Expression of Naked DNA in Human, Pig, and Mouse Skin

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Abstract

The insertion and expression of genes in the epidermis may have a variety of therapeutic uses, including the treatment of skin diseases. Here we show that when both human skin organ cultures and human skin grafts on immunocompromised mice are injected with naked DNA, the DNA is taken-up and genes are expressed in the epidermis in a manner similar to both pig skin injected in vivo and injected pig skin organ cultures. In contrast, DNA injected into mouse skin is expressed not just in the epidermis, but also in the dermis and underlying fat and muscle tissue, and is expressed at lower levels. These findings suggest that genes can be expressed in human skin, after injection of naked DNA, and indicate that pig skin is an appropriate model for the study of DNA uptake and gene expression in human skin. The organ cultures of human and pig skin may be useful in understanding how naked DNA is internalized and expressed after in vivo injections. Additionally, skin obtained from patients with skin disease maybe studied as skin grafts and organ cultures to help optimize genetic approaches for the treatment of skin diseases prior to clinical trials, by determining if the injected gene can provide a therapeutic benefit. (*J. Clin. Invest.* 1996. 97:2911–2916.)
Key words: epidermis • organ culture • plasmid DNA • gene expression

Introduction

The epidermis is an especially attractive target for genetic manipulation because it is readily accessible and can be easily monitored for both the presence and the expression of the inserted genes. Previously, we demonstrated that pig skin, when injected with naked DNA, transiently expresses the injected gene at high levels in the epidermis and produces a biologically active protein (1). A variety of other tissues including muscle, thyroid, liver, and synovial cells are also able to take-up and express naked DNA (2–5). Potential therapeutic uses of directly injecting DNA into the skin include expression of cytokines and other biologically active molecules for the treatment of skin lesions. For example, genes encoding for alpha-interferon could be injected to treat skin tumors and viral lesions (Kaposi's sarcoma, basal cell carcinoma, cutaneous squamous cell carcinoma, papilloma) by evoking a biological response against these lesions (6). The direct injection method could also be used for DNA vaccination by directly expressing genes of pathogens in the epidermis where large numbers of dendritic antigen-processing cells called Langerhans cells (LC) exist (7). Indeed, when naked DNA expressing the nucleoprotein of influenza is injected into mouse tail skin, long-lasting protective cellular and humoral immune responses result (8).

Because of these many potential clinical uses, we wanted to determine if human epidermis is capable of taking-up and expressing naked DNA, and investigate if skin organ culture is a reliable model for DNA uptake by skin in vivo. Here we show that, when both organ cultures of human skin and human skin grafted to immunocompromised mice are injected with naked DNA, the DNA is internalized and genes are expressed in the epidermis, just as pig skin and pig skin organ cultures express injected DNA in the epidermis. Because mice are widely used as an in vivo model for both skin and gene therapy research, we have also investigated and characterized gene expression in mouse skin following the direct injection of naked DNA in vivo. In contrast to human and pig skin, mouse skin expresses injected DNA in the epidermis, dermis, and underlying fat and muscle layers. These results indicate that organ cultures can take-up and express injected DNA similar to skin injected in vivo. Furthermore, human epidermis, like pig epidermis, is able to express naked DNA after local injection.

Methods

DNA injection. Plasmid DNA (pCMV:βGal, Clontech, Palo Alto, CA), containing the CMV promoter and the β-galactosidase indicator gene (9), was injected into the trunk skin of 2–8-mo-old inbred miniature swine from the NIH herd (Poolesville, MD) after anesthesia with ketamine, xylazine, butorphan, atropine (1:1:1:1). The plasmid DNA was diluted in phosphate-buffered saline to the concentrations indicated. Injection was performed into the superficial dermis using a glass syringe (Hamilton, Reno, NV) and a 30 gauge needle. The injected volume was 50 μl per injection site. Eight mm punch biopsies encompassing the injected site were obtained at 12 h after injection.

Ex vivo injections into the dermis of pig and human skin specimens (human skin from different body sites like scalp, face, extremities, and trunk was obtained from discarded specimens after unrelated surgical procedures) were performed under sterile conditions using a dissecting microscope (Stemi SV 11; Zeiss). After injection, human organ culture skin specimens ($\sim 2 \times 1$ cm in size) were incubated submerged in six-well plates (Greiner) using keratinocyte media (Keratinocyte Media SFM; GIBCO BRL, Gaithersburg, MD) supplemented with epidermal growth factor (5 ng/ml) and bovine pituitary extract (50 μg/ml) for up to 24 h at 37°C at 5% CO_2 (10).

