

In Vivo Gene Transfer Method in Keratinocyte Gene Therapy: Intradermal Injection of DNA Complexed with High Mobility Group-1 Protein in Rats

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In order to develop a more efficient method of introducing genes into keratinocytes *in vivo*, we intradermally injected DNA bound to high mobility group 1 protein, thereby taking advantages of the naked DNA and hemagglutinating virus of the Japan-liposome method reported previously. First we performed a gel mobility shift assay, which confirmed DNA binding to high mobility group 1. Then we injected β -galactosidase expression vector complexed with high mobility group 1 into the rat skin and the activity of sample with the protein was 2–3 times higher than that without the protein as control. Semiquantification of transferred-DNA content using polymerase chain reaction and a time course of transgene expression in keratinocytes suggested that high mobility group 1 protein increased transfer of the DNA from the cytoplasm to the nucleus. Direct injection of the DNA-high-mobility-group-1 complex is a highly efficient method for introducing genes into keratinocytes in connection with gene therapy.

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Gene therapy is defined as the introduction of an exogenous gene into a host cell in order to obtain a therapeutic benefit (1). One of the target cells in gene therapy for skin diseases is the epidermal keratinocyte. Keratinocyte gene therapy may be applied to intractable genetic, neoplastic and inflammatory skin diseases. In order to achieve keratinocyte gene therapy for use in clinical practice, highly efficient methods of gene transfer to keratinocytes need to be developed. There are 2 basic means for gene transfer, *ex vivo* and *in vivo* systems (2). Because the *ex vivo* approach requires culture of patient cells and *in vitro* transfection before returning cells to the patient, an *in vivo* approach, enabling direct gene transfer to the patients, would be advantageous.

Recently, we succeeded in transferring genes to keratinocytes *in vivo* using hemagglutinating virus of the Japan (HVJ) liposome method (3). The HVJ-liposome method (4) involves encapsulation of DNA in liposomes with HVJ, which enhances fusion of the liposome with cell membranes. Furthermore, complexing DNA with high mobility group (HMG)-1 protein increases the amount of DNA transported into the nucleus (5). HMG-1, the ubiquitous non-histone component of chromatin, is considered to be implicated in DNA replication and cellular differentiation (6). HMG-1 contains an HMG box, a conserved domain of about 80

amino acids that mediates the DNA binding and nuclear localization signal that keeps HMG-1 inside the nucleus. When the plasmid DNA and nuclear protein were co-introduced into cells in rat liver by injection into the portal veins of adult rats, the plasmid DNA was carried efficiently into liver cell nuclei by nuclear protein (4).

The various other methods for *in vivo* gene transfer to keratinocytes include the particle bombardment (7), the adenoviral (8) and the naked DNA (9) methods. Of these methods, the naked DNA method is the simplest and is also relatively efficient. It does not require special equipment and was shown recently to be useful for gene transfer to human keratinocytes *in vivo* (10).

In this study, we examined a modified method, whereby the DNA-HMG-1 complex was injected intradermally, making use of the HVJ-liposome method and the naked DNA method. We demonstrated that keratinocytes incorporated the intradermally-injected complex and that transgene expression was higher in DNA with than without HMG-1.

MATERIAL AND METHODS

Plasmids

Plasmid pCAGGS, a gift from Professor J. Miyazaki, contains the chicken β -actin promoter, the cytomegalovirus-immediate early enhancer, and the 3'-flanking sequence of the β -globin gene (11). pCAGGS exhibits strong expression of the inserted gene in keratinocytes *in vivo* (12). Plasmid pCAGGS-lacZ was constructed by inserting the β -gal gene into pCAGGS. Plasmid pNASS- β (CLONTECH, Palo Alto, CA, USA) is a promoterless β -gal expression vector used as a control.

Gel mobility shift assay

A 567-bp DNA fragment that corresponds to the lacZ gene sequences from bp 1430 to 1996 (13) was amplified by PCR with the primers: 5'-GACGATGGTGCAGGATATCC3' and 5'-TACTGACGAAACGCCTGCCAGT3', since molecular-size change of DNA by binding of HMG-1 is more detectable in short DNA fragments than in long ones. The PCR product was incubated with various doses of HMG-1 for 30 min at 4°C. HMG-1 was a gift from Professor Y. Kaneda (4). After the binding reaction, the DNA-HMG-1 complexes were fractionated on a 1.5% agarose gel by electrophoresis at 4°C at a constant 50 V. The gel was stained with ethidium bromide.

