Efficient Gene Expression in Skin Wound Sites Following Local Plasmid Injection

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Transfection of the skin by local gene delivery, as well as widespread transfection of systemic tissues following intravenous injection of cationic liposome/DNA complexes have been reported. Here, we show that surgically wounded mouse skin can be transfected either by local injection of DNA alone or by intravenous injection of optimized cationic liposome/DNA complexes; however, direct cutaneous injection produces much higher levels of gene expression in the skin, which is targeted to dermal and subdermal layers. High levels of chloramphenicol acetyltransferase activity were present from 3 h to 2 wk following direct injection of a gene expression plasmid into wounded skin and were maintained at detectable levels up to 8 wk after injection. Expression of transferred chloramphenicol acetyltransferase as well as β-GAL genes was localized to fibroblasts, macrophages, and adipocytes as determined by histochemistry and immunohistochemistry. Furthermore, local injection of a human granulocyte-colony-stimulating factor gene expression plasmid produced high levels of the biologically relevant human granulocyte-colony-stimulating factor protein in wounded mouse skin. This efficient and simple method of site-specific gene transfer into wounds may lead to the development of cutaneous gene therapy directed against disorders of abnormal cutaneous wound healing. ***Key words: cationic liposomes/cutaneous gene transfer/gene therapy/wound healing. J Invest Dermatol 116:131–135, 2001***

The skin is in many ways an ideal target site for gene delivery. It is readily accessible, and gene expression may be restricted not only to the skin (potentially avoiding nonspecific toxicity), but also to specific layers and cell types within it (Greenhalgh *et al*, 1994). The site of cutaneous gene expression will depend on the route of gene delivery. In the skin, at least three different routes must be considered: (i) topical application; (ii) intradermal injection; and (iii) systemic delivery. The first two approaches are of natural importance as local/regional treatment strategies. Some of the most serious dermatologic diseases that could benefit from gene therapy however, have both widespread cutaneous involvement and extracutaneous involvement. Therefore, successful treatment of these diseases may require systemic delivery that can achieve efficient gene transfer to the skin.

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Abbreviations: CAT, chloramphenicol acetyltransferase; CLDC, cationic liposome/DNA complexes; G-CSF, granulocyte-colony-stimulating factor; GAL, galactosidase; PMN, polymorphonuclear neutrophils.

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Gene delivery to the skin has been accomplished by several different approaches. These include topical application of non-cationic liposome–DNA complex (Li and Hoffman, 1995), cationic liposome–DNA complex (Nabel *et al*, 1993; Alexander and Akhurst, 1995; Yu *et al*, 1999), particle-mediated (gene gun) (Yang *et al*, 1990; Benn *et al*, 1996) and puncture-mediated (Ciernik *et al*, 1996) gene transfections, and viral approaches (Setoguchi *et al*, 1994). Gene products have been detected in a number of cell types, including basal keratinocytes, following epithelial cells, sebaceous gland cells and dermal fibroblasts and dermal macrophages after gene delivery.

Poor wound healing has been related to many factors, such as radiotherapy (Miller and Rudolph, 1990), steroids (Di Rosa *et al*, 1986), ultraviolet radiation (Davidson *et al*, 1991), and microvascular diseases (Ehrlichman *et al*, 1991). In these conditions, neutropenia and neutrophil dysfunction have been found to predispose to bacterial infection and have been associated with delayed wound healing. Granulocyte-colony-stimulating factor (G-CSF), a white blood cell hematopoietic growth factor, upregulates the production of polymorphonuclear neutrophils (PMN) (Anderlini *et al*, 1996). Importantly, in addition to promoting the production of PMN, there is increasing evidence that G-CSF may significantly enhance PMN function (Betsuyacu *et al*, 1999). Favorable effects of treating cutaneous wound sites with recombinant G-CSF have been reported (Yalcin *et al*, 1997; Harada *et al*, 1998). Recently, administration of recombinant human G-