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Received for publication 11 September 1995 and accepted in revised form 22 March 1996.

1. *Abbreviations used in this paper:* CMV, cytomegalovirus; β-Gal, β-Galactosidase.

The Journal of Clinical Investigation
Volume 97, Number 12, June 1996, 2911–2916

Adult female mice (BALB/c, C57BL/6, BALB/c nu/nu) of 8–12 wk of age were obtained from NIH Animal Production (Frederick, MD). Before injection, the mice were anesthetized with ketamine and xylazine (10:1) and the hair was shaved. Injections were performed superficially into the trunk skin. 12 h thereafter, the mice were killed by cervical dislocation and skin or lymph nodes were obtained. Animals were maintained in accordance with NIH Guide, USDA and Animal Welfare Act guidelines and housed in AALAC accredited housing.

Skin grafts. Human skin was grafted according to a described method (11). After dissecting away the underlying soft tissue, human skin grafts were placed subcutaneously in the nude mice. An incision was made along the lateral flank through the dermis and the panniculus carnosus. The graft bed was prepared by separating the panniculus carnosus from the underlying fascia by blunt dissection. The skin graft was placed on the fascia with the epidermal side up and the mouse skin flap was closed with 5-0 Vicryl. 3–5 d after grafting the human skin graft was exposed to the air by surgically removing overlying mouse skin. At various time points, pCMV:βGal plasmid DNA was injected into the human skin grafts as described above. One day after DNA injection, the mice were killed (with CO_2, and the human skin was excised together with adjacent mouse skin, and analyzed histochemically for β-galactosidase expression.

Detection of β-galactosidase activity. A histochemical staining method was used to detect β-Gal activity in the skin specimens as previously described (1). Briefly, specimens were fixed with paraformaldehyde, washed in PBS, and incubated with Bluo-Gal staining solution at room temperature for 8–16 h. Epidermal sheets were obtained from in vivo skin biopsies by incubation with 10 U/ml dispase (Boehringer Mannheim, Indianapolis, IA) for 1 h at 37°C.

Quantitative assay for β-galactosidase activity. A chemiluminescence assay was used to quantitate the β-galactosidase activity in the epidermis (12). Briefly, epidermal sheets were obtained 12 h after ex vivo injection (12.5 μg pCMV:β-Gal) of pig and human organ culture and from in vivo injected mouse skin by dispase treatment and lysed in lysis buffer using a mortar and pestle. After centrifugation, a 2-μl aliquot of the cell extract was mixed with reaction buffer (0.035 mM Galacton chemiluminescent substrate) (Tropix, Bedford, MA). After incubation, Emerald luminescence amplifier (Tropix, Bedford, MA) was added immediately before measurement of the chemiluminescence in a chemiluminometer (Monolight 1005; Analytical Luminescence, San Diego, CA). The β-galactosidase specific activity in epidermal extracts was calculated as the ratio of light units divided by the protein content (Biorad protein assay kit, Hercules, CA). Background β-Gal specific activity in epidermal extracts was determined by injecting non-β-Gal expressing plasmids (e.g., vector DNA) with the background activity being subtracted from positive samples. Assays were performed in duplicates and compared to a standard curve obtained by spiking β-galactosidase-negative cell extract with known amounts of purified β-galactosidase (No. G5635; Sigma Chemical Co., St. Louis, MO).

Results

In vivo and organ culture expression of β-Gal in pig skin. The expression of genes in skin may have many therapeutic uses in