In vivo introduction of genes into keratinocytes

Hirosaki hairless rats (14) were used, and the range of body weights was 180–200 g. Plasmid was incubated with various doses of HMG-1 for 30 min at 4°C. After the binding reaction, the mixture was diluted with phosphate-buffered saline (PBS) to a DNA concentration of

0.2 µg/µl, and 50 µl was injected as superficially as possible into the subepidermal dermis using a 30-gauge needle.

Detection of β-gal activity

We introduced DNA into 5 areas that gave 5 different biopsy specimens. An epidermal sheet obtained from biopsy specimen by dispase (1,000 pU/ml, Godo Shusei, Japan) treatment (2 h at 37°C) was lysed in 200 µl lysis buffer (0.1 M potassium phosphate, pH 7.8, 0.2% Triton X-100 and 1 mM dithiothreitol), centrifuged at 12,000 rpm for 2 min, and a 5 µl aliquot of the cell extract was assayed using the Luminescent β-Galactosidase Genetic Reporter System (Clontech, Palo Alto, CA, USA). Measurements were obtained using a chemiluminometer. β-Gal activity was expressed as light units per µg protein (Protein Assay Kit, BioRad, Hercules, CA, USA) and obtained from 5 individual samples.

Biopsy specimens were embedded in OCT compound, and 10 µm sections were cut and placed in fixative at 4°C for 20 min. All sections were incubated uniformly with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) staining buffer (5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆, 1 mM MgCl₂ and 100 µg/ml X-gal) at 37°C for 2 h and counterstained with nuclear fast red.

Semiquantification of transgenes in keratinocytes using PCR

We introduced DNA into 2 areas that gave 2 different biopsy specimens. Epidermal sheets from the biopsy specimens were obtained after dispase treatment, then digested with trypsin, washed extensively 3 times with PBS, lysed in 40 µl lysis buffer and centrifuged for 2 min (12). A 2 µl aliquot of each cell extract was used as the template for PCR to semiquantify the *lacZ* gene in the cells. We used the primers described in the "gel shift mobility assay". The amount of each PCR product was estimated after electrophoresis on a 1.5% agarose gel and ethidium bromide staining. Before the experiment, we established PCR conditions that gave exponential amplification (11). PCR consisted of 28 cycles of denaturation (94°C, 1 min), annealing (57°C, 1 min) and extension (94°C, 1 min).

RESULTS

HMG-1 binds DNA

In order to examine the binding of HMG-1 to DNA, we performed a gel mobility shift assay. After incubation of 50, 5, 0.5, and 0.05 ng of HMG-1 with 200 ng of the 567-bp PCR fragment, the complexes were fractionated by electrophoresis. A sample with 50 ng albumin was used as a negative control. A band shift was observed in the samples with 50, 5, and 0.5 ng of HMG-1, but little or no shift in the samples with 50 ng albumin and 0.05 ng HMG-1 (Fig. 1). The sample with 50 ng HMG-1 showed a clearly-shifted single band, indicating that binding of HMG-1 to the DNA fragment was saturated (Fig. 1). The binding increased with the dose of HMG-1.

Binding of HMG-1 increases transgene expression

The results of the gel mobility shift assay indicated that 200 ng of DNA was saturated with 50 ng HMG-1. Next, we incubated 2.5 µg HMG-1 or 5 µg albumin with 10 µg pCAGGS-lacZ, injected these complexes intradermally, and obtained skin biopsies 24 h after injection. In both skin sections, β-gal-positive keratinocytes were observed in the epidermis of the injected area and little expression was found in the dermis (Fig. 2). The results demonstrated that HMG-1 did not lead to a higher frequency of expressing cells and also

