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ORIGINAL PAPER

Masanori Sato · Daisuke Sawamura · Shinsuke Ina
Tadashi Yaguchi · Kastumi Hanada · Isao Hashimoto

In vivo introduction of the interleukin 6 gene into human keratinocytes: induction of epidermal proliferation by the fully spliced form of interleukin 6, but not by the alternatively spliced form

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Abstract **The achievement of keratinocyte gene therapy in clinical practice requires fundamental experiments using human keratinocytes or skin. We have recently demonstrated that the in vivo introduction of the interleukin 6 (IL-6) gene into rat keratinocytes induces epidermal proliferation and lymphocyte infiltration into the skin. In this study, we first amplified the human IL-6 cDNA from oligo-dT-primed keratinocyte cDNA and then detected the fully spliced (FS) form and the alternatively spliced (AS) form of IL-6 cDNA. Sequence analysis showed that the AS form, which was composed of the IL-6 coding region with all of exon II deleted except for the first guanine, was identical to that reported to be present in lymphocytes. We constructed the expression vectors phIL6 of the FS form and phIL6S of the AS form. We transplanted human skin onto nude rats and introduced phIL6 and phIL6S into the human keratinocytes using the naked DNA method. Keratinocytes prepared 24 h after introduction from the areas treated with them were examined by reverse transcriptase (RT)-PCR and enzyme linked immunosorbent assay (ELISA). RT-PCR showed that the amounts of FS IL-6 mRNA and AS IL-6 mRNA were similar, whereas the ELISA showed that the amount of FS IL-6 peptide was four times that of the AS IL-6 peptide. Histological examination 48 h after introduction showed that the FS form had induced epidermal proliferation, whereas the AS form had not. The epidermal thickening without lymphocyte infiltration induce by the FS form indicates that keratinocyte proliferation is caused by a direct effect of overexpressed IL-6, and not by a secondary effect of infiltrating lymphocytes. This is the first report of the introduction of a human gene into human keratinocytes to produce a biologically active transgenic gene product in human skin using the naked DNA method.**

Key words Interleukin 6 · Keratinocyte · Gene therapy · Alternative splicing

M. Sato · D. Sawamura (✉) · S. Ina · T. Yaguchi · K. Hanada
I. Hashimoto
Department of Dermatology,
Hirosaki University School of Medicine, 5 Zaifu-cho,
Hirosaki 036–8562, Japan
e-mail: smartdai@hirosaki-u.ac.jp
Tel. +81-172-39-5087; Fax +81-172-37-6060

Introduction

Intractable skin diseases are potential candidates for the use of keratinocyte gene therapy. To realize keratinocyte gene therapy in clinical practice, we need to develop highly efficient methods of gene transfer to keratinocytes. Various in vivo methods including particle bombardment [1], adenoviral [2], naked DNA [3] and hemagglutinating of Japan-liposome [4] methods have been reported. Subcutaneous injection of naked DNA has been shown to transfer genes efficiently and preferentially into keratinocytes in vivo [3, 4]. This method is simple, does not require special equipment and can introduce DNA into the keratinocytes of fully developed adult animals. Hennge et al. [5] were the first to succeed in transferring the bacterial β-galactosidase (β-gal) gene into human keratinocytes in vivo using this method. Although this is the first report of the introduction of genes into human keratinocytes using an in vivo method, the biological effect of the human transgenic protein produced from a transgene in human keratinocytes has yet to be reported.

IL-6 is a cytokine that exerts various effects on a wide range of target cells [6–8]. Grossman et al. [9] have demonstrated that IL-6 stimulates the proliferation of cultured human keratinocytes and that it is strongly expressed in the skin lesions of psoriasis, a common skin disease characterized by epidermal hyperplasia and infiltration by polymorphonuclear leukocytes and T cells. We recently introduced the IL-6 gene into rat keratinocytes in vivo by the naked DNA method; the overexpression of IL-6 induced macroscopic erythema, histologically evident epi-

dermal proliferation and lymphocytic infiltration of the treated area of the skin [10].