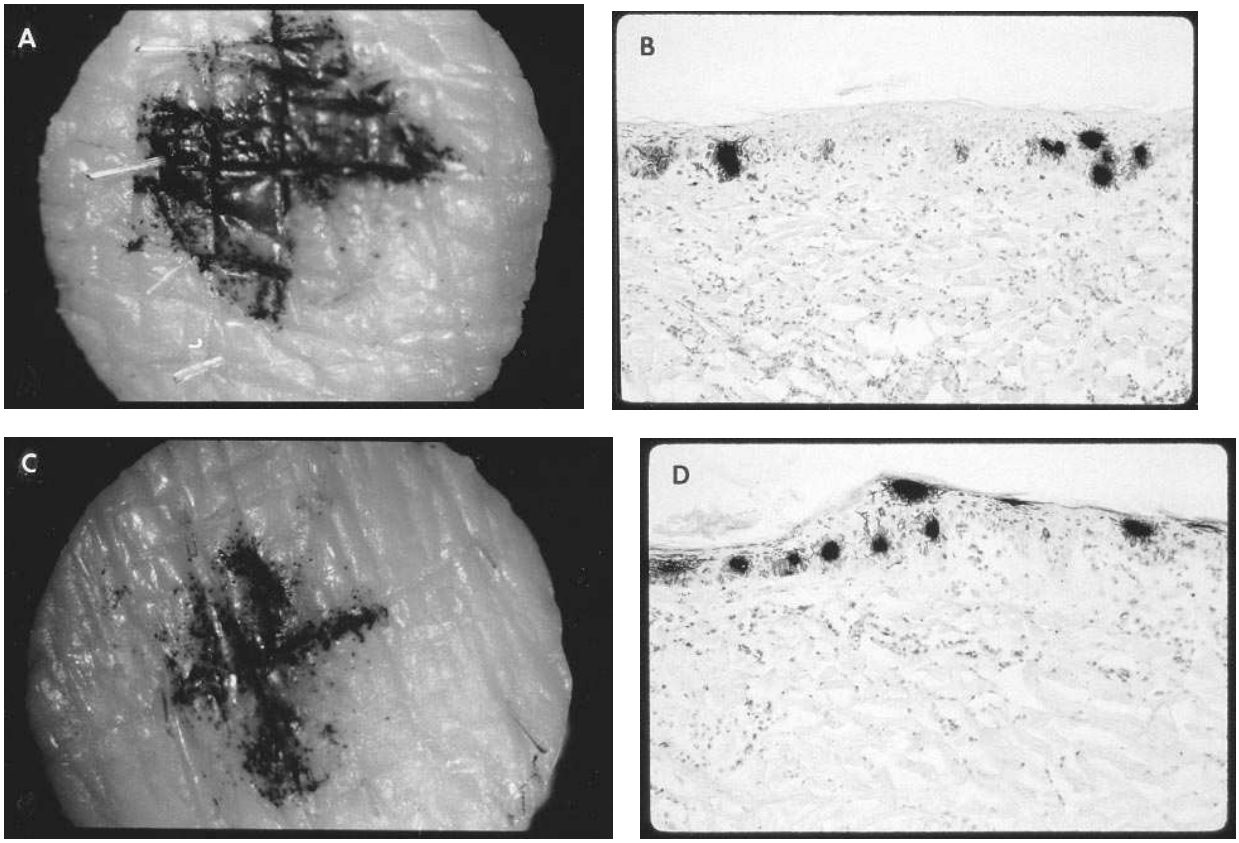

Figure 1. Expression of β-Gal in the epidermis of pig and human skin after injection of pCMV:β-Gal into the superficial dermis. (*A*) Gross β-Gal staining in 8 mm punch biopsy of pig skin injected in vivo with 20 μg of pCMV:β-Gal at 12 h after injection (×8.5). (*B*) Vertical section of in vivo injected pig skin at 12 h after injection (×200). Staining is observed in basal and suprabasal layers of the epidermis. Very little staining is seen in the dermis, where the plasmid DNA has been injected. (*C*) Gross β-Gal staining in 8 mm punch biopsy of pig skin organ culture at 12 h after injection with 20 μg of pCMV:β-Gal (×8.5). (*D*) Vertical section of injected pig skin organ culture at 12 h after injection (×200). Staining is seen in various layers of the epidermis in a distribution similar to pig skin injected in vivo, but almost absent in the dermis.

clinical medicine, and we wanted to determine if human skin is able to express injected DNA, and also analyze the pattern of expression. Since human skin cannot be injected with DNA in vivo, both human skin organ cultures and grafted human skin models were analyzed instead. To test the feasibility of using skin organ cultures as a representative model of in vivo skin, we first compared pig skin organ cultures to pig skin injected in vivo, which were both injected with naked DNA (20 μg of pCMV:βGal). This direct comparison would allow assessment of the quality and structural integrity of skin organ cultures as well as the quantity and pattern of gene expression. Histologically, skin organ cultures were indistinguishable from in vivo injected specimens at 12 h. Expression of β-Gal was analyzed by in situ β-Gal histochemical staining using the chromogen Bluo-gal, and by a quantitative chemiluminescent assay for β-Gal activity in cellular extracts from epidermis. Interestingly, 12 h after injection of pCMV:βGal, organ cultures of pig skin expressed β-Gal in the epidermis in a distribution similar to pig skin injected in vivo (Fig. 1, *A–D*). As seen on the vertical sections, the β-Gal staining was detectable at all layers of the epidermis, most abundantly in the postmitotic stratum spinosum, where keratinocytes differentiate. Little β-Gal staining was observed in the cells of the dermis. Therefore, organ cultures of pig skin appear to be a representative model for the in vivo uptake and expression of naked DNA.