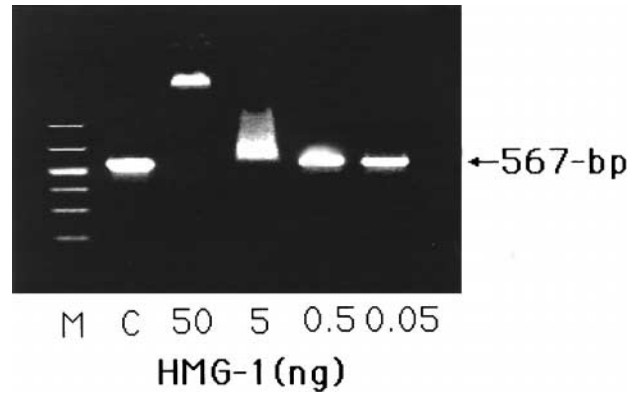


Fig. 1. Gel mobility assay shows binding of high mobility group (HMG)-1 to DNA. A 567-bp DNA fragment from the *lacZ* gene was amplified using PCR, and 200 ng of PCR product was incubated with 50, 5, 0.5, or 0.05 ng HMG-1 for 30 min at 4°C. A sample with 50 ng albumin was used as a negative control. After the binding reaction, the DNA-HMG-1 complex was fractionated on a 1.2% agarose gel by electrophoresis. M: size marker.

did not alter the localization pattern of the β-gal positive cells in the skin histologically. However, examination of several sections revealed that the β-gal activity in sections with HMG-1 seemed to be higher than those without HMG-1.

In the next step, we performed a quantitative assay for β-gal activity. We incubated 10, 1, 0.1 and 0.01 µg HMG-1 and 5 µg albumin with 10 µg pCAGGS-lacZ, injected these complexes intradermally, prepared keratinocytes from the treated skin 24 h after the injection, and measured β-gal activity. The activities of 10, 1, and 0.1 µg HMG-1 samples were 2–3 times higher than 0.01 µg HMG-1 and albumin samples (Fig. 3). The sample of 1 µg pNASS-β plus 1 µg HMG-1 showed no or little activity. The 1:10 HMG-1 to DNA ratio, (1 µg HMG-1: 10 µg pCAGGS-lacZ) gave the highest level of expression.

Next, to determine how HMG-1 binding increases transgene expression, we semiquantified the amount of transferred DNA in keratinocytes 24 h after injection of complexes containing 10 µg and 1 µg HMG-1 or 5 µg albumin with 10 µg pCAGGS-lacZ and 1 µg HMG-1 with 10 µg pNASS-β using PCR. The results showed no clear differences among the band intensities of the PCR products (Fig. 4), indicating that binding of HMG-1 did not alter the amount of DNA transferred to keratinocytes. Furthermore, we examined the time course of activity in samples containing 1 µg HMG-1 or 5 µg albumin with 10 µg pCAGGS-lacZ. The β-gal activities of the samples with and without HMG-1 on days 3 and 7 were expressed relative to the activities on day 1. The results showed that β-gal expression declined gradually in both samples, and no differences were found in the rate of decline between samples (Fig. 5), indicating that binding of HMG-1 did not prolong expression of the plasmid.

DISCUSSION

Recently, the naked DNA (9) and HVJ-liposome (3, 4) methods were reported to be useful for *in vivo* gene transfer to keratinocytes. In HVJ-liposome method, 3–10 times more DNA was transferred to the nucleus when it was bound to HMG-1 than when introduced alone. HMG-1 binds DNA,

