Introduction

Gene therapy is a promising technique to treat patients with intractable diseases and is now approved worldwide [1]. One of main target cells in gene therapy for skin diseases is the epidermal keratinocyte. Keratinocyte gene therapy is primarily directed toward skin diseases with a single gene deficiency, but is also applicable to skin cancers and intractable inflammatory skin diseases [2,3]. Keratinocyte gene therapy can also be extended to systemic diseases in an attempt to deliver gene products into the general circulation [2,4]. Successful keratinocyte gene therapy requires the development of highly efficient methods of gene transfer into keratinocytes. Several methods including viral- [5] and non-viral- [6] mediated transduction have been reported during *in vivo* gene transfer.

Among those methods, a simple, safe and relatively efficient method is the direct injection of naked DNA [7], in which intradermally injected plasmid DNA is taken up and expressed by keratinocytes *in vivo*. This method can directly introduce the gene even into human keratinocytes [8,9] and can initiate biological effects from the gene product within the human skin [10]. Although this method is thought to involve a complex processes, the detailed mechanisms of uptake of DNA by keratinocytes have not yet been fully elucidated.

The naked DNA injection method can also be used to transfer genes into myocytes. Intramuscular injection of plasmid DNA expressing cytokine genes into patients with critical limb ischemia has provided favorable results by increasing the rate of angiogenesis [11,12]. Thus, gene transfer into keratinocytes using the naked DNA method also harbors great potential for treating intractable skin diseases. However, plasmid DNA may induce different types of reactions since the skin has more immunological functions than muscle. Towards the clinical use of this method for gene transduction in keratinocyte gene therapy, we have examined whether the injection of naked plasmid DNA could induce any adverse dermatological effects.

Materials and Methods

Preparation of plasmid DNA

Plasmid Bluescript II KS(+) (Stratagene, La Jolla, CA: GenBank accession no. X52327) was used for these experiments. The Bluescript II (BS) is a cloning vector designed to simplify commonly used cloning and sequencing procedures, and is used worldwide.

We obtained plasmid DNA (Ordinary BS) utilizing ordinary preparation techniques with cultured bacteria using a Qiagen Plasmid Kit (Qiagen, Germany). Endofree BS was also prepared using an Endofree Plasmid Kit (Qiagen), in which bacterial lysates are cleared by filtration with QIA filter cartridges and plasmid DNA is purified by gravity-flow QIAGEN anion-exchange tips. The instructions mention that plasmid DNA purified with this kit contains only negligible amounts of endotoxin. To further reduce the endotoxin contamination, we utilized endotoxin-removing gel, Detox-Gel (Pierce, Rockford, IL) in which polymixin B immobilized in agarose gel inactivates endotoxin. We obtained Gel-treated BS by treating Endofree BS with column chromatography containing the removing gel. Calf thymus DNA (Calf DNA: SIGMA, St Luis, IL) was used as the control mammalian DNA. Endotoxin contamination was assayed by the Limulus test (WAKO, Osaka, Japan) and the values represents the mean $\pm$ SD of the 5 samples.

Injection of plasmid DNA to the skin

To evaluate the effects of plasmid DNA in the skin, we injected DNA into Hirosaki hairless rats and control human individuals. The DNA samples were diluted in saline to obtain plasmid DNA at an appropriate concentration and were then intradermally injected into the rat and human skin using a 29 G needle. We injected saline only as control. Since we needed to inject plasmid DNA as superficially as possible into the dermis for the proper transfer of DNA into rat keratinocytes [13], we also tried to inject superficially in this study. The injected volume was 30 μl per injection site. The injected site was observed carefully 48 hr after the injection and the skin condition was graded according to the following scores, 0: no reaction, 1: slight erythema, 2: erythema, 3: indurated erythema, 4: erythema with a hemorrhage or blister. We used 6 rats for each experiment and three series of dilution were injected into the back skin of each rat. Also three

normal human volunteers were enrolled for each experiment and three series of dilution were injected into bilateral flexor aspects of forearms. The inflammatory index was calculated as the mean of scores of three areas. The values shown in this study represents the mean ± SD of 6 individual indexes. Experiments were repeated three times. Skin biopsy specimens were taken from the treated sites several times after introduction. This study was approved by the Ethical Committee at Hokkaido University Graduate School of Medicine. Informed consent was obtained from all participating individuals.