Expression of β-Gal in human skin organ culture and skin grafts. To determine if human skin can express injected DNA, organ cultures of human skin were also injected with naked DNA and found to express injected DNA in a manner similar to pig skin organ culture at 12 h after injection (Fig. 2, *A* and *B*). Only rarely was β-Gal expression seen in the dermis, either because relatively few cells such as fibroblasts and endothelial cells are present in the dermis or because these dermal cells may be unable to efficiently take-up and express naked DNA. If these results on injected human skin organ cultures are predictive of in vivo results, then human skin, when injected in vivo, would be expected to also express the injected DNA in the epidermis.

Expression of DNA in human skin was assayed in an in vivo skin graft model by grafting human skin specimens onto immunocompromised mice (BALB/c nu/nu and SCID). Plasmid DNA (pCMV:βGal) injections into human skin grafts were performed between 1 and 5 d after air-exposure, and samples were analyzed histochemically for expression of β-Gal 24 h after injection. Although less reproducible than human skin organ cultures, scattered areas of β-Gal expression were grossly visible in the human skin grafts, following injection in approximately half (18/34) of the injected samples (Fig. 2 *C*). Histologically, β-Gal was again detected primarily in the epidermis (Fig. 2 *D*), similar to the injected human skin organ cultures.

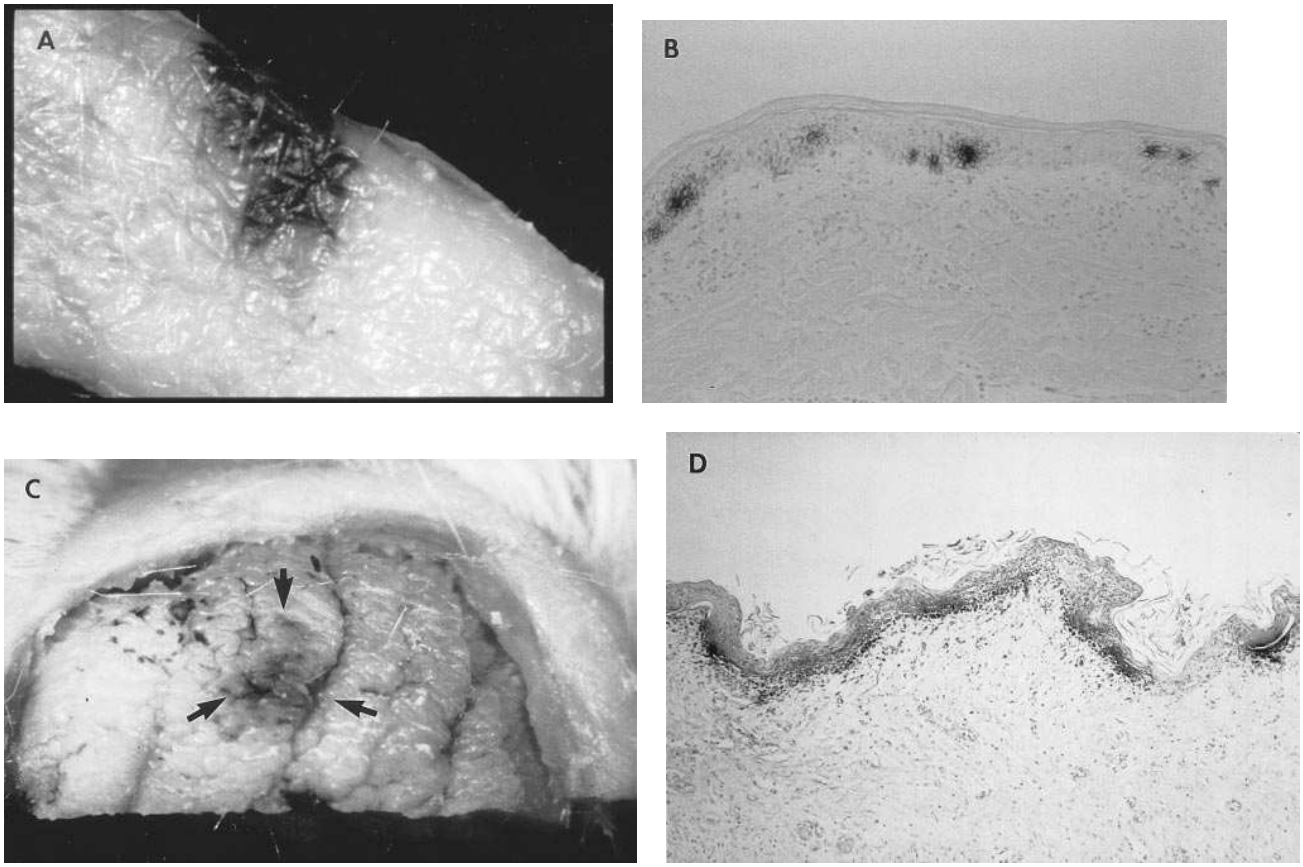

Figure 2. Expression of β-Gal in human skin organ culture and skin grafts. (*A*) Gross view of β-Gal staining in a 15×8 mm excision biopsy of human skin with 20 μg of pCMV:β-Gal at 12 h after injection (×8.5). (*B*) Vertical section of injected human skin organ culture at 12 h after injection (×200) showing β-Gal staining in various layers of the epidermis similar to injected pig skin with β-Gal staining absent in the dermis. (*C*) Gross β-Gal staining of human trunk skin injected 8 d after grafting onto an immunocompromised mouse with 20 μg of pCMV:β-Gal at 24 h after injection (×5) (bracketed by arrows). (*D*) Vertical section of in vivo injected human facial skin at 24 h after injection (×200). Note the β-Gal staining in the epidermis.

Expression of β-Gal was also analyzed quantitatively with a chemiluminescent assay of β-Gal activity in epidermal protein extracts following injection of DNA (12.5 μg of pCMV:βGal) into both human and pig skin organ cultures (Fig. 3). Although the β-Gal activity for injected human and pig skin organ cultures do overlap, the average amount of β-Gal activity in the epidermis of human organ cultures (37×10^3 light units per μg protein extract) was less than the average β-Gal activity found in the epidermis of pig skin injected in vivo (100×10^3 light units per μg