In this study, we grafted human skin onto nude rats and introduced expression vectors of human fully spliced (FS) or alternatively spliced (AS) IL-6 cDNA into the human keratinocytes using the naked DNA method. We observed epidermal proliferation in the skin treated with the FS IL-6 cDNA gene. This is the first report of the introduction of a human gene into human keratinocytes to produce a biologically active transgenic gene product in human skin using the naked DNA method. Our results suggest that the naked DNA method has the potential to be used for keratinocyte gene therapy to treat human skin diseases in the future.

Materials and methods

Construction of human expression vectors

To amplify the 639-bp coding region of the human IL-6 cDNA by PCR, we synthesized two primers, 5′-GGGAAGCTTGCTATGAACTCCTCCTCCACA-3′ and 5′-GGGGAATTCATGCTACATTTGCCGAAGAGC-3′, based on the sequence of the human IL-6 gene [11]. The primers had restriction enzyme sites at their 5 ends for subcloning. PCR was performed with oligo-dT-primed human keratinocyte cDNAs as the templates and consisted of cycles of denaturation (94 °C, 1 min), annealing (57 °C, 1 min), and extension (72 °C, 1 min). The DNA fragments were subcloned into TA cloning vector (Invitrogen, SanDiego, Calif.) and sequence analysis of the DNA fragments was performed. They were digested with *EcoRI* and *HindIII*, and subcloned into a pCY4B expression vector, which contains the cytomegalovirus immediate early enhancer, the modified β-actin promoter and the 3′ flanking sequence of the β-globin gene. The pCY4B plasmid was constructed by deleting the SV40 origin of replication from pCAGGS, but their functional fragments were all the same in terms of gene expression [12]. We have previously shown that these expression vectors express strongly the inserted gene in keratinocytes in vivo [13]. The combination of the naked DNA method and these strong vectors results in the strong expression of the IL-10 gene in keratinocytes in vivo, and the transgenic IL-10 peptide can be detected in the bloodstream and exerts biological effects in distant areas of the skin [14]. A plasmid containing the human IL-6 cDNA without the eukaryotic promoter ph(–), was used as a control.

Skin graft

Nude rats (F344/N Jcl-rnu) were obtained commercially (Clea Japan, Japan). Human skin was excised, dissected from the underlying soft tissue, and transplanted into the back skin of the rats according to the method of Lane et al. [15]. The site for transplantation was first prepared by excising an area of rat epidermis and dermis equal to the size of the human graft. The graft was placed on the prepared site and an occlusive dressing was quickly placed over the graft to hold it in position and to prevent it from drying. The dressing was removed after 5 days and gene introduction was carried out after 7 days.

In vivo DNA transfer into keratinocytes

A plasmid was injected into the graft on the back of each nude rat, which had been anesthetized with 3.6% chloral hydrate at 1 ml/100 g body weight. Each plasmid DNA was diluted in PBS(–) to 0.2 μg/μl. The DNA was injected using a 29 G needle and the injected volume was 40 μl per injection site. Skin biopsy specimens were taken from the injection sites 24 h and 48 h after injection.

Assay for human IL-6 mRNA and protein

IL-6 expression vector (8 μg) was injected into the grafted human skin and the injection site was biopsied 24 h later. Biopsy specimens were treated with dispase to obtain epidermal sheets [4]. Total RNA was extracted from each epidermal sheet. To remove contaminating plasmid DNA, the RNA sample was incubated with 50 U/ml RNase-free DNase for 2 h at 37 °C [3], followed by phenol/chloroform extraction and ethanol precipitation. Oligo-dT-primed keratinocyte cDNA was synthesized with reverse transcriptase (RT). PCR was performed using the above primers for human IL-6 cDNA and consisted of 29 cycles of denaturation (94 °C, 1 min), annealing (57 °C, 1 min), and extension (72 °C, 1 min). PCR products were fractionated by agarose gel electrophoresis.