Migration assay

Human monocytes and neutrophils were obtained from venous blood from normal volunteers by a routine method. Briefly, erythrocytes were sedimented by addition of 6% dextran saline solution, and then the neutrophil and mononuclear cell fractions were obtained by centrifugation with Lymphoprep (Nycomed Pharma, Oslo, Norway). The mononuclear cells were further allowed to adhere to sterile tissue culture plates for 30 min and were then treated with 1 mM EDTA-PBS containing 5% serum. Monocyte or neutrophil migration was performed using 24-well transwell plate with a 5-nm pore size, untreated (monocyte) or endothelialized (neutrophil) polycarbonate membrane. Plasmid DNA or Calf DNA (25 μg/ml) was placed in the upper, lower or both upper and lower chambers, and then 1 x 10^5 monocytes, or 1 x 10^6 neutrophils, were seeded in the upper chamber. Neither plasmid DNA nor calf thymus DNA was added in control. After incubation for 3 h (monocyte) or 1.5 h (neutrophil), migrated cells were counted.

Monocytes IL-6 production

Human monocytes were prepared by the methods mentioned above. The cells were prepared at a concentration of 2.0 x 10^5/ml and were cultured in 24-well plates in the presence or the absence of plasmid DNA for 24 h. The supernatants were harvested, filtered (using 0.2-μm filters) and stored at -80°C. By comparing the OD of the samples with a standard curve, the concentration of the IL-6 in the culture supernatant was determined. The ELISA kit (R&D Systems: Minneapolis, MN) was performed according to the manufacturer's instruction.

Neutrophil reactive oxygen production

Human neutrophils were prepared by the same method as mentioned above. Production of O_2^- was measured by the rate of reduction of ferricytochrome *c* [14]. Approximately 2.0 x 10^5 cells were incubated at 37°C in Tyrode's buffer (2.6 mM KCl, 1 mM $MgCl_2$, 137 mM NaCl, 6 mM $CaCl_2$, 0.1% glucose, and 1 mM Tris, pH 7.4) containing 80 μM cytochrome *c* with or without plasmid DNA. The absorbance of each supernatant was measured in a spectrophotometer at 550 nm. The extinction coefficient of ferricytochrome *c* at 550 nm was taken as 2.1 x 10^4 M^{-1} cm^{-1}. O_2^- production was expressed as a nanomolar concentration of cytochrome *c* reduced per the cell.

Inflammatory reaction in TLR-9 knockout mouse

The inflammatory reaction induced by plasmid DNA was examined using a knockout TLR9 gene mouse [15]. We injected 10 μl of plasmid solution into the dorsal and ventral aspects of the right ears of the knockout mice (TLR9 -/-) and wild type littermates (TLR9 +/+) whereas the left ears were treated with saline. We used 7 mice for each experiment. The ear thickness of the mice was then measured using an engineer's micrometer 48 h after the initial injection. The inflammatory reaction was then evaluated by measuring the difference in thickness between the right and left ears. The values represents the mean ± SD of 7 individual animal's differences in ear thickness. Experiments were repeated three times.

Statistical analysis of data

Significant differences between groups were evaluated using the Student's *t* test.

Results

Plasmid DNA induces an inflammatory reaction in rat skin

First, we prepared Ordinary BS and Endofree BS, subsequently injected various doses of the samples into rat skin intradermally and observed the treated sites 48h after the initial treatment. The introduction of the genes into rat keratinocytes using this method, usually involved the injection of 3-6 μg plasmid DNA into the site [13]. A dose of 10 μg caused slight erythema using the Ordinary BS sample while the sites of the Endofree BS and Calf DNA samples failed to show any inflammatory reactions (Fig 1A). The inflammatory index (see materials and methods) using 10 μg of Ordinary BS was 0.75. However, the inflammatory reaction using the Ordinary BS sites increased with the DNA dose. Furthermore, an increase in the dose, induced a greater erythematous reaction when using the Endofree BS plasmid (Fig. 1A). We did not observe any reaction in the injection sites when Calf DNA (mammalian DNA control) or saline (DNA free vehicle control) was performed (data not shown).

Next, we observed over a time course for the histological changes in the site of the inflammatory reaction induced by plasmid DNA. Endofree BS (30 μg) was injected into rat skin and the injected site was histologically examined at various time points after injection. We found infiltration of leukocytes in the upper dermis 24h after gene transfer (Fig 2). The 48h sample demonstrated significant leukocyte infiltration, telangiectasia and intracellular edema of the epidermis. Afterwards the inflammation gradually decreased.