protein) and injected pig skin organ cultures (320×10^3 light units per μg protein) (Fig. 3). The 37×10^3 light units per μg protein found in human skin represents ~10 ng of β-Gal protein (1). The lower β-Gal activity in the human skin organ cultures might be due, in part, to the lower quality of the human skin samples, caused by the time delay between obtaining the skin at surgery and the actual time of injection. In contrast, the pig skin organ culture specimens were of uniformly high quality since they were obtained, processed, and injected within 1 h of biopsy. It should be noted that the β-Gal activity obtained from injecting pig skin organ culture with 12.5 μg β-Gal DNA compares favorably to the average obtained from injecting pig skin in vivo, providing further evidence that the organ culture quantitative assay is a valid indicator for in vivo studies.

Expression of β-Gal in mouse skin. Since mice are frequently used in gene therapy research, we wanted to determine if mouse skin can take-up and express DNA injected in vivo and assess how this expression compares, qualitatively and quantitatively, to pig and human skin. Murine skin is structurally different from pig and human skin, having a thin epidermis consisting of fewer layers of keratinocytes, and displaying a thinner dermis with numerous hair follicles, which penetrate into the sub-dermal adipose tissue as well as structural differences in accessory glands (13). The thinness of mouse skin makes it technically difficult to perform intradermal injections. Despite these difficulties, β-Gal expression has been obtained in different mouse strains after in vivo injection of naked DNA into trunk skin, as shown grossly in Fig. 4 *A*, but the reproducibility and consistency of expression is less than pig skin injected in vivo. Unlike pig and human skin, vertical histology sections of injected mouse skin show β-Gal expression not only in the epidermis, but frequently in the dermis, fat tissue and underlying muscle fascicles (Fig. 4, *B* and *C*). Injection of DNA vector controls did not yield any β-Gal staining (Fig. 4 *D*). The β-Gal expression seen in the dermis was present at different locations, consistent with staining in fibroblasts and endothelial cells. Although it should not be a surprise that muscle cells take-up and express naked DNA (2), adipocytes have not been described to absorb and express DNA, but this was a common feature in the mouse skin we injected. Mouse skin from other locations, such as ear, tail, and footpad, also expressed injected naked DNA (data not shown). Time course studies were not performed to determine the duration of expression in mouse skin because the cell-types expressing β-Gal vary in different injections of mouse skin and different cell-types may have different durations of expression (1, 14).

