Demonstration of the pathogenic effect of point mutated keratin 9 in vivo

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Abstract A wild type keratin 9 (K9) cDNA and a point mutated keratin 9 cDNA were injected subcutaneously into mouse skin. The hemagglutinin tag staining of the wild type K9 cDNA injected specimens mainly showed a homogeneous pattern, whereas the point mutated K9 cDNA injected specimens mainly showed a granular pattern in the suprabasal cells. Double staining of K9 and the endogenous keratin revealed the incorporation of de novo synthesized K9 into the keratin network. These results demonstrate that (1) a naked DNA transfection into mouse skin can detect the pathogenic changes of point mutated keratin in vivo and (2) the keratin 9 mutation disrupts the keratin network formation in the suprabasal cells in vivo.

Key words: Keratin 9; Keratin intermediate filament; Naked DNA transfection; In vivo assay; Point mutation; Immunofluorescence

1. Introduction

Keratins are the major structural proteins of the epidermis [1]. The 10 nm keratin filaments belong to the intermediate filament superfamily. All the intermediate filaments have a central α-helical domain, the rod, which is flanked by a non-helical head and tail domain. Keratins assemble in vitro as obligatory heteropolymers. The highly conserved rod ends play a crucial role in intermediate filament network formations [2–4]. A point mutation in the keratin perturbs the keratin filament structure, causing hereditary diseases of the skin [3–8], such as an epidermolytic palmoplantar keratoderma [8–14]. Several methods have been proposed for detecting the effect of pathogenicity caused by the point mutation seen in hereditary diseases such as epidermolysis bullosa simplex and epidermolytic hyperkeratosis. There are two types of assays in vitro to detect the effect of such point mutations. The transfection of the wild type and point mutated human keratin cDNA into cells [15], and the reconstitution of the keratin filament with or without the point mutated keratin, are methods to detect the pathogenic changes of point mutation in vitro [16]. While transgenic mice with mutant keratin cDNA have been used to demonstrate change of the keratin filament structure in vivo [8], much work is needed to construct transgenic mice. In naked DNA transfection, the skin is a useful target because it is accessible and easily monitored for the presence and expression of a gene [17,18]. The direct transfection of a gene into the skin is possible by needle injection [19,20], and the direct injection of a naked plasmid DNA into the cutaneous is a simple and direct in vivo technique for introducing and expressing a gene in the epidermis [20]. We found a G to A transition in the K9 gene, resulting in the substitution of glutamine for arginine in all patients with a pedigree of epidermolytic palmoplantar keratoderma, and reported that the K9 point mutation seen in such patients was responsible for the pathogenicity in transfected cultured cells with a degenerative change of the keratin intermediate filament structure [8]. We have tested whether the functional change can be detected by naked DNA transfection in vivo. Two types of naked DNA plasmids, normal K9 cDNA and point mutated K9 cDNA, were injected subcutaneously into mice. Here we demonstrate that a point mutation in K9 causes the effect of pathogenicity in the upper epidermal layers in vivo.

2. Materials and methods

2.1. Expression vectors

Expression vectors were constructed as described previously [8]. In short, the pCMX-HA contained a cytomegalovirus promoter, a hemagglutinin protein of influenza (HA) tag and a SV40 poly(A) signal. A normal human K9 cDNA was inserted into the pCMX-HA to construct the plasmid pCMX-K9HA. The plasmid pCMX-K9R162QHA has a point mutated K9 cDNA existing in the epidermolytic palmoplantar keratoderma patient. These plasmid DNAs were purified by affinity chromatography (Qiagen, Chatsworth, CA, USA) followed by column chromatography with Bio-Gel 150 (Bio-Rad, Boston, MA, USA). Plasmid DNAs were suspended in distilled water and stored at −20°C.

2.2. Animals and gene transfer

Non-pregnant hairless mice were purchased from Shimizu Co. Ltd. (Kyoto, Japan). With a 1 ml syringe and a 30 gauge needle, hairless mice were injected subcutaneously with 20 μg of each plasmid DNA in a total volume of 50 μl daily for 3 days. Five days after the first injection, the injected skin was removed and frozen immediately in OCT Compound (Sakura Finetechnical, Tokyo, Japan), cut into 6 μm thick sections and air dried for 30 min. Sections were rinsed with phosphate buffered saline (PBS) and processed for immunostaining.