The epidermal sheets were also suspended in 0.25 *M* Tris (pH 7.8), lysed by three cycles of freeze-thawing and centrifuged at 5000 *g*. The concentration of human IL-6 in the supernatant was measured using a human IL-6 ELISA kit (Cytoscreen, Camarillo, Calif.) and is expressed as picograms per nanogram protein (Protein Assay Kit, Bio-Rad, Hercules, Calif.). The assay reactions were performed in triplicate.

Immunohistochemical staining

phIL6 (8 μg) was injected into the grafted human skin and skin biopsy specimens were taken from the treated sites 24 h and 48 h after introduction. The specimens were fixed, paraffin-embedded, and 5 μm sections cut. A monoclonal antibody of proliferating cell nuclear antigen (PCNA) (YLEM, Roma, Italy) was used for immunostaining [16]. An antihuman IgG mouse monoclonal antibody was used as an isotype control. Amplification of staining using diaminobenzidine was performed with a PAP Kit (Dako, Carpinteria, Calif.). The remaining operations were carried out according to the manufacture's instructions.

Results

Amplification of human IL-6 cDNA by PCR

After PCR had been performed with oligo-dT-primed human keratinocyte cDNAs as templates, the PCR product was electrophoresed on a 1.5% agarose gel. Two single clear bands were detected by 37 cycles of PCR, whereas 29 cycles did not show and bands. The stronger band was about 0.65 kb in size and the weaker one was about 0.45 kb in size (Fig. 1). After subcloning these DNA fragments into TA cloning vector, we performed sequence analysis. The 0.65 kb DNA fragment corresponded to the coding area of the human IL-6 cDNA [11]. The 0.45 kb fragment was identical to the IL-6 coding region with all of exon II deleted except for the first guanine [17]. In spite of this alternative splicing, the frame between exons I and III remained and the isoform of IL-6 was synthesized from the AS transcript [17]. We constructed expression vectors containing the FS cDNA (0.65 kb) and AS cDNA (0.45 kb) and called them phIL6 and phIL6S, respectively.

Detection of IL-6 mRNA and protein expression

We injected phIL6, phIL6S, and ph(–) into the grafts, and 24 h later took a biopsy, extracted the total keratinocyte RNA, prepared oligo-dT-primed cDNAs and performed

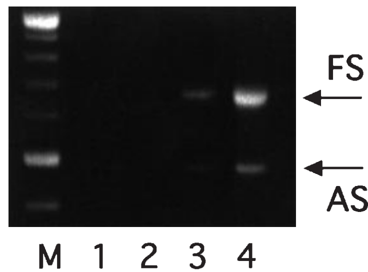

Fig. 1 Detection of FS and AS IL-6 mRNA in keratinocytes by RT-PCR. Total RNA was extracted from keratinocytes and reverse transcriptase-PCR was performed using primers to amplify the human IL-6 cDNA. PCR consisted of cycles of denaturation (94 °C, 1 min), annealing (57 °C, 1 min), and extension (72 °C, 1 min). Two single bands, about 0.65 kb (the FS from) and 0.45 kb (the AS form) in size, were detected. PCR cycles for lanes 1, 2, 3 and 4 were 25, 29, 33 and 37, respectively (*M*, size marker)

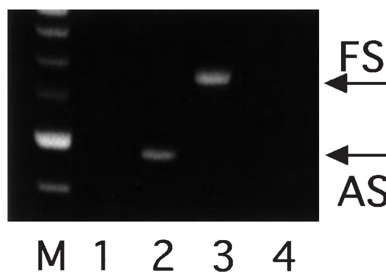

Fig. 2 Detection of FS and AS forms of IL-6 mRNA after the introduction of phIL6 and phIL6S, respectively. We prepared oligo-dT-primed cDNA from treated rat keratinocytes 24 h after the introduction of phIL6, phIL6S and ph(–) and performed PCR using primers to amplify the human IL-6 cDNA. PCR consisted of 29 cycles of denaturation (94 °C, 1 min), annealing (57 °C, 1 min), and extension (72 °C, 1 min). The expected 0.45-kb (AS) and 0.65-kb (FS) bands were observed in the samples treated with phIL6 (*lane 2*) and phIL6S (*lane 3*), respectively, whereas no band was evident from ph(–)-transfected keratinocytes (*lane 4*). Although we also performed PCR using DNase-treated RNA from the phIL6 sample, no amplification was observed (*lane 1*) (*M* size marker)

