benign EB [2] . In this aspect, EBS with pyloric atresia in this study would belong to the variants.

Molecular mechanism of PA development even in JEB-PA has not been fully elucidated yet although phenotype and genotype information of this disease has been accumulated. Targeted ablation of both the *ITGA4* and *ITGB6* genes in mice clearly induced separation of the epidermis from the dermis as seen in the skin of JEB-PA [38,39,40]. In contrast, those model mice could not show straightforward evidence concerning mechanism of PA. Further studies are needed to clarify the pathogenesis of PA in all patients with EBS-PA as well as those with JEB-PA.

In this study, we have demonstrated for the first time that *PLEC1* mutations induce novel subtype of EB, EBS-PA. No definite conclusion has yet been reached regarding the occurrence of late onset muscular dystrophy phenotype of our three cases, since two patients have already died and one patient is still a neonate. However, this study furthers our understanding of the possible range of pathophysiology in EB and of the biology of the cutaneous basement membrane. In the previously reported 4 cases of EBS-PA [11,12,13,14], 2 cases (11, 12) also died shortly after birth although a search for plectin mutations were not carried out. In terms of clinical prognosis, this novel subtype EBS-PA is the lethal variant in the EBS category and will become a real target for gene therapy in the near future.

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FIGURE LEGENDS

Fig. 1 Clinical, X-ray and ultrastructural findings of proband 1 in pedigree 1. A: Sharply demarcated erosions and ulcers on the trunk, genitalia, and lower extremity. **B:** Marked ulcers on the lower extremities. **C:** A single abdominal bubble of gas in an abdominal X-ray of probnad 1. **D** and **E:** The same clinical manifestation of his elder brother. **F:** Electron microscopy of the skin from proband 1 shows tissue separation occurring at the base of the basal keratinocytes (stars). Keratin filaments are sparse, and thin and not well associated with the hemidesmosome (HD) inner plaque (full arrowheads). Reduced numbers of hypoplastic HDs are recognized (triangles) and occasional HDs can be observed associated with thin bundles of keratin filaments within the basal keratinocyte (open arrow). The lamina densa (LD) and lamina lucida (LL) are present in the papillary dermis. Bar: 500nm.

Fig. 2 Clinical and ultrastructural findings of proband 2 in pedigree 2. A: Clearly demarcated blisters and ulcers on the scalp, abdomen and extremities. **B:** Ulcers on the left arm. **C:** Localized erosions on the scalp. **D:** Ulcers on the left leg. **E:** Electron microscopy of the skin shows basal cell debris including flat electron densities (representing remnant hemidesmosome outer plaques (arrows) can be seen at the base of the intraepidermal split (asterisk). Bar: 1 μm.

Fig. 3 Immunofluorescence using antibodies against basement membrane zone components. Staining using HD1-121, K15,

10F6 and 5B3 monoclonal antibodies (mAbs) for plectin were markedly attenuated in the proband 1 and completely loss in the proband 2. Immunostaining for other proteins including α6 and β4 integrins, laminin 5, type VII and IV collagens were normal in both probands and controls.

Fig. 4 Mutation detection in pedigree 1. The proband harbored a G→A transition at position c.1344 in exon 12 within an intron-exon border (middle-right panel). He also possessed a heterozygous transition 913C→T (exon 9), leading to the substitution of glutamine 305 with a nonsense codon (Q305X). *HphI* digestion of the 428-bp fragment with and without the 1344G->A mutation product resulted in single band of 428 bp and double bands of 221 and 207-bp, respectively (left panel). The 387-pb PCR fragment containing the Q305X mutation was not digested by *PstI* whereas the digestion of the fragment without the mutation showed two bands of 240 and 147 bp (right panel). The 1344G→A mutation was paternal and the Q305X mutation was maternal.

Fig. 5 Mutation detection in pedigree 2. The proband 2 harbored a homozygous transition 3565C→T (exon 27) at codon 1189 producing a nonsense codon instead of arginine (R1189X) (middle panel). She also harbored a heterozygous C→T (c.7612) substitution (exon 32) in codon 2538, replacing glutamine with a nonsense codon (Q2538X). The R1189X mutation caused the generation of site for the *Tsp45I* restriction enzyme. The 771 bp PCR product with the mutation was digested by *Tsp45I* resulting in 424-bp and 347-bp bands (left panel). Since no proper restriction enzyme site was found around the Q2538X mutation, we changed one base of the PCR primer from the original sequence to create a site for *AluI* (see Materials and Methods). The digestion of 225-bp PCR product with Q2538X produced 70-bp band (right panel). The father and the mother are heterozygous

for R1189X and Q2538X mutations, respectively.