Presence of endotoxin contamination in plasmid samples

Since we found erythematous reactions even in the Endofree sample, we examined the concentration of endotoxin in the plasmid samples. A high level of endotoxin contamination was observed in the Ordinary BS, while the Endofree BS samples showed only a small amount of endotoxin (Table 1). There was no detectable endotoxin in the Calf DNA and the Gel-treated BS samples. These samples were obtained by passing the Endofree BS samples through a column chromatography to remove the endotoxin contaminants.

Plasmid DNA induces an inflammatory reaction in the human skin

Since a high level of endotoxin contamination was observed in the Ordinary BS, we used only the Endofree BS and the Gel-treated BS for the following human experiments. Various does of the plasmid samples were injected into the flexor aspects of forearms of three volunteers. At a maximum dose of 16 μg, erythema was observed at the sites of the Endofree and Gel-treated BS, and inflammation at the site of the Endofree treatment was considerably stronger that that of the Gel-treated BS site (Fig 1B). The erythematous reaction of both samples became weaker with decreasing DNA concentrations. The sites of Calf DNA and saline did not show any reaction. Histological examination of the 16 μg Endofree section demonstrated dermal infiltration of neutrophils and mononuclear cells (Fig. 3). The majority of the infiltrating mononuclear cells were CD3 (-), CD4 (-) and CD8 (-) while expression of CD68 was found, indicating that these cells were monocytes (Fig.3). Histological findings of the 16 μg Gel-treated BS sections were basically the same as that of the Endofree BS sections, but the extent of infiltration at the Endofree injection sites was much greater that that of the Gel-treated BS.

Activation of monocytes by plasmid samples

Since we had identified the infiltrating cells as predominantly monocytes and neutrophils, we then examined which subsets of cells responded primarily to the plasmid DNA samples. Our *in vivo* experiments had suggested that any contamination of endotoxin in the Endofree BS sample could enhance plasmid-DNA-induced inflammation. We used the Gel-treated BS samples in the next experiment, a cell migration assay. Plasmid DNA was added to the upper, lower, or both upper and lower chambers. Migration assays using monocytes showed that the cell numbers of the samples with plasmid in upper and upper/lower chambers increased whereas that in lower camber was constant (Fig 4). Calf DNA had no effect on the cell number when compared with control samples. These results indicated that plasmid DNA enhanced monocyte chemokinesis rather than chemotaxis. However, neutrophil migration assays showed no changes in the cell numbers. Collectively, plasmid DNA directly activates or modulates only monocyte chemokinesis, and not transendothelial neutrophil migration *in vitro*.

To further confirm the extent of monocyte involvement in the

inflammatory reactions, we examined what effects the Gel-treated BS has on monocyte IL-6 production and reactive oxygen production by neutrophils. The results suggested that the plasmid DNA increased IL-6 production, while reactive oxygen production was not affected by plasmid DNA (Fig 5). These results also suggested that the primary cells that responded to the plasmid DNA were monocytes rather than neutrophils.

Involvement of TLR9

It was recently shown that cellular responses to the cytosine-guanosine dinucleotide (CpG) motif in bacterial DNA were mediated by TLR9. We therefore examined whether there was any involvement of the TRL9 in plasmid-DNA-induced skin inflammation using a TLR9 knockout mouse. We intradermally injected Gel-treated BS plasmid samples and calf thymus DNA into the ear of TLR9 knockout mice and measured the thickness of the treated ear. An approximate 40 % decrease in the ear swelling was noted in the TLR9 deficient (-/-) mice in the plasmid sample as compared with TRL9 (+/+) littermates. Histological examination of the treated ears also showed similar results (Fig. 7). Although prominent leukocyte infiltration and skin thickening was observed in littermates treated with plasmid, this inflammation was clearly inhibited in TLR9 (-/-) mice. This result suggested that plasmid DNA-induced dermatitis was also mediated by TLR9 expressed by monocytes.

Discussion

Intradermally injected plasmid DNA is taken up and expressed by keratinocytes *in vivo*. This technique alone or combined with the other methods are promising candidate clinical treatments for keratinocyte gene therapy. In fact, the injection of plasmid DNA encoding vascular endothelial growth factor into the skeletal muscle has been successful in patients with critical limb ischemia, resulting in clinical improvement [11]. However, bacterial DNA including many plasmids also induces a strong inflammatory reaction in the respiratory tract and knee joint when applied to these areas [16,17]. Therefore, in this study we examined whether bacterial plasmid DNA introduced into the skin would induce any adverse cutaneous effects.