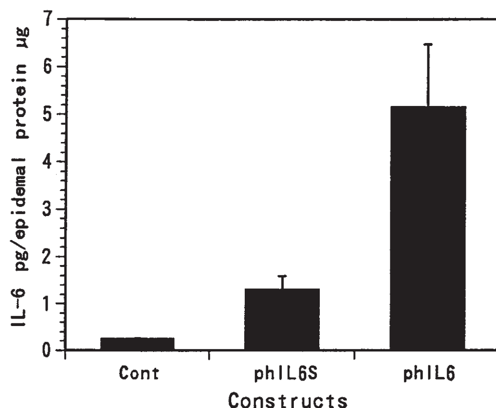

Fig. 3 Measurement of human transgenic IL-6 protein after the introduction of phIL6 and phIL6S. Epidermal sheets were prepared 24 h after the introduction of phIL6 and phIL6S. The IL-6 level in the epidermal extract was assayed by ELISA for human IL-6. Each value shown represents the mean ± SD of three individual tissue samples

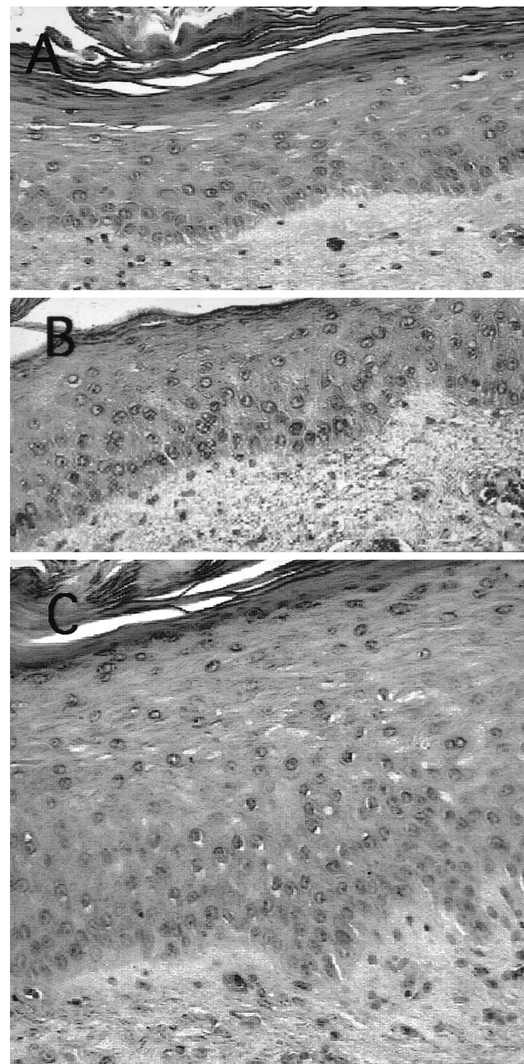

Fig. 4 **A–C** Histological changes in human skin after the introduction of phIL6S and phIL6. Biopsy specimens were obtained 48 h after the introduction of ph(–) (**A**), phIL6S (**B**) and phIL6 (**C**). Magnification, × 100

RT-PCR. PCR consisted of 29 cycles of denaturation (94 °C, 1 min), annealing (57 °C, 1 min), and extension (72 °C, 1 min). These PCR conditions are not be enough for amplification of endogenous the FS and AS cDNAs. The expected 0.65-kb and 0.45-kb bands were observed in the samples treated with phIL6 and phIL6S, respectively, whereas no band was evident in the ph(–)-transfected keratinocytes (Fig. 2). RT-PCR also showed that the mRNA amounts from these transgenes were similar.