Fig. 6 Detection of uniparental isodisomy. Comparison of 8 intragenic polymorphisms around the homozygous R1189 mutation showed that the father and the mother were heterozygous for 2/8 and 6/8 markers. The mutation R1189X and intron 28/10648 T →A were informative for the absence of a maternal allele in the proband.

Figure 7 Database showing the position of mutations in *PLEC1*. Each functional domain is shown in the schematic model of plectin structure. The cDNA and the amino acids of the protein are numbered based on the previous sequence information (GenBank accession no. AH003623)(17). Amino-terminal actin binding domain, amino-terminal globular domain, β4 integrin binding sites, rod domain and carboxyl-terminal globular domain are shown.

Table 1 Epidermolysis bullosa(EB) classification and the causative genes [1] .

Major EB type	Major EB subtypes	Involved genes/protein
EB Simplex (EBS)	Dowling-Meara EBS	K5, K14
	Koebner EBS	K5, K14
	Weber-Cockayne EBS	K5, K14
	EBS with muscular dystrophy	Plectin
Junctional EB (JEB)	Herlitz JEB	Laminin 5
	Non- Herlitz JEB	Laminin 5, BPAG2
	JEB with pyloric atresia	α6β4 integrin
Dystrophic EB (DEB)	dominant DEB	type VII collagen
	Hallopeau-Siemens recessive DEB	type VII collagen
	Non Hallopeau-Siemens recessive DEB	type VII collagen

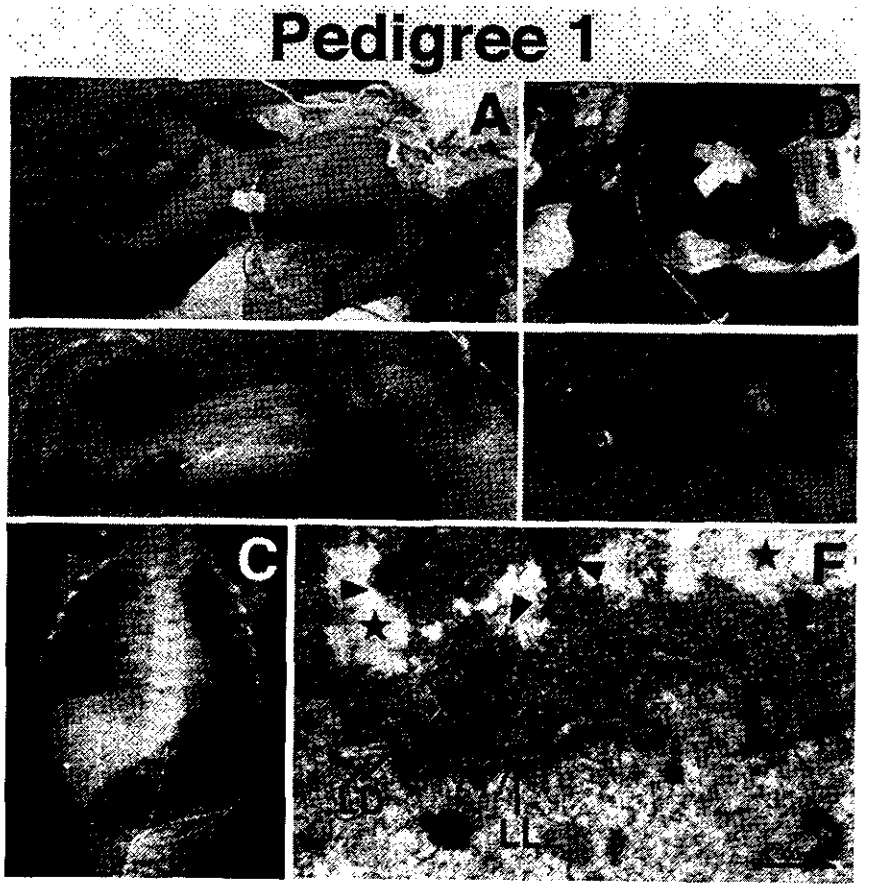


Fig. 1 Nakamura et al.