One of the problems in the use of bacterial DNA is endotoxin contamination,

which produces an apparent dermatitis [18]. Thus, attempts should be made to decrease the levels of endotoxin contamination in plasmid DNA preparations. In clinical trials using intramuscular injection, the vascular endothelial growth factor gene plasmids were obtained using a QIAGEN preparation kit [11]. In this study, we first injected the Ordinary and Endofree BS into normal rat skin. Both samples elicited cutaneous inflammation, although the inflammatory reaction in the Ordinary BS treatment was stronger than that of Endofree BS sites. We hypothesized that the endotoxin remaining in plasmid samples was likely to be at least partially responsible for the dermatitis and we therefore measured the endotoxin content in these samples. The results showed that a high and low level of endotoxin was detected in both the Ordinary and Endofree samples, respectively. There was no detectable endotoxin in the Calf DNA and the Gel-treated BS. Previous reports have applied plasmid DNA at 50 μg in 100 μl solution (0.5 μg/μl) but have failed to describe any skin changes after delivery [19, 20]. This study also found that Endofree BS plasmids induced an erythematous reaction not at 10 μg in 30μl solution (0.33 μg/μl), but at 20 μg in 30μl solution (0.67 μg/μl).

In human skin, a maximum dose of 16 μg plasmid DNA induced an erythematous reaction at sites of both the Endofree BS and Gel-treated BS samples. Inflammation at the sites of the Endofree samples was much greater than that of the Gel-treated BS (Fig 1). The reaction at injection sites of Calf DNA and saline failed to show any inflammation. The results indicated that plasmid DNA itself, could cause an inflammatory skin reaction while endotoxin remaining in the Endofree BS samples only marginally worsened the reaction. Histological examination of these inflammation sites showed infiltrations of neutrophils and mononuclear cells in the dermis, and further immunohistochemistry demonstrated that the infiltrating mononuclear cells were predominantly CD3 (-), CD4 (-), CD8 (-), CD68 (+) compatible with monocytes (Fig.3).

To further determine which immune cells played a major role in this plasmid DNA induced dermatitis, we performed several experiments including migration, IL-6 production, and reactive oxygen species production assays. The results indicated that the primary responsible cells were monocytes rather than neutrophils. A previous paper has showed that arthritis can be induced by bacterial DNA and that this is also mediated by monocytes [17]. This suggests that innate immunity related to monocytes might be involved in plasmid-induced

dermatitis rather than the acquired immunity related with lymphocytes.

As mentioned above, bacterial DNA is a potent stimulus for vertebrate immune cells and systems. Specifically, the presence of unmethylated cytosine-guanosine dinucleotide (CpG) sequence motifs is responsible for its immunostimulatory activity [21,22]. CpG motifs are present more frequently in bacterial DNA than vertebrate DNA, and the cytosines in CpG dinucleotide sequences in vertebrate DNA are highly methylated in contrast to bacterial DNA. Recent findings indicate that the toll-like receptor (TLR) 9 is critical in the recognition of CpG motifs of bacterial DNA [15]. In fact, oligonucleotides with CpG motifs were shown to stimulate IL-6 and IL-12 production by monocytes[15]. So far, ten TLRs have been identified in mammalian systems [23,24]. TLRs are broadly expressed in various tissues and the complete panel of TLR mRNA is expressed in the spleen and peripheral blood [25,26]. The greatest variety of TLR mRNAs is found in specialized phagocytes, suggesting a key role for TLRs in innate immunity. Endotoxin, a compound that is known to cause skin inflammation is recognized by TLR4. In general, the recognition of ligands by TLRs causes stimulation and activation of signaling pathways that end in NF-kB activation, resulting in the induction of many immunomodulating molecules.

