junction (Ortiz-Urda *et al,* 2003; Woodley *et al,* 2003). In addition, using an intradermal injection of lentivirus with *COL7A1* induced the expression of collagen VII in fibroblasts and endothelial cells, resulting in collagen VII accumulation in the grafted DEB skin on the host animal (Woodley *et al,* 2004).

In this study we have established a retroviral method to transfer *COL7A1* into DEB keratinocytes and fibroblasts. Next, we produced the skin grafts with gene-transferred keratinocytes or fibroblasts, and transplanted them into nude rats. Examination of collagen VII graft expression revealed that gene transferred fibroblasts assembled more collagen VII in the form of anchoring fibrils beneath the basement membrane than gene-transferred keratinocytes. We conclude that fibroblasts are a more ideally suited

target for *COL7A1* gene transfer than keratinocytes using retroviral gene therapy for the treatment of DEB.

RESULTS

Successful transfer COL7A1 using retroviral systems

We employed two retroviral vectors, pLIXN and pDON-AI, 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). Several series of preliminary experiments demonstrated that retronectin (TAKARA) increased attachment of virus to keratinocytes and fibroblasts. Also, use of plasmid pVSV-G (Pantropic System: Clontech) enabled concentration of viral particles by ultracentrifugation, resulting in an increase of transfer efficacy. After transfection of plasmids pLI-COL, pDON-COL and pDON(Δ)-COL to 293 packaging cells, the culture media were collected. The virus titers (mean + SD X 10^6/ml) of pLI-COL, pDON-COL and pDON(Δ)-COL were 1.1 + 0.35, 1.6 + 0.46 and 2.7 + 0.55 , respectively.

We transferred *COL7A1* into cultured DEB keratinocytes and fibroblasts using the retroviral system. Transfection experiments showed that retroviral methods using pLI-COL and pDON-COL failed to introduce *COL7A1* to DEB cells (data not shown). Plasmid pDON(Δ)-COL with the VSV-G system allowed to increase allowed a greater gene transfer after further concentration of the virus particles increased the transfer rate. (Fig 2). Immunostaining revealed that the transfer rates in DEB keratinocytes and fibroblasts were almost the same (Fig 2, 3A), and immunoblotting demonstrated that the amount of collagen VII in their culture media was also identical (Fig 3B). The average copy number per cell of the *COL7A1* cDNA was evaluated by Southern blot analysis of genomic DNA extracted from the transduced cells. The result indicated that the intensities of 7.2 kb band from the integrated cDNA were the same in treated keratinocytes and fibroblasts, suggesting integration copies for keratinocytes and fibroblasts was almost equal (Fig 3C). The copy number was estimated 2~3 by comparing with a serial dilution standard (data not shown).

In vivo COL7A1 expression in epidermis is higher than that in dermis

To determine the *COL7A1* expression level in the epidermis and dermis *in vivo,* we separated the epidermis from the dermis, and measured *COL7A1* mRNA levels using real time PCR. Real time PCR demonstrated that the *COL7A1* specific signal (per RNA) of the epidermis was higher than that of the dermis by 3.2 fold (Fig 4). 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 by 3.4 fold (Fig 4). Thus, the quantity of *COL7A1* expression in epidermis was significantly higher than that in dermis *in vivo*.

Gene-transferred fibroblasts can supply more collagen VII to the basement membrane zone than gene-transferred keratinocytes

We transplanted the gene-transferred DEB keratinocytes and fibroblasts into the wound of nude rats, and then observed *COL7A1* deposition 3, 6 and 9 weeks after transplantation. In the skin graft with gene-transferred keratinocytes and untreated fibroblasts, the *COL7A1* deposition was detectable in the basement membrane zone at 3 week and maintained this expression at least until 9 weeks (Fig 5). However, we found a greater accumulation of collagen VII in dermal-epidermal junction of the grafts using untreated keratinocytes and gene-transferred fibroblasts 3 weeks after transplantation. Furthermore, DEB fibroblasts transfected with *COL7A1* demonstrated more dermal epidermal junction collagen VII staining than *COL7A1* transfected DEB keratinocytes / untreated fibroblast (Fig 5) from 6 to 9 weeks. The grafts of DEB keratinocytes and fibroblasts as controls demonstrated no deposition (Fig 5). Semiquantification of *COL7A1* deposition in basement membrane zone in each point showed DEB fibroblasts with COL7A1 can supply higher amount of collagen VII to the basement membrane zone than DEB keratinocytes with *COL7A1* (Fig 6). Significant differences were found between the keratinocytes and fibroblasts samples at 3W and 9W points.

Collagen VII released from gene-transferred cells forms ultrastructurally normal anchoring fibrils

We examined the ultrastructural formation of anchoring fibrils in the graft. The grafts with both gene-transferred keratinocytes and fibroblasts demonstrated filamentous loop structures just beneath the lamina densa which were corresponding to anchoring fibrils (Fig 7). We could not see any filamentous structures in control (untransfected) samples without *COL7A1*.