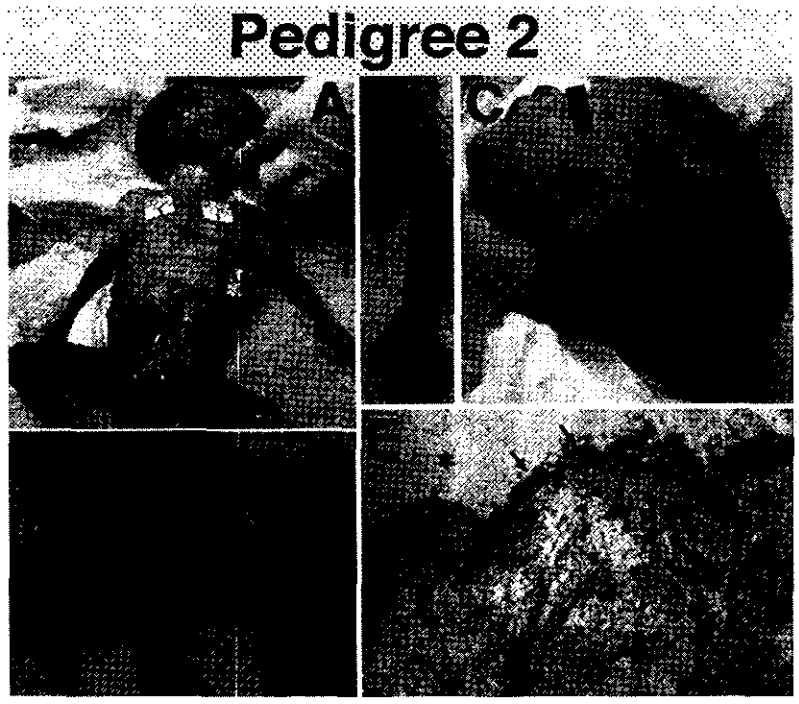


Fig. 2 Nakamura et al.

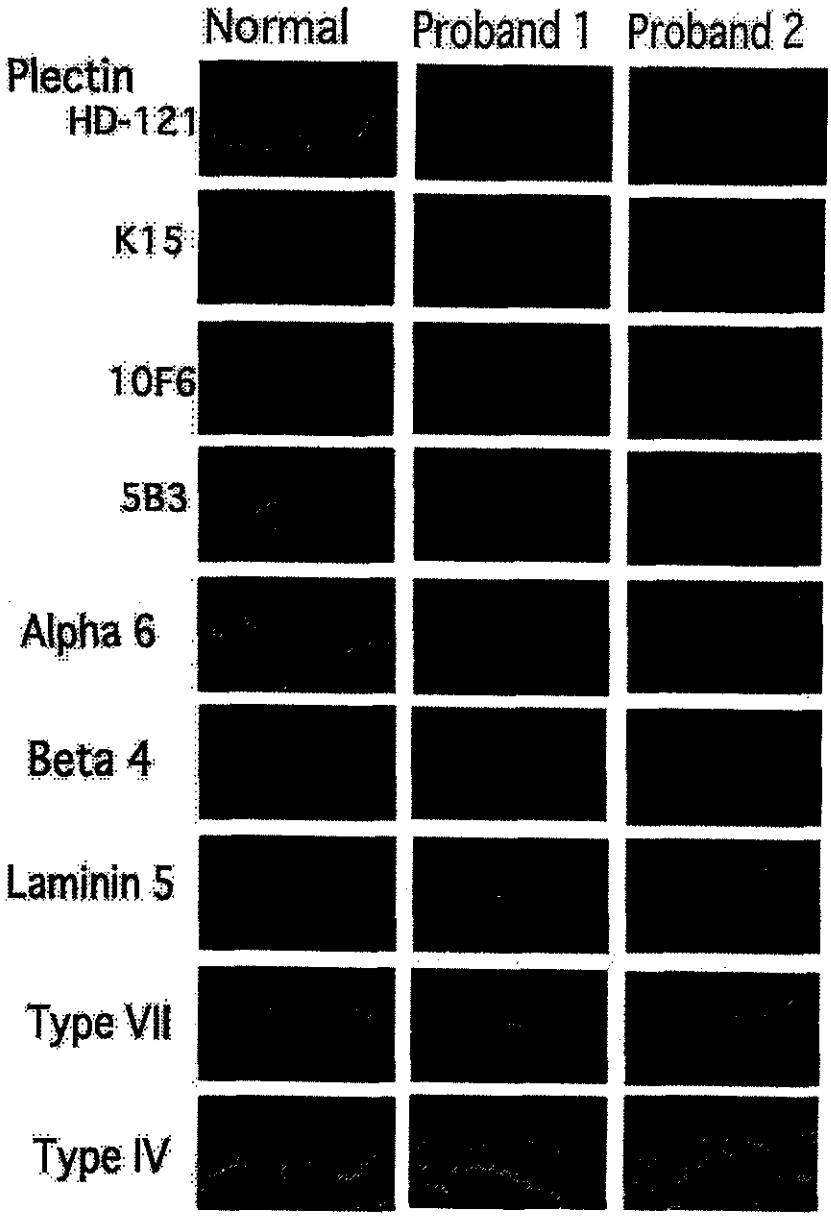


Fig. 3 Nakamura et al.