2.3. Cells and transfection

Madin-Darby canine kidney cells (MDCK cells) were grown in Dulbecco's MEM supplemented with 10% fetal calf serum (FCS) and 100 U/ml penicillin and 100 μg/ml streptomycin. MDCK cells were grown on glass coverslips in 6-well plates (Iwaki, Kyoto, Japan). The cells were transfected with plasmid DNA using Lipofectamine reagent (Gibco-BRL, Bethesda, MD, USA) according to the distributor's recommendation. At 24 h after transfection, cells were fixed in 3% paraformaldehyde for 10 min and permeabilized by incubation with 0.5% Triton-X for 10 min. Transfected cells were then processed for immunostaining.

2.4. Immunofluorescence

To detect transfected cells, an anti-HA mouse monoclonal antibody (clone 12CA5) (Boehringer Mannheim, Indianapolis, IN, USA) was used to recognize the HA tag as a primary antibody and a fluorescein-conjugated goat IgG fraction to mouse IgG (Organon Teknika, NC, USA) was used as a secondary antibody. Sections were incubated at room temperature with a primary antibody (1:400 dilution) for 60 min, rinsed, subsequently incubated with a secondary antibody (1:120 dilution) for 60 min, rinsed again, and finally mounted with PPD-glycerin (*p*-phenylenediamine-glycerin). The following antibodies

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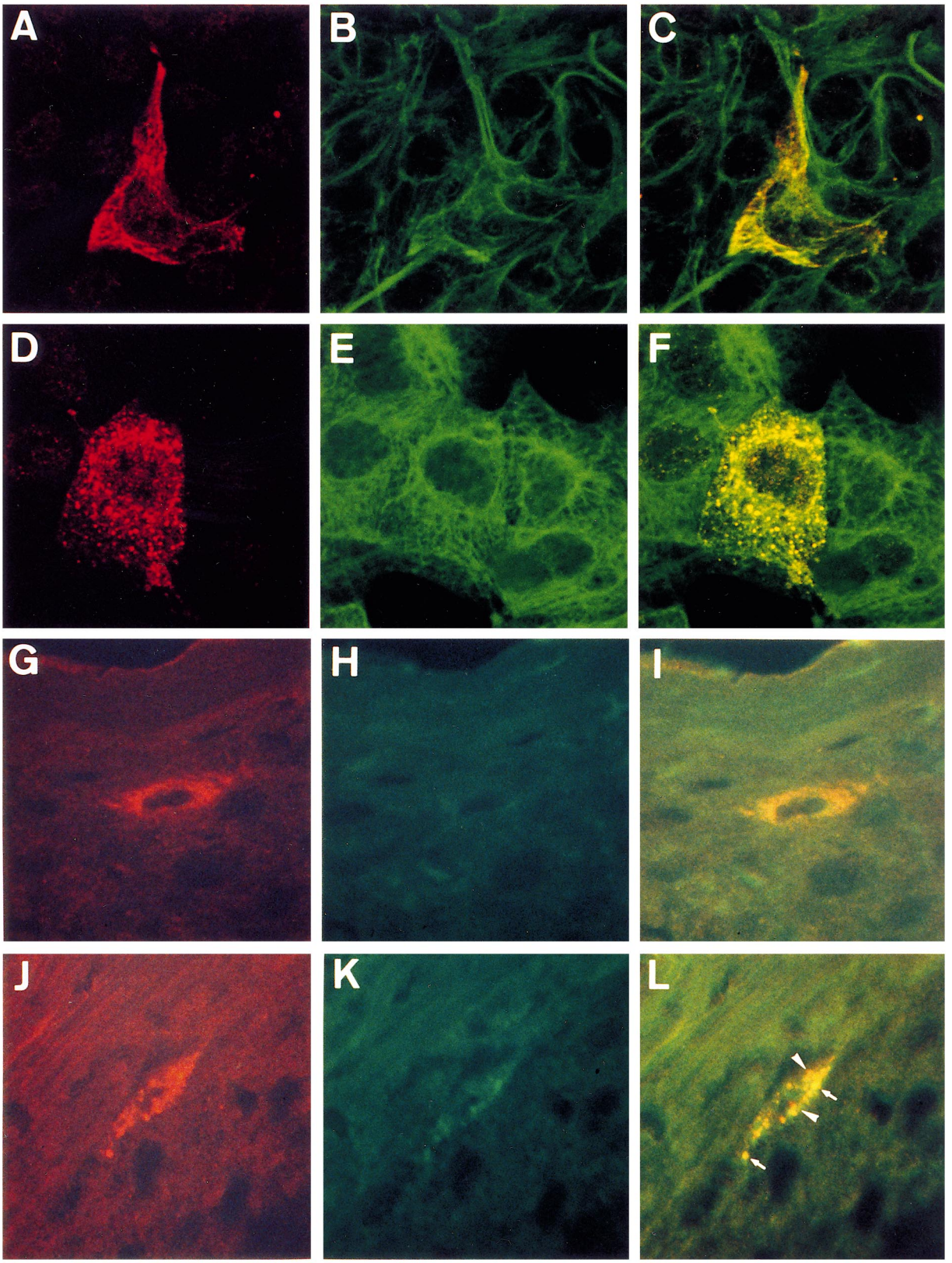