In this study, we intradermally injected plasmid DNA into ear skin, and noted an approximately 40 % decrease in the ear swelling in TLR9 knockout mice compared with TRL9 (+/+) littermates. This inhibition suggested that plasmid DNA-induced dermatitis was mediated mostly by TLR9. However, a decrease of 40 % implies that pathways other than TLR9 might be involved in the reaction. In this study, we are aware that even small amounts of endotoxin contamination can worsen the skin plasmid DNA reaction. Although endotoxin and plasmid DNA basically interact with different TLR, TLR4 and TLR 9, respectively, both stimulate the same signaling pathway resulting in NF-kB activation. Therefore, increases in endotoxin levels can additively or synergistically worsen the skin reaction caused by plasmid DNA. We therefore insist on the strict avoidance of any endotoxin contamination in the clinical use of the naked plasmid DNA injection technique, especially in the skin. We recommend that clinical grade plasmids should be used for even experimental animals in similar future experiments. Furthermore, since the

reaction was dose dependent on the amount of plasmid DNA, it seems important that we should determine the precise minimal dose of DNA for the most efficient expression in human keratinocytes.

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References

1. Edelstein ML, Abedi MR, Wixon J, *et al.* Gene therapy clinical trials worldwide 1989-2004-an overview. *J Gene Med* 2004; **6**: 597-602.

2. Vogel JC. Keratinocyte gene therapy. *Arch Deramtol* 1993; **129**: 1478-1483.

3. Khavari PA. Gene therapy for genetic skin disease. *J Invest Dermatol* 1998; **110**: 462-467.

4. Meng X, Sawamura D, Tamai K, *et al.* Keratinocyte gene therapy for systemic diseases: Circulating interleukin-10 released from gene-transferred keratinocytes inhibits contact hypersensitivity at distant areas of the skin. *J Clin Invest* 1998; **101**: 1462-1467.

5. Ghazizadeh S, Taichman LB. Virus-mediated gene transfer for cutaneous

gene therapy. *Hum Gene Ther* 2000; **11**: 2247-2251.

6. Vogel JC. Nonviral skin gene therapy. Hum Gene Ther 2000; **11**: 2253-2259.

7. Hengge UR, Chan EF, Foster RA, *et al.* Cytokine gene expression in epidermis with biological effects following injection of naked DNA. *Nature Genet* 1995;**10**: 161-166.

8. Hengge UR, Walker PS, Vogel JC. Expression of naked DNA in human, pig, and mouse skin. *J Clin Invest* 1996; **97**: 2911-2916.

9. Sawamura D, Ina S, Itai K, *et al.* In vivo gene introduction into keratinocytes using jet injection. *Gene Ther* 1999; **6**: 1785-1987.

10. Sato M, Sawamura D, Ina S, *et al.* In vivo introduction of the interleukin-6 (IL-6) gene into human keratinocytes: Introduction of epidermal proliferation by the fully spliced form of IL-6, but not by the alternative spliced form. *Arch Dermatol Res* 1999; **291**: 400-404.

11. Baumgartner I, Pieczek A, Manor O, *et al.* Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation* 1998; **97**: 1114-1123.

12. Vale PR, Losordo DW, Milliken CE, *et al.* Left ventricular electromechanical mapping to assess efficacy of phVEGF(165) gene transfer for therapeutic angiogenesis in chronic myocardial ischemia. *Circulation* 200; **102**: 965-974.

13. Sawamura D, Meng X, Ina S, *et al.* In vivo transfer of a foreign gene to keratinocytes using the hemagglutinating virus of Japan-liposome method. *J Invest Dermatol* 1997; **108**: 195-199.

14. Guthrie LA, McPhail LC, Henson PM, *et al.* Priming of neutrophils for enhanced release of oxygen metabolites by bacterial lipopolysaccharide. J Exp Med 1984; 160: 1656-1571.

15. Hemmi H, Takeuchi O, Kawai T, *et al.* A Toll-like receptor recognizes bacterial DNA. *Nature* 2000; **408**: 740-745.

16. Schwartz DA, Quinn TJ, Thorne PS, *et al.* CpG motifs in bacterial DNA cause inflammation in the lower respiratory tract. *J Clin Invest* 1997; **100**: 68-73.

17. Deng GM, Nilsson IM, Verdrengh M, *et al.* Intra-articularly localized bacterial DNA containing CpG motifs induces arthritis. *Nat Med* 1999; **5**: 702-705.

18. Ishikawa Y, Kirikae T, Hirata M, *et al.* Local skin response in mice induced by a single intradermal injection of bacterial lipopolysaccharide and lipid A. *Infect Immun* 1991; **59**: 1954-1960.

19. Glasspool-Malone J, Somiari S, Drabick JJ, *et al.* Efficient nonviral cutaneous transfection. *Mol Ther*. 2000;**2**:140-146.