A quantitative analysis of β-Gal expression in mouse skin, which has been injected in vivo, would allow a more accurate determination of how well mouse skin expresses genes after naked DNA injection compared to pig skin (both in vivo and organ culture injections) and human skin (organ culture injections). The quantitative chemiluminescence assay was used to analyze β-Gal expression 12 h after injection of pCMV:β-Gal plasmid in whole mouse skin as well as in separated epidermal and dermal components of mouse skin, since β-Gal is expressed in both the epidermis and dermis of mice (Fig. 5). In general, whole mouse skin, mouse epidermis, and mouse dermis expressed much lower amounts of β-Gal (15×10^3 light units per μg protein extract) than injected pig skin (Fig. 2 and Fig. 5). Additionally, the individual β-Gal activities shown in Fig. 5 reveal the variable level of expression in mouse skin, with many values overlapping those of the negative (vector DNA-injected) control specimens. Upon statistical analysis, only mouse dermis was significantly different from vector con-

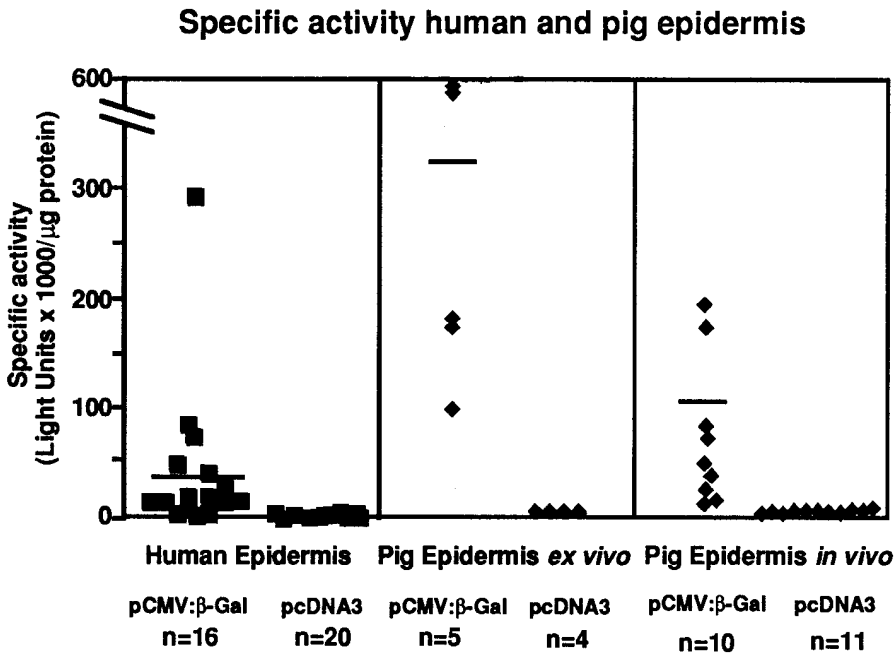

Figure 3. Specific β-Gal activity (chemiluminescent light units per μg protein) present in epidermal sheets of injected human organ cultures (*left column*), injected pig skin organ cultures (*middle column*), and pig skin injected in vivo (*right column*). 12.5 μg of pCMV:β-Gal or vector control DNA (pcDNA3) were injected intradermally and epidermal extracts prepared from epidermal sheets 12 h after injection. *n* is the number of independent samples. Bars indicate the mean of each group. Statistical analysis using the students *t* test revealed a significant difference for pCMV:β-Gal injected human skin organ cultures ($P < 0.003$), injected pig skin organ cultures ($P < 0.039$) and pig skin injected in vivo ($P < 0.006$) over vector control injected specimens.