To confirm the presence of transgenic human IL-6 peptide in the treated skin, we also injected phIL6, phIL6S, and ph(–) into the grafts, prepared epidermal lysates and measured the human IL-6 by ELISA. As shown in Fig. 3, a high level of IL-6 expression was detected in the phIL6 samples, whereas the phIL6S sample s showed a relatively low expression level. Expression of FS IL-6 was four times higher than that of AS IL-6. We detected little or no expression in the phIL(–) samples.

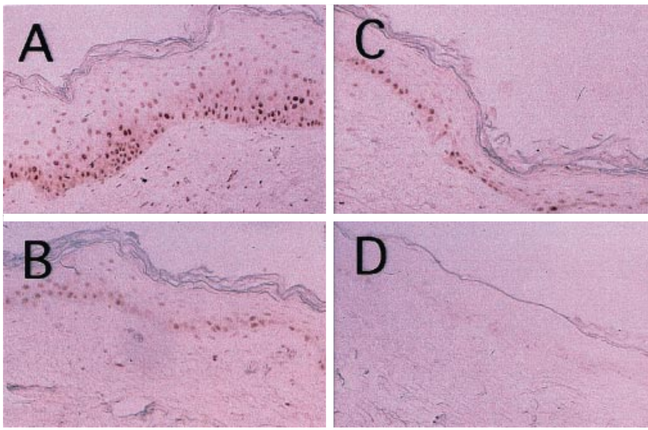

Fig. 5 PCNA expression by human skin after the introduction of phIL6S and phIL6. We injected 8 μg phIL6, phIL6S, and ph(–) into the grafts, and took biopsy specimens from the treated areas 48 h after injection. PCNA expression was examined by immunohistochemistry with PCNA antibody (**A**, **B** and **C** samples with phIL6, phIL6S and ph(–); respectively. **D** ph(–) sample reacted with control antibody; × 100)

Induction of histological changes by the introduction of phIL6 and phIL6S

We injected 8 μg of phIL6, phIL6S, and ph(–) into the grafts and 48 h later took a biopsy from the treated areas. After routine staining of the sections, we examined these plasmids for histological changes. Epidermal thickening was found in focal areas of the phIL6-treated skin, whereas no clear changes were observed in the phIL6S- and ph(–)-treated skin (Fig. 4). These results indicate that phIL6- transformed human keratinocytes overexpress the transgenic FS IL-6, which induces keratinocyte proliferation. Lymphocytic infiltration was not observed in any of the graft specimens.

PCNA expression

Since the introduction of phIL6 induced epidermal proliferation, we examined PCNA expression using immunohistochemistry. We injected 8 μg of phIL6, phIL6S, and ph(–) into the grafts, and took a biopsy from the treated areas 24 and 48 h after injection. In the untreated and 24-h specimens, PCNA-positive keratinocytes were seen in the basal and suprabasal layers of the epidermis (data not shown). In the 48-h phIL6 specimens, keratinocytes in several layers of the lower epidermis were positive in the area of epidermal thickening. In the 48-h phIL6S and ph(–) specimens, the cells in the basal and suprabasal layers were positive (Fig. 5). There were no positive cells in the specimens reacted with antihuman IgG mouse monoclonal antibody used as a control (Fig. 5).