20. Dujardin N, Van Der Smissen P, Preat V. Topical gene transfer into rat skin using electroporation. *Pharm Res* 2001;**18**:61-66.

21. Krieg AM. Direct immunologic activities of CpG DNA and implications for gene therapy. *J Gene Med* 1999; **1** :56-63.

22. Yamamoto S, Yamamoto T, Tokunaga T. Oligodeoxyribonucleotides with 5'-ACGT-3' or 5'-TCGA-3' sequence induce production of interferons. *Curr Top Microbiol Immunol* 2000; **247**: 23-39.

23. Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol* 2003; **21**:335-376.

24. Underhill DM. Toll-like receptors: networking for success. *Eur J Immunol* 2003; **33**: 1767-1775

25. Zarember KA, Godowski PJ. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J Immunol* 2002; **168:** 554-561

26. O'Neill LA. Immunology. After the toll rush. *Science* 2004; **303:** 1481-1482.

Figure legends

Fig. 1 Plasmid DNA induces an inflammatory reaction in rat and human skin. **A:** We prepared the Ordinary BS and Endofree BS plasmid DNA. Various doses of the samples were injected into rat skin and the injected site was carefully observed 48 h after the first injection. The values of the inflammatory index shown represents the mean ± SD of 6 individual indexes. At a dose of 10 μg, slight erythema was observed in the Ordinary BS sample. An increased dose induced an erythematous reaction in the Endofree BS plasmid site. No reaction was observed in the calf DNA injection site (a control for mammalian DNA). Significant differences between the Ordinary and Endofree BS groups in 10, 20 and 30 μg, $^{※}p<0.01$. **B:** We prepared plasmid DNA using the Endofree BS and Gel-treated BS systems. Various does of the samples were injected into human skin and the injected site was carefully observed 48 h after the injection. The inflammatory index values shown represent the mean ± SD of 6 individual indexes. At a maximum dose of 16 μg, erythema was observed at sites prepare using the Endofree and Gel-treated BS, and the inflammation of the Endofree was much stronger that that of the Gel-treated BS. No reaction was observed in the Calf DNA treated site representing control mammalian DNA. Significant differences between the Endofree and Gel-treated BS groups in 16 μg, $^{※}p<0.01$.

Fig. 2 Time course showing the histological changes in the inflammatory reaction induced by plasmid DNA. Endofree BS plasmid DNA (30 μg) was injected into rat skin and the injected site was examined histologically (a) before, and 24h (b), 48h (c), 72h (d) and 96h (e) after the injection. The 48h sample demonstrated strong leukocyte infiltration, telangiectasia and intracellular edema of the epidermis.

Fig. 3 Plasmid DNA induced-reaction in human skin. a, b) Histological examination of the 16 μg Endofree (a) and Gel-treated (b) BS injected sites demonstrated a dermal infiltration of neutrophils and mononuclear cells. c,d): The majority of the infiltrating mononuclear cells were CD3(-) (c) while there was expression of CD68 positive cells (d).

Fig. 4 Plasmid DNA activates only monocyte chemokinesis, not transendothelial neutrophils migration.

Human monocytes and neutrophils, obtained from venous blood from normal control volunteers, were subjected to migration assays using the Gel-treated BS. The values were expressed as the mean ± SD of between four-six samples. A) The migration assays showed that the cell numbers of the samples with the plasmid in upper and upper/lower chambers increased whereas that in lower camber was constant. The Calf DNA did not show any effect on the cell number. Control: no DNA samples. Significant differences between the upper and lower, and between the lower and upper/lower groups,※ $p<0.01$.

B) The neutrophil migration assay failed to show any change the number of cells.

Fig. 5 Plasmid DNA increases IL-6 production, but does not affect reactive oxygen production. We incubated monocytes and neutrophils with the Gel-treated BS, and then measured IL-6 production and reactive oxygen production, respectively. The values were expressed as the mean ± SD of four-six samples. The results showed that the plasmid DNA increased IL-6 production in a dose dependent manner (A), while reactive oxygen production was not affected by plasmid DNA concentration (B). PMA: positive control. Significant differences between the 10μg/ml and 100μg/ml groups, ※$p<0.02$.