Fig. 1. Confocal laser microphotographs of double-immunofluorescent colocalization of the HA tag and endogenous keratins. MDCK cells were transfected with pCMX-K9HA (A–C) and pCMX-K9R162QHA (D–F). Mouse skin was injected with pCMX-K9HA (G–I) and pCMX-K9R162QHA (J–L). The red color of 543 nm laser light demonstrates the localization of K9HA product (A,G) or K9R162QHA product (D,J) while the green of 488 nm demonstrates endogenous keratins (B,E,H,K). C, F, I and L show the superpositioning of colocalization through overlaying of the 488 nm and 543 nm light.

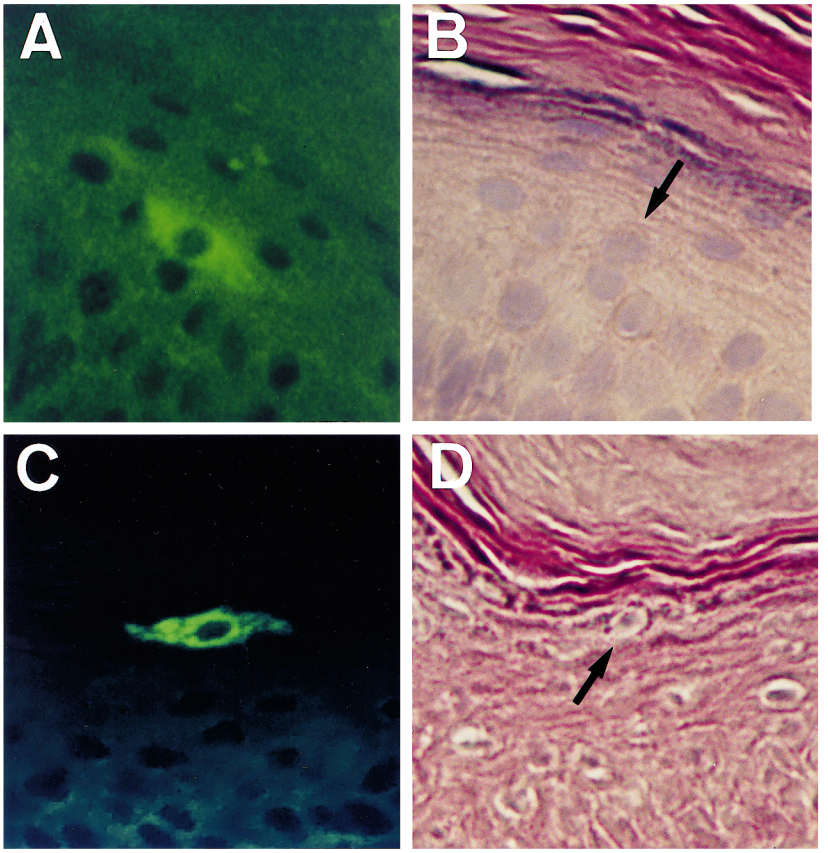

Fig. 2. Confocal laser microphotographs reveal localization of pCMX-K9HA and pCMX-K9R162QHA DNA products by staining with the anti-HA tag. The mice injected with the pCMX-K9HA mainly showed a homogeneous pattern (A) while those given pCMX-K9R162QHA mainly showed a granular pattern (C). The same samples were reused for the HE stain for the microscopic observation. There were no morphological changes (B,D).