CSF (hG-CSF) has been reported to accelerate wound healing in a patient with a persistent pharyngocutaneous fistula following laryngectomy (Cody *et al*, 1999). Therefore, it is reasonable to hypothesize that topical delivery of therapeutically relevant genes to wounded skin may help accelerate wound healing, thus potentially reducing the high cost associated with recombinant protein administration. In this study, we first assessed the efficiency, time course, and localization of gene expression following both local and systemic gene delivery with either plasmid DNA alone or with cationic liposome DNA complexes (CLDC) specifically to sites of wounded skin. We then successfully delivered plasmid DNA coding the hG-CSF gene into surgical wound sites, resulting in significant G-CSF expression.

MATERIALS AND METHODS

Plasmids, liposomes, and complexes The hCMV chloramphenicol acetyltransferase (CAT) plasmid, p4119, the hCMV-β-GAL plasmid, pCMVβ, and the hCMV-hG-CSF plasmid have all been described previously (Liu *et al*, 1995, 1997). Plasmid DNA was purified and DNA quantitated as previously described (Liu *et al*, 1995). Multilamellar liposomes containing the cationic lipid 1-[2-(9(2)-Octadecencyloxy)-ethyl]-2-[S(2)-heptadecenyl]-3-(2-hydroxyethyl)-imidazolinium chloride (Solodin *et al*, 1995) in a 1 : 1 molar ratio with cholesterol were prepared as previously described (Liu *et al*, 1997). Complexes of the liposomes with plasmid were prepared as previously described (Liu *et al*, 1995).

Animal studies Two month old female ICR mice obtained from Simonsen Labs (Gilroy CA), were used in all experiments. For skin injection, in order to mimic a surgical wound, mice were first anesthetized by metofane inhalation, their backs shaved and disinfected with 70% ethanol, and then a 1 cm long, full thickness surgical incision was made at four individual sites on the dorsum of the back of each animal. Immediately thereafter, 25 μg of the expression plasmid per wound, either uncomplexed or as CLDC, was injected in 50 μl 5% wt/vol glucose into the wound edges of each of the four wound sites on each animal in groups of two, using a 0.5 ml syringe and a 28.5G needle. Wounds were closed using a metal clip. For intravenous injection (tail vein), mice in groups of four each received 60 μg of plasmid DNA, either uncomplexed or as CLDC, in 200 μl 5% wt/vol glucose. The mice were killed in a CO_2 chamber, and skin samples as well as liver, spleen, heart, lungs, and lymph nodes were harvested at 0 (control), 1, 3, 6, and 24 h and 1, 2, 5, and 8 wk after injection.

Tissues were processed and analyzed for CAT enzymatic activity as previously described (Liu *et al*, 1995), except that the [^{14}C]-labeled chloramphenicol–tissue extract mixture was allowed to react for 2 rather than 8 h. Tissues were analyzed for hG-CSF protein by enzyme-linked immunosorbent assay (Liu *et al*, 1995). Histochemical staining for β-GAL activity (Liu *et al*, 1997), and immunohistochemical staining for CAT antigen (Zhu *et al*, 1993) were each performed as described previously. Statistical significance of differences between the different levels of expression was assessed by a two-sided Student's test.

RESULTS AND DISCUSSION

We first determined dose–response relationships between the amount of DNA injected and the level of CAT gene expression in skin, 24 h following local cutaneous injection into wound sites of CLDC containing from 5 μg to 200 μg of a CMV-CAT plasmid at a 2 : 1 DNA/lipid ratio (μg DNA/nmole lipid) (**Fig 1*a***). We found