Fig. 6 Involvement of TLR9 in the plasmid DNA induced-reaction. We injected the Gel-treated BS and calf thymus DNA to the right ears of TLR9 knockout mice (TLR9 -/-) and wild type littermates (TLR9 +/+) whereas the left ears were injected with saline alone. The ear thickness of each mouse was measured 48 h after the first injection. The inflammatory reaction was then evaluated by measuring the difference of thickness between the right and left ears. The values represents the mean ± SD of 7 individual differences. A significant decrease in the ear swelling in TLR9 mice (-/-) was observed in

plasmid samples as compared with TRL9 (+/+) mice. ※p<0.01.

Fig 7 Histological demonstration of a decrease in the plasmid DNA induced-inflammation reaction in TLR9 knockout mice . We injected the Gel-treated BS and calf thymus DNA to the right ears of TLR9 knockout mice (TLR9 -/-) and wild type littermates (TLR9 +/+), and the treated ears were examined histologically 48 h after the injection. a) TLR9+/+ with plasmid, b) TLR9 -/- with plasmid, c) TLR9+/+ with calf DNA, d) TLR9 -/- with calf DNA.

Table 1 Contamination of endotoxin in plasmid preparations

Plasmid DNA	Amount of Endotoxin ± SD (EU /μg plasmid DNA)
Ordinary BS	8.5 U / μg ± 1.2
Endofree BS	0.1 U / μg ± 0.02
Gel-treated BS	< 0.001 U/ μg
Calf thymus DNA	< 0.001 U/ μg
Saline	< 0.001 U/ μg

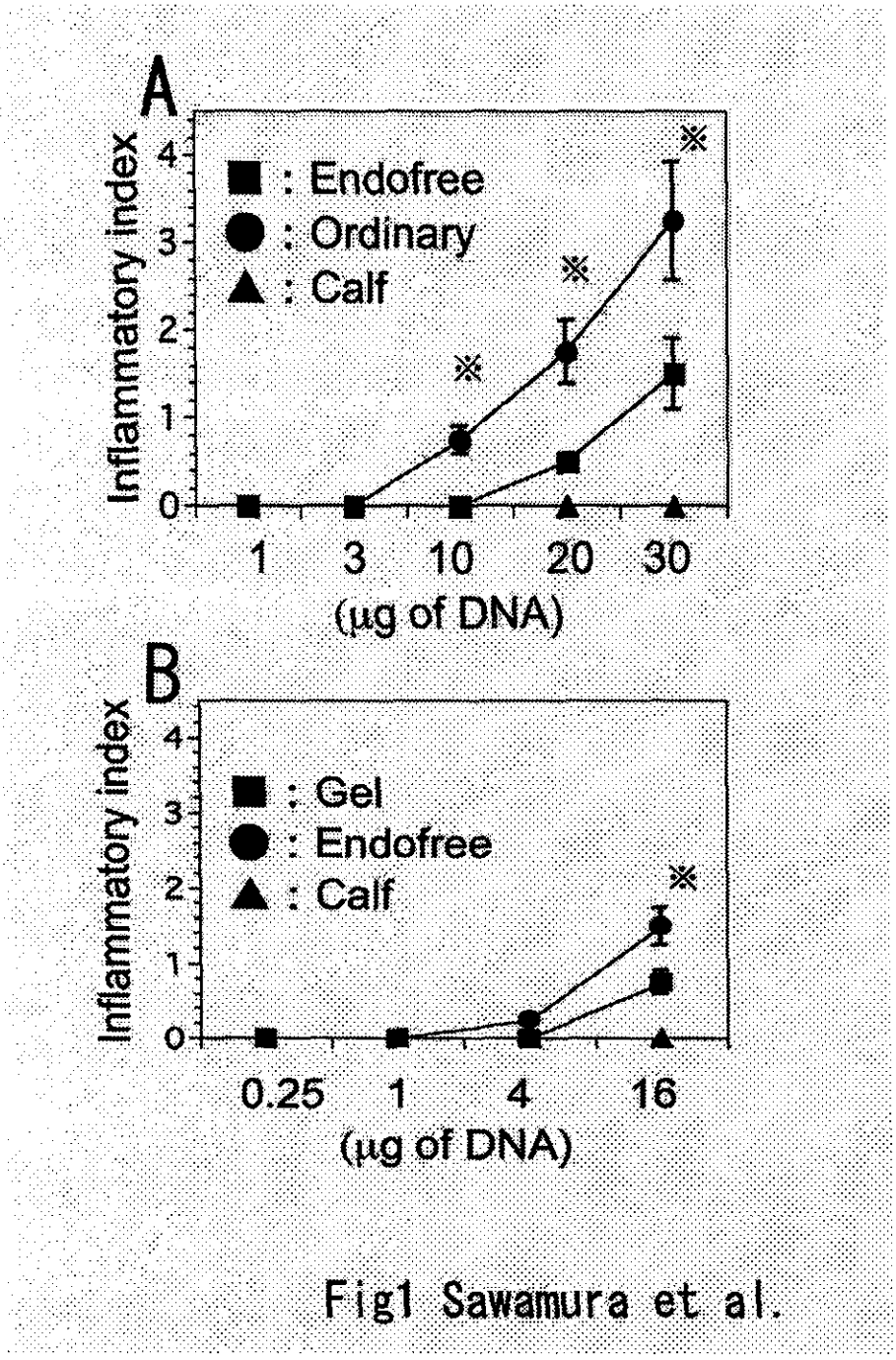
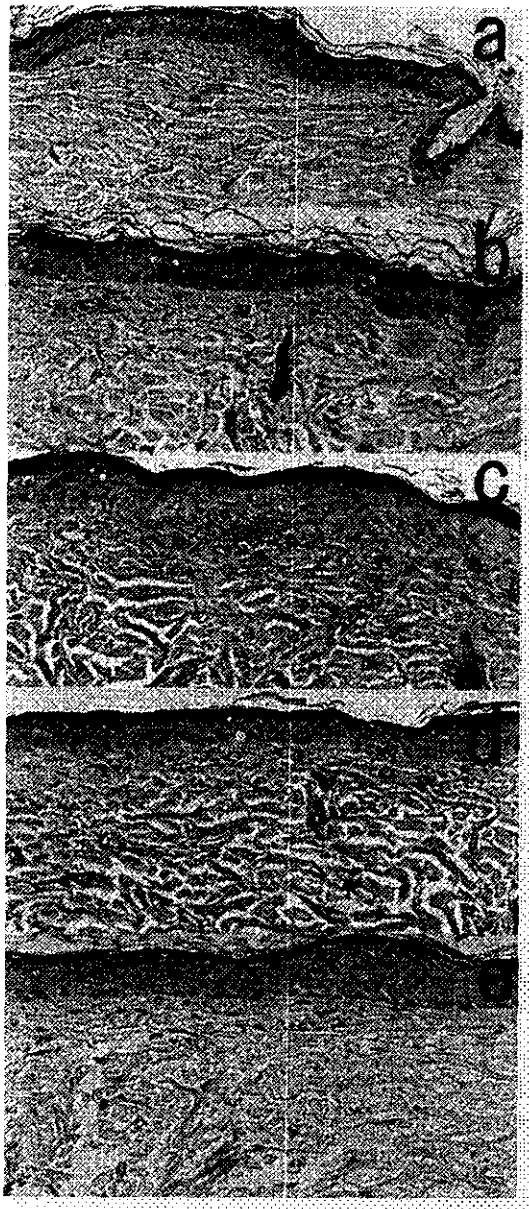