were used for further double staining studies. In MDCK cell staining, a 1:400 diluted anti-HA.11 rabbit polyclonal antibody (Berkeley Antibody, CA, USA) and a 1:100 diluted rhodamine-conjugated swine anti-rabbit IgG (Dakopatts, Glostrup, Denmark) was used for the HA tag staining. Subsequently endogenous keratin network of MDCK cells were stained with a 1:1000 diluted MAB1629 (mouse monoclonal anti-cytokeratin 5, 8; Chemicon, CA, USA) and a 1:100 diluted fluorescein-conjugated goat IgG fraction to mouse IgG (Organon Teknika, NC, USA). To examine the expression of HA and keratin in naked DNA transfected mice skin sections the following antibodies were used: a 1:400 diluted anti-HA mouse monoclonal antibody, a 1:120 diluted rhodamine-conjugated swine anti-mouse IgG (Dakopatts, Glostrup, Denmark), a 1:10 diluted rabbit anti-cytokeratin (Zymed Laboratories, CA, USA) and a 1:120 diluted fluorescein-conjugated swine anti-rabbit IgG (Dakopatts, Glostrup, Denmark). All sections were observed with a Carl Zeiss LSM 410 confocal laser scanning microscope.

2.5. HE stain analysis

After the immunofluorescence study, slides were incubated with 10% formalin for 3 days. The coverslips were removed and rinsed with PBS. The sections were stained with hematoxylin for 1 min, rinsed with tap water and stained with eosin for 7 min.

3. Results and discussion

The phenotypic changes of naked DNA transfected epidermal cells were compared with the DNA transfected culture cells. We transfected the wild type K9 cDNA (pCMX-K9HA) or the point mutated K9 cDNA (pCMX-K9R162QHA) into MDCK cells and performed an analysis by double staining with a confocal laser scanning microscope using 488 nm and 543 nm laser light. The red signal (HeNe 543 nm laser) in Fig. 1 demonstrates the staining on the tag sequence (HA) derived from transfected pCMX-K9HA (Fig. 1A,G) and pCMX-K9R162QHA (Fig. 1D,J) cDNAs and the green signal (argon 488 nm laser) shows the endogenous keratin network by the keratin staining (Fig. 1B,E,H,K). As shown by the red signal, the K9 protein showed droplet formation in the mutant K9 transfectant cells (Fig. 1D), whereas it showed the filament formation in the wild type cDNA transfected cells (Fig. 1A). The specimens for the naked DNA transfection showed essentially the same results. The wild type transfectants mainly showed a homogeneous staining pattern (Fig. 1G), while the mutant transfectants mainly exhibited a granular pattern (Fig. 1J). In cultured cells, both the filament and droplet formation of endogenous keratin were observed in the mutant transfectant (Fig. 1E). In the endogenous keratin staining of the specimens subjected to naked DNA transfection, there was no difference between pCMX-K9HA (wild type) and pCMX-K9R162QHA (point mutated type) (Fig. 1H,K), moreover, there was no difference between the transfectant and the non-transfectant. These observations suggest that disruption of internal keratin is not detectable with a single staining by keratin in vivo. The ability of K9 to integrate into the endogenous keratin network was examined by superpositioning because a dominant-negative effect of the mutant keratin is thought to be the pathomecha-

Table 1
The ratio of types of keratin filaments in the epidermal keratinocytes

	pCMX-HA	pCMX-K9HA	pCMX-K9R162QHA
Granular	9.3 ± 6.8 (33.3%)	12.3 ± 4.1 (37.4%)	21.7 ± 5.7 (62.5%)
Homogeneous	18.7 ± 9.0 (66.7%)	20.7 ± 1.6 (62.6%)	13.0 ± 3.6 (37.5%)

Fluorescent positive cells were counted according to the granular/homogeneous appearance. Figures in parentheses show % of each staining pattern. Values are expressed as cells/HA positive cells ± S.D. Results represent the means of three experiments.