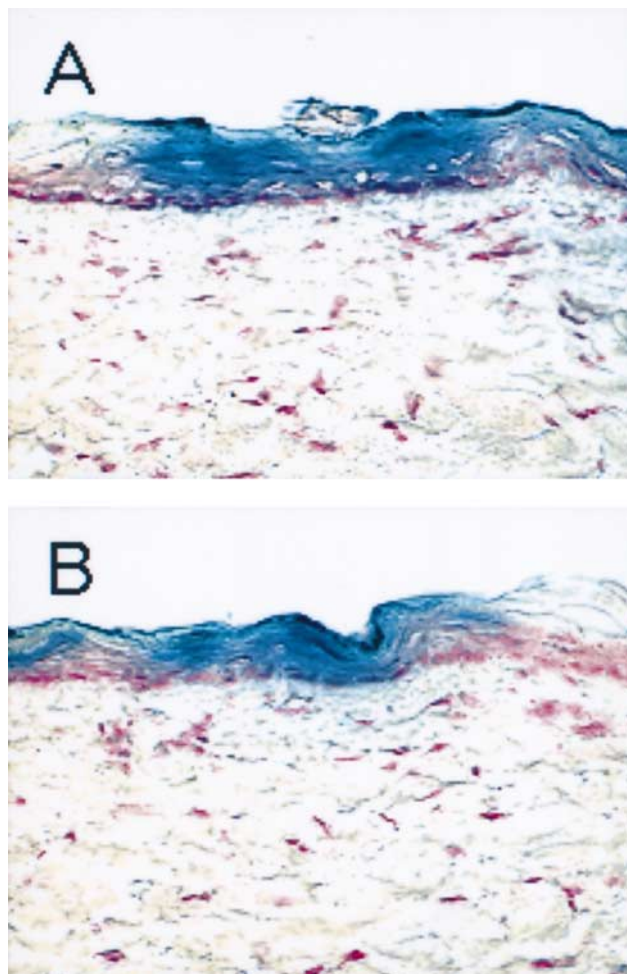


Fig. 2. Keratinocytes take up and express high mobility group (HMG)-1-bound DNA. We injected 2.5 µg HMG-1 or 5 µg albumin complexed with 10 µg pCAGGS-lacZ, obtained skin biopsy 24 h after transfection, and performed β-gal staining. Samples with (A) HMG-1 and (B) albumin.

stabilizes its molecular structure, and contributes to the transfer of DNA to the nucleus through the nuclear localization signal (5). For gene transfer efficiency to keratinocytes, the naked DNA method is better than the HVJ-liposome method (3). We hypothesized that co-introduction of DNA with HMG-1 might increase the transfer efficiency of the naked DNA method. However, the molecular size and ionic charge of the DNA-HMG-1 complex are different from those of DNA alone, and we did not know if the intradermally-injected complex could transfer to the epidermis and go through the keratinocyte membrane.

First, to determine the binding of DNA with HMG-1, we incubated the 567-bp PCR fragment with several doses of HMG-1 and performed a gel mobility shift assay. HMG-1 binds DNA, not in a sequence-specific, but in a structure-specific manner, and induces a considerable bend in the DNA by making contact primarily in the minor groove of DNA (15). This indicates that the binding of HMG-1 does not depend on length and sequence of DNA. Also, a molecular size change of DNA by binding to HMG-1 is more apparent in a short PCR fragment than in a long one. Therefore we used relatively short DNA fragments for a gel mobility shift

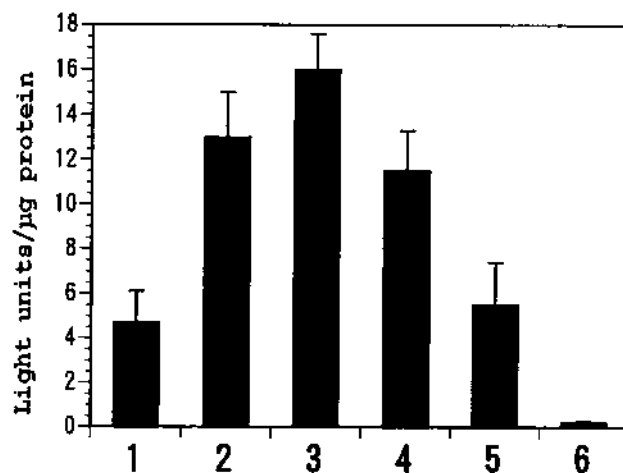


Fig. 3. HMG-1 increases transgene expression. We injected 10, 1, 0.1 and 0.01 µg HMG-1 or 5 µg albumin complexed with 10 µg pCAGGS-lacZ, obtained keratinocytes from the treated area 24 h after injection, and performed a β-gal assay. Each value shown represents the mean $\pm$ SD of 5 individual samples. pCAGGS-lacZ with 5 µg of albumin (1), pCAGGS-lacZ with 10 µg (2), 1 µg (3), 0.1 µg (4) and 0.01 µg (5) of HMG-1, and pNASS-β with 1 µg of HMG-1 (6).

assay. The results showed a band shift, which confirmed the binding of DNA to HMG-1 and suggested that a saturation of the binding occurred in a sample consisting of 50 ng HMG-1 and 200 ng DNA (Fig. 1). Next, we incubated 2.5 µg HMG-1 or 5 µg albumin with 10 µg pCAGGS-lacZ, injected these complexes intradermally, obtained skin biopsies 24 h after injection, and then performed β-gal staining of each skin section. The presence of HMG-1 did not alter the number and the localization pattern of the β-gal positive cells in the skin histologically. Since histological examination is not suitable for quantitative assay, we measured β-gal activity in the epidermis of the treated rat skin. The activities of HMG-1 samples were 2–3 times higher than those of the albumin sample as a negative control (Fig. 3) and the 1:10 HMG-1 to DNA ratio, (1 µg HMG-1: 10 µg pCAGGS-lacZ) gave the highest level of expression.