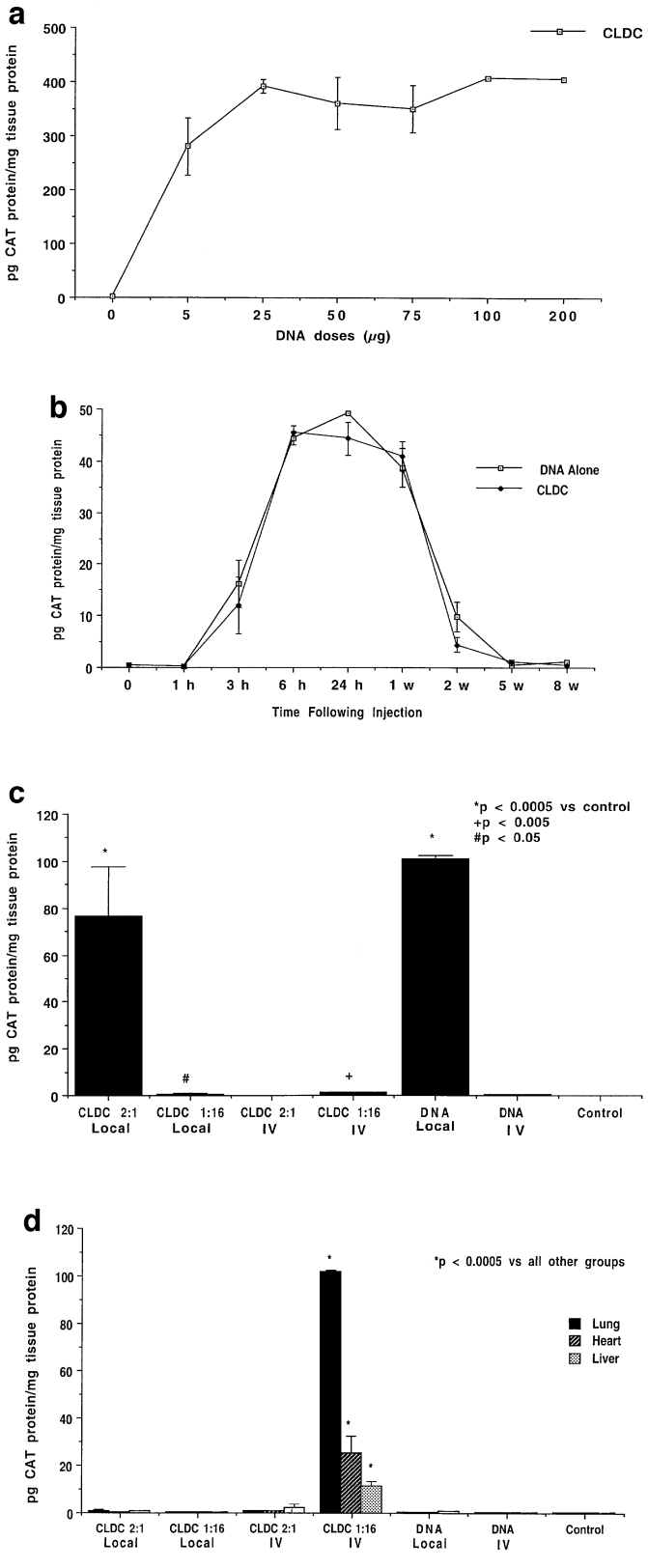

Figure 1. CAT expression in mice following injection of p4119, a CMV-CAT expression plasmid. (*a*) DNA dose-gene expression response relationships in mice receiving local cutaneous injection of CLDC. CAT activity in skin was determined 24 hours after local cutaneous injection per wound of 0, 5, 25, 50, 75, 100, or 200 μg of a CMV-CAT plasmid (p4119) complexed to cationic liposomes at a ratio of 2 : 1 (μg DNA:nmole lipid). The values shown are the mean of eight individual samples per dose ± SEM. (*b*) Time course of expression following local injection of 25 μg per wound of p4119, either as DNA alone or as CLDC. The values shown are the mean of eight individual samples per time point ± SEM. CAT activity in skin extracts (*c*), or in lung, heart, and liver extracts (*d*), 24 h after local or intravenous injection of DNA alone or CLDC. Groups of four mice received (i) CLDC containing 25 μg of p4119 at 2:1 or 1:16 (μg DNA/nmol lipid) ratios by direct injection into wounded skin, or CLDC containing 60 μg of p4119 at 2:1 or 1:16 DNA/lipid ratios by intravenous tail vein injection; or (ii) 25 μg per wound of p4119 as naked DNA, injected into cutaneous wound sites; or (iii) 60 μg of p4119 alone by intravenous tail vein injection. All mice were killed 24 h after injection.