Fig1 Sawamura et al.



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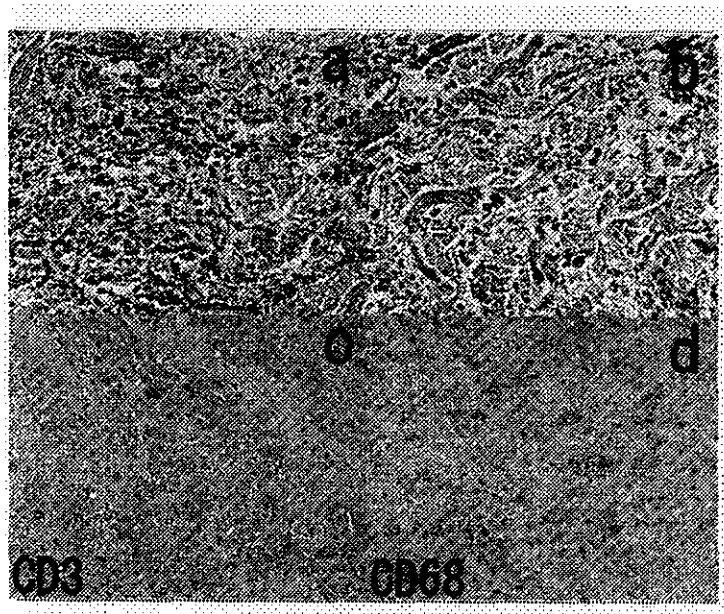

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