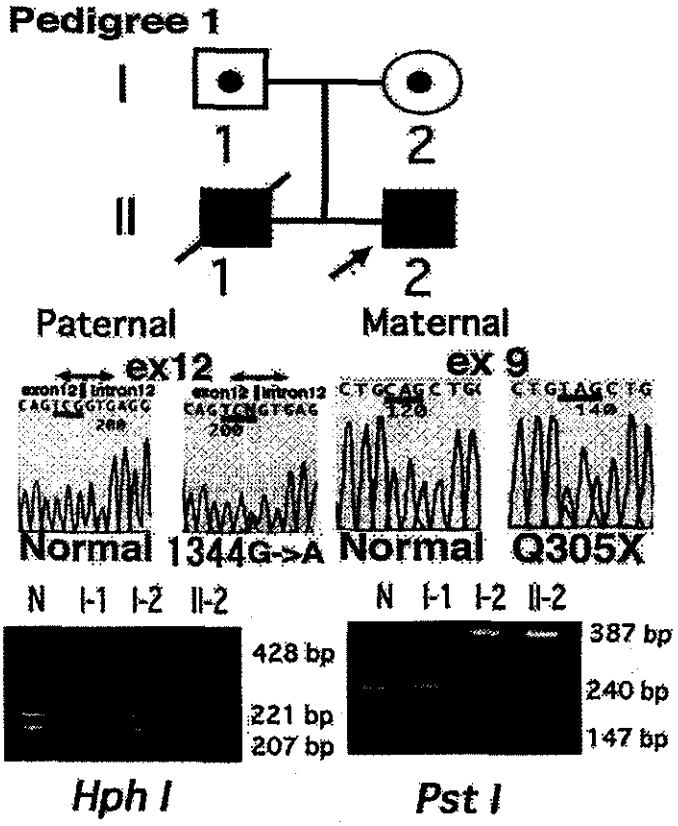


Fig. 4 Nakamura et al.

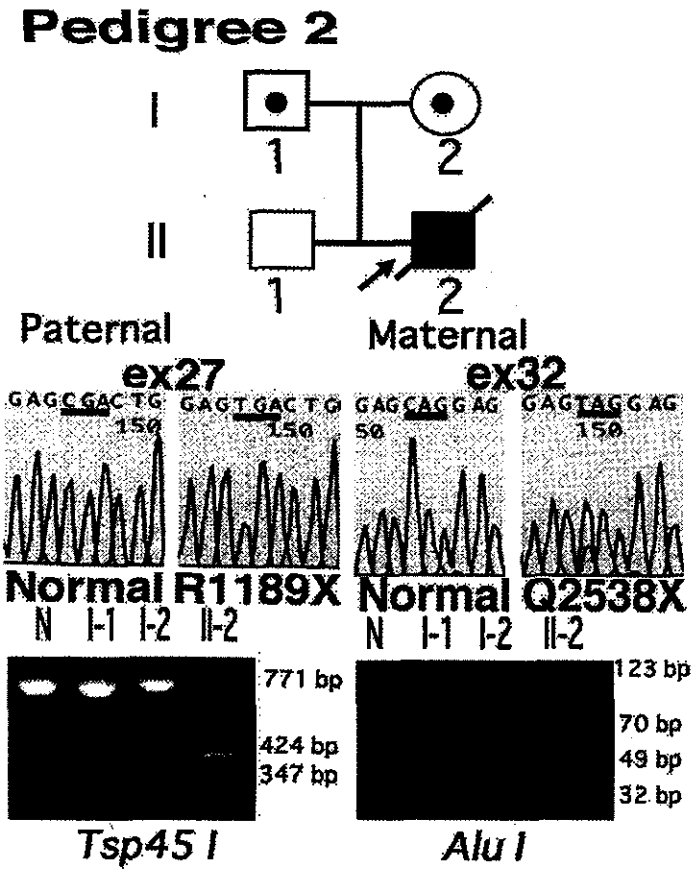
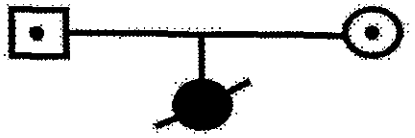


Fig. 5 Nakamura et al.