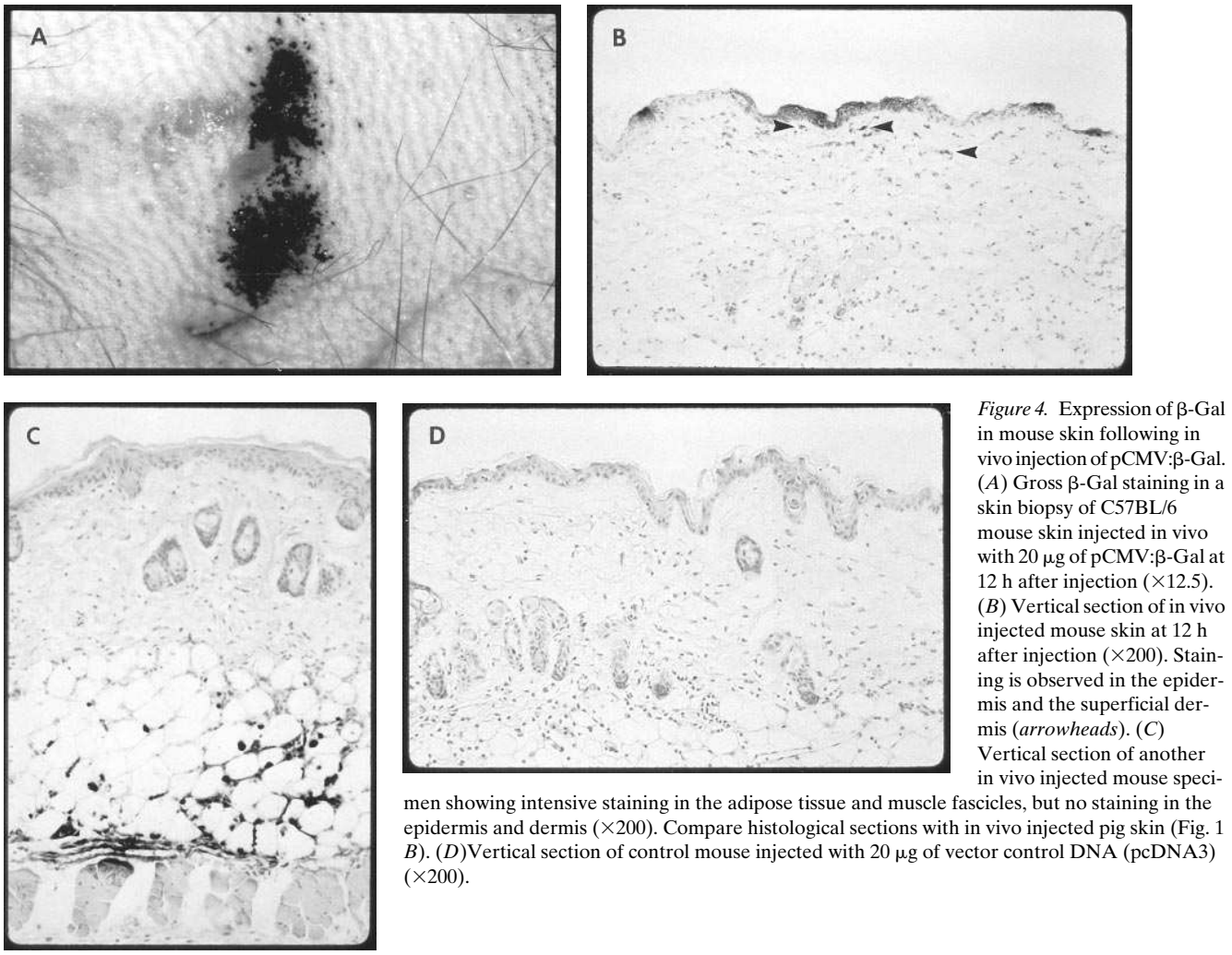

Figure 4. Expression of β-Gal in mouse skin following in vivo injection of pCMV:β-Gal. (*A*) Gross β-Gal staining in a skin biopsy of C57BL/6 mouse skin injected in vivo with 20 μg of pCMV:β-Gal at 12 h after injection (×12.5). (*B*) Vertical section of in vivo injected mouse skin at 12 h after injection (×200). Staining is observed in the epidermis and the superficial dermis (*arrowheads*). (*C*) Vertical section of another in vivo injected mouse specimen showing intensive staining in the adipose tissue and muscle fascicles, but no staining in the epidermis and dermis (×200). Compare histological sections with in vivo injected pig skin (Fig. 1 *B*). (*D*)Vertical section of control mouse injected with 20 μg of vector control DNA (pcDNA3) (×200).

trol ($P < 0.05$), while both injected mouse whole skin and mouse epidermis were not significantly different from the background controls. Taken together, mouse skin is capable of expressing injected naked DNA, but expression is variable, and when present, it is at low levels compared with pig skin.

Discussion

The present study demonstrates that both human skin organ cultures and human skin grafts internalize and express injected DNA in the epidermis in a histological pattern similar to injected pig skin and pig skin organ culture. These results suggest that human skin should also take-up and express naked DNA after in vivo injection. Thus, human skin organ culture and human skin grafts may be of great utility to gene therapists since they appear to provide appropriate models for the expression of exogenous genes in human skin injected in vivo. Human skin obtained from patients with skin diseases could be used to determine if a particular gene can be expressed and provide a therapeutic benefit. For example, skin organ cultures from patients with a genetic skin disease (dystrophic epidermolysis bullosa) could be injected with the corrective gene (collagen type VII), and the skin assessed for therapeutic improvement. Additionally, since pig and human skin have similar structures, and comparable abilities to express exogenous genes in the epidermis following DNA injection, pig skin should be a good model for human skin when conditions for gene expression are being optimized in vivo.