We revealed that co-introduction of HMG-1 increased transgene expression about 3-fold. It is possible that HMG-1-bound DNA crossed the keratinocyte membrane easier than

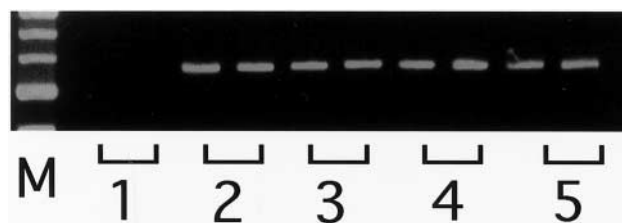


Fig. 4. High mobility group (HMG)-1 does not change the amount of transferred DNA. We injected 10 and 1 µg HMG-1 or 5 µg albumin complexed with 10 µg pCAGGS-lacZ and 1 µg HMG-1 with 10 µg pNASS-β, obtained keratinocyte lysate from the treated area 24 h after injection, and performed PCR to semiquantify the gene amount. pCAGGS was also injected as control vector without the lac Z. pCAGGS plus 1 µg of HMG-1 (1), pCAGGS-lacZ plus 10 µg (2) and 1 µg (3) of HMG-1, pCAGGS-lacZ with 5 µg of albumin (4), and pNASS-β with 1 µg of HMG-1 (5).

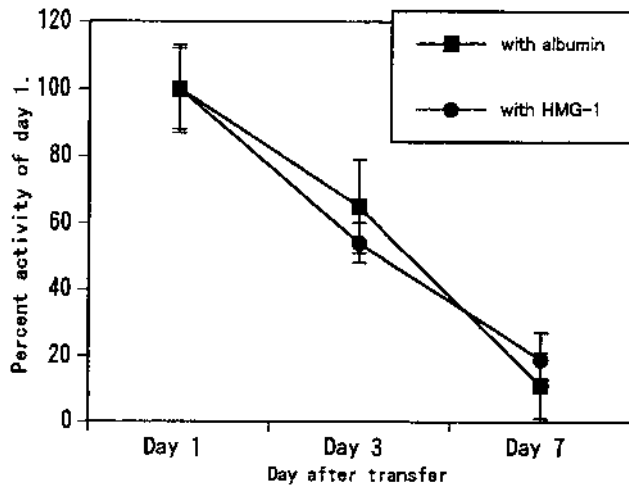


Fig. 5. High mobility group (HMG)-1 does not confer prolonged expression of the plasmid. We injected 1 μ g HMG-1 or 5 μ g albumin (control) complexed with 10 μ g pCAGGS-lacZ, obtained keratinocytes from the treated area 1, 3, and 7 days after injection and performed a β -gal assay. The β -gal activities of the samples with and without HMG-1 on days 3 and 7 are expressed relative to those on day 1. Each value represents the mean $\pm$ SD of 5 individual samples.

DNA alone. To measure the amount of transgene in the cell, we carried out PCR experiment. Although we first prepared genomic DNA from treated keratinocytes and performed Southern blot analysis with the β -gal gene as a probe, we could not detect clear bands (data not shown). PCR showed no differences between samples with or without HMG-1, suggesting that the amounts of the transgene in keratinocytes did not differ. Another possibility is that HMG-1 stabilizes DNA and prevents DNA from degradation in keratinocytes. If this is true, co-introduction of HMG-1 causes prolongation of transgene expression. However, the time course of activity in samples with or without HMG-1 showed no differences in the rate of decline. It has been shown that 3–10 times more DNA was transferred to the nucleus when it was bound to HMG-1 than when introduced alone in HVJ-liposome method (5). We suggest that also when injected into the skin, DNA-bound HMG-1 enhanced transfer of DNA from the cytoplasm to the nucleus, resulting in an increase in transgene expression.

Our method may be of future value in keratinocyte gene therapy. Furthermore, a recombinant fusion protein consisting of HMG-1 and a domain with a special function could be added to DNA using HMG-1 as glue. In keratinocyte gene therapy, it is important to introduce genes into basal keratinocytes or keratinocyte stem cells. In theory, if one injects a complex of DNA and a fusion protein that contains ligands to receptors of these cells, it should be possible to transfer genes specifically to basal keratinocytes or keratinocyte stem cells.

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