Exon 5/ 749 T→C	+	+	+	−	+	−
Intron 9/ 3023 C→G	+	+	+	−	+	−
Exon 15/ 4766 C→T	+	−	+	−	+	+
Intron 19/ 7407 T→C	+	+	+	−	+	−
Exon 27/ 10113(**R1189X**)	+	−	+	+	−	−
Intron 28/ 10648 T→A	+	+	+	+	−	−
Exon 31/ 12020 C→T	+	−	+	−	+	+
Exon 31/ 12271 T→C	+	+	+	−	+	−
Exon 32/ 15497(**Q2538X**)	−	−	−	+	+	−
Exon 32/ 15547 A→C	+	+	+	−	+	−

Fig. 6 Nakamura et al.

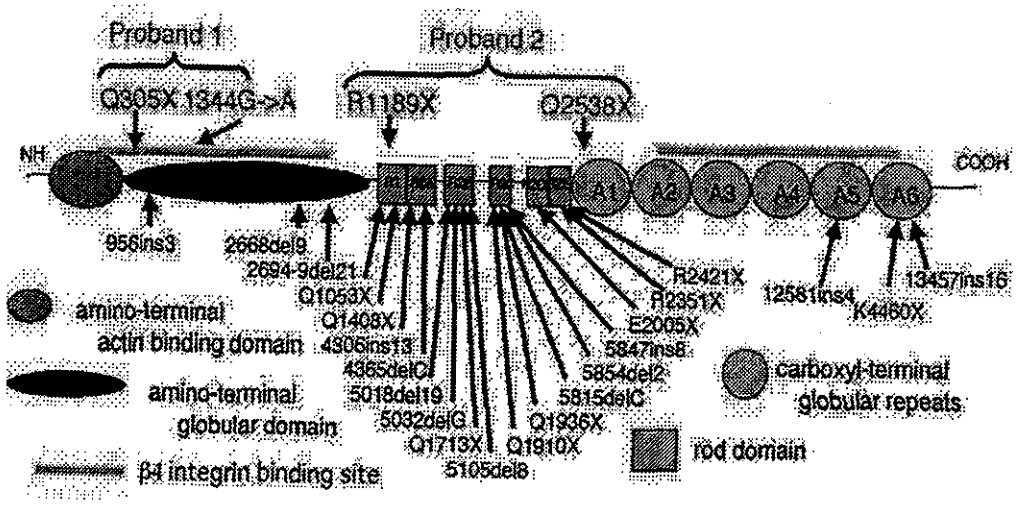


Fig. 7 Nakamura et al.

Article for Journal of Gene Medicine

Direct injection of plasmid DNA into the skin induces dermatitis by activation of monocytes through toll-like receptor 9

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Running title: Plasmid DNA induces dermatitis

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Abbreviations: BS: bluescript II, CpG: cytosine-guanosine dinucleotide, IL-6: interleukin-6, TLR: toll-like receptor

Abstract

Background Direct injection of naked DNA into skin can be efficiently used to transfer genes into keratinocytes *in vivo*. However, bacterial DNA is known to be a potent stimulus for vertebrate immune cells and immune systems. Towards the clinical use of this method, this study examined whether the application of plasmid DNA by direct injection induces any adverse skin effects.

Methods Several plasmid preparations were prepared and directly injected into rat and human skin. Migration, IL-6 production, and reactive oxygen production assays were performed to determine the type of the primary cells responsible for the reaction. Involvement of toll-like receptor (TLR) 9 was examined by experiments using TLR9-knockout mice.

Results Injection of several plasmid preparations into rat and human skin resulted in an inflammatory reaction at the treated site. Contamination by endotoxin in the plasmid preparation was shown to worsen this skin inflammation reaction. Immunohistochemical analysis showed that the infiltrating cells in the skin lesions were predominantly monocytes and neutrophils. Further experiments examining migration, IL-6 production, and reactive oxygen production assays indicated that the primary responsible cells were monocytes rather than neutrophils. Since it was recently shown that cytosine-guanosine dinucleotide (CpG) motif is critical for immune reaction induction in bacterial DNA and cellular responses were mediated by TLR9, we injected plasmids into the ear skin of TLR9- knockout mice. A decrease in ear swelling was noted in the knockout mice, compared to controls suggesting that plasmid DNA-induced dermatitis was mediated mostly by TLR9.

Conclusion This study demonstrates that injection of plasmid DNA induces skin inflammation initiated by monocyte activation via TRL9. We should therefore attempt to counteract this dermatitis during the clinical use of the naked DNA injection method in skin.

Key words: gene therapy, keratinocyte, naked DNA