Discussion

To achieve keratinocyte gene therapy in the future, fundamental experiments have to be performed using human skin. Hennge et al. transplanted human skin onto a SCID mouse and succeeded in transferring the bacterial-gal gene into the human keratinocytes in vivo using the naked DNA method [5]. In our preliminary experiments, we transplanted human skin onto a nude rat, injected a β-gal expression vector into the human skin, and then detected the activity in human keratinocytes of the transplanted skin (data not shown). This result confirms that the naked DNA method is useful for the in vivo introduction of genes into human keratinocytes. However, these experiments using the bacterial gene did not demonstrate that the transgenic protein produced from the transgene was biologically active in the skin tissue. We recently introduced the IL-6 gene into rat keratinocytes using the naked DNA method and overexpression of the IL-6 gene induced epidermal proliferation and lymphocytic infiltration into the upper dermis [10]. In this study, we examined whether these skin changes would occur in human skin transfected with the human IL-6 gene.

First, we constructed a human IL-6 expression vector. PCR amplification of keratinocyte cDNA produced two DNA fragments, which were demonstrated to be the FS and AS forms of the IL-6 cDNA by sequence analysis. Recently, Kestler et al. have identified an AS form which lacks exon II in peripheral blood mononuclear cells and they have shown that this sequence change does not cause a frame-shift between exons I and III [17]. They also examined the mRNA of the human bladder carcinoma cell line 5637 which produces IL-6 constitutvely, but the AS form was not detected in the 5637 cells. In this study, we detected the same AS form of the IL-6 transcript in keratinocytes. IL-6 interacts with two distinct receptor subunits, IL-6Rα and gp130. Recently it has been predicted that IL-6 possess three topologically distinct receptor binding sites: site 1, which binds to the subunit-specific chain, IL-6Rα, and sites 2 and 3, which interact with two subunits of the signaling chain, gp 130 [18, 19]. The truncated IL-6 protein translated from the AS mRNA would lack the gp130 interactive domain of the IL-6 (site 2) [16]. We introduced the AS IL-6 cDNA (phIL6S) or the FS IL-6 cDNA (phIL6) into human keratinocytes. RT-PCR showed that the mRNA amounts transcribed from these constructs were similar. We detected the transgenic IL-6 protein product of each of these cDNAs, but FS IL-6 was expressed at a level that was four times higher than that of AS IL-6. Since we had observed an erythematous reaction in rat skin into which we had injected 0.5 μg phIL6 [10], we thought that the amount of AS IL-6 was enough for inducing histological changes. However, we did not observe histological changes in the human skin grafts after the introduction of phIL6S, suggesting that AS IL-6 lacks biological activity in human skin. Further investigation is required to determine the biological significance of this truncated IL-6.

We have recently demonstrated that the in vivo introduction of the human IL-6 gene into rat keratinocytes induces epidermal thickening and lymphocyte infiltration in the rat skin [10]. Here, we injected the same gene into human skin grafts on nude rats and observed epidermal thickening, but not lymphocytic infiltration into the upper dermis. This difference might have been due to the rat lymphocytes, activated by human IL-6, not recognizing the human skin components. Interspecies differences between adhesion molecules has been suggested between rat lymphocytes and human skin cells. The thickening of the rat epidermis observed in our previous study, after the introduction of the IL-6 gene, might have resulted from either of two mechanisms: a direct effect of IL-6 and a secondary effect of cytokines released from dermal cells such as lymphocytes, endothelial cells and fibroblasts. However, the epidermal thickening without lymphocyte infiltration observed in the present study suggests that IL-6 causes keratinocyte proliferation directly. PCNA is an acidic nonhistone nuclear protein and appears in proliferating cells. PCNA expression of keratinocytes in normal and abnormal states has been investigated [20, 21]. Immunohistochemical study using anti-PCNA antibody showed that PCNA was strongly expressed in the area of epidermal thickening (Fig. 5). Another immunohistochemical study using antihuman keratin K10, which did not react with rat keratin K10, demonstrated that the examined keratinocytes were human and not rat (data not shown).

This study first introduced a human gene into human keratinocytes and determined the biological activity of the transgenic protein using the naked DNA method. This system is useful for examining the biological functions of various proteins in human keratinocytes or skin. Furthermore, the naked DNA method may be used in the future to transfer genes into keratinocytes in keratinocyte gene therapy.

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