In contrast to human and pig skin, where injected genes are expressed predominantly in the epidermis, mouse skin appears to take-up and express injected plasmid DNA at multiple sites including epidermis, dermis, adipocytes, and muscle fascicles underlying the dermis, as previously described (2). The reasons for these different patterns of expression in mouse skin, compared to human or pig skin, are not clear, but need to be considered when mice are used as a model in biological studies utilizing direct injection of plasmid DNA. Although multiple types of cells in mouse skin can internalize and express naked DNA, the quantitative expression of injected β-Gal plasmid DNA in mouse skin is significantly lower than pig skin, and the level of expression is more variable. One potential explanation for the different patterns and low levels of expression relate to structural differences between mice and human or pig skin (13). Mouse skin is more difficult to inject because it is thin, and even though many different cell types appear to take-up and express DNA, either fewer total cells are expressing DNA or the level of expression per cell is much lower than in pig or human skin.

The finding that organ cultures of both human and pig skin take-up and express injected DNA in the epidermis is, to our

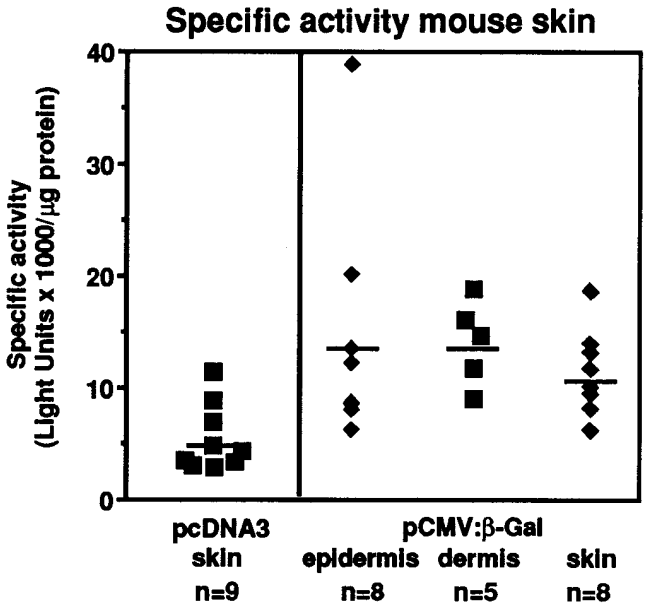

Figure 5. Specific β-Gal activity (chemiluminescent light units per mg protein) of in vivo injected BALB/c mouse skin. 12.5 μg of pCMV:β-Gal or vector control DNA (pcDNA3) were injected superficially into mouse trunk skin. Specific β-Gal activity was assessed 12 h after injection of pCMV:β-Gal in whole skin ($n = 8$), epidermal ($n = 8$) and dermal ($n = 5$) protein extracts, and in whole skin ($n = 9$) for vector control DNA samples, respectively, as described above. Bars indicate the mean of each group. Statistical analysis using the students' *t* test revealed that only pCMV:β-Gal injected dermis was significantly different ($P < 0.05$) from vector control specimens. Both whole mouse skin and mouse epidermis were not significantly different.

knowledge, the first description of naked DNA uptake in a tissue culture setting. Since keratinocyte monolayers do not take-up naked DNA (data not shown), this suggests that keratinocytes must be in an organized epidermis for DNA uptake to occur. The process of how naked uncomplexed DNA is internalized by cells is intriguing and not yet understood, but may involve potocytosis which uses caveolae, or semi-permanent membrane vesicles, for uptake of extracellular molecules via a nonendocytic pathway (15, 16). Skin organ cultures provide a controlled in vitro setting in which DNA uptake and expression can be studied and manipulated, for example, by injecting controlled amounts of labeled DNA and following its uptake into keratinocytes over time.

DNA vaccines are one exciting potential use for the direct introduction and expression of DNA in the skin. Interest in the intradermal approach of DNA vaccination has significantly increased and our data provides useful information about where injected DNA is expressed in the skin of different animal models. Furthermore, the organ culture models may be useful in understanding the mechanisms of how the injected DNA gene product is presented to the immune system.

Genetic approaches to treatment of human skin diseases will be practical if prolonged high level expression of exogenous genes can be achieved using simple delivery systems (such as direct injection of DNA). Optimization of the necessary methodology will be facilitated if pre-clinical studies can be carried out in organ culture or animal models. We suggest that human skin organ cultures and skin grafts along with pig skin in vivo represent appropriate model systems for studies of human keratinocyte gene therapy.

Acknowledgments

The authors would like to thank Mark Udey, Stephen Katz, and Kim Yancey for helpful discussions and criticisms, and Al Harrington and William James for providing surgical specimens. We are also grateful to Victoria Hampshire and Melissa Williams for veterinarian assistance and Mitra Hengge for reading the pathology.

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