nism of an epidermolytic palmoplantar keratoderma, i.e. the incorporation of de novo synthesized mutant keratin is needed to disrupt the keratin filament network. We performed an analysis by using 488 nm and 543 nm laser light. In cultured cells the yellow colored filament formation in Fig. 1C clearly demonstrates the colocalization of pCMX-K9HA product and endogenous keratin. Similarly the yellow droplet formation in Fig. 1F demonstrates the colocalization of pCMX-K9R162QHA product and the endogenous keratin, indicating de novo synthesized K9 filament integrated into the endogenous keratin network. Specimens of the naked DNA transfection revealed similar results. The yellow in pCMX-K9HA and pCMX-K9R162QHA transfectant cells indicates that exogenous DNA products were integrated into the endogenous keratin filaments (Fig. 1I,L). However, in addition to the granules of yellow signal (Fig. 1L, arrow), those of red signal (Fig. 1L, arrowhead) were observed in pCMX-K9R162QHA transfectant, indicating that newly synthesized K9R162QHA products are not completely integrated in the endogenous keratins (Fig. 1L) in vivo. These results show that the naked DNA transfection assay can detect pathogenic change to the same degree as the transfected cell culture assay by staining with HA, and at the point mutated K9 integrates endogenous keratin network not only in the basal cell layer but also in the suprabasal cell layer.

To test the relationship between the granule formation in the immunostaining and HE staining, wild type K9 cDNA (pCMX-K9HA), point mutated K9 cDNA (pCMX-K9R162QHA) and negative control plasmid without K9 sequence (pCMX-HA) were injected subcutaneously into mice at days 1, 2 and 3. Although an epidermolytic palmoplantar keratoderma with the K9 point mutation is clinically characterized by a diffuse thickening of palmar and plantar epidermis [21,22], there was no clinical change in the skin of mice at day 5. As shown in Figs. 1 and 2 by immunofluorescence detection, expression of the exogenous DNA in the keratinocyte occurred only once to several in each specimen which may reflect low efficiency of DNA uptake in the keratinocyte and may reflect no clinical change in the skin. There are no naked DNA uptake in fibroblast.

The immunofluorescence study revealed that the specimens injected with the pCMX-K9HA plasmid and pCMX-HA (data not shown) mainly showed a homogeneous pattern of expression (Fig. 2A) and those injected with the pCMX-K9R162QHA plasmid a granular pattern (Fig. 2C). The same samples were subjected to hematoxylin eosin staining (HE stain) for microscopic observation. Fig. 2B,D shows the HE stains of specimens corresponding to Fig. 2A,C. No morphological changes such as individual cell keratinization were observed in the fluorescence positive cells, suggesting that there was no cell damage caused by the aggregation of the keratin intermediate filaments. After the HE stain analysis, we subjected the samples to ultrastructural analysis by transmission electron microscopy, but were unable to observe any clear changes in keratin intermediate filaments such as clear vacuolated areas in their cytoplasm or clumps of coarse tonofilaments (data not shown) [22], because samples had been badly damaged by the immunofluorescence and HE stain analysis.

The staining ratio of each pattern was determined through the detection of the HA tag derived from the pCMX-K9HA and pCMX-K9R162QHA cDNAs. The pCMX-HA showed 66.7% (18.7 ± 9.0; mean ± S.D. is cells per HA positive cells) homogeneous (H) and 33.3% (9.3 ± 6.8) granular (G) staining. The pCMX-K9HA showed 62.6% (20.7 ± 1.6) H and 37.4% (12.3 ± 4.1) G patterned staining. The pCMX-K9R162QHA showed 37.5% (13.0 ± 3.6) H and 62.5% (21.7 ± 5.7) G staining (Table 1). Thus, pCMX-K9HA injected specimens showed twice the number of H than G. Reciprocally, pCMX-K9R162QHA injected specimens revealed twice number of G than H. These results show that the point mutated K9 cDNA affected the keratin intermediate filament assembly.

Our results revealed that naked DNA transfection into mouse skin produced an effect of pathogenicity similar to that in transfected cultured cells, and the K9R162Q mutation caused the aggregation of the keratin intermediate filament in vivo.

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