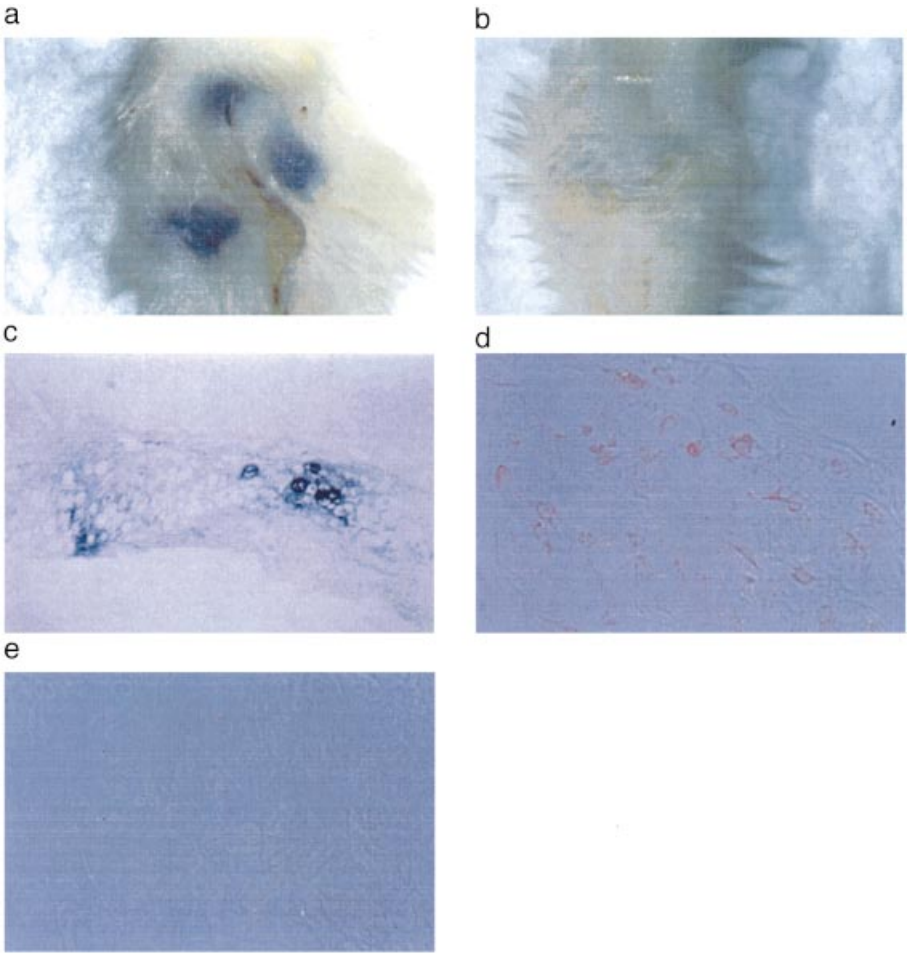

Figure 2. Localization of β-galactosidase or CAT expression following plasmid injection into wounded skin. (*a*) Macroscopic aspect of wounded skin. Blue foci near wound edges demonstrate the β-galactosidase gene product, present in sites where CMV-β-galactosidase expression plasmid was injected intradermally 24 h earlier. (*b*) In a control area of wounded skin, injected with CMV-CAT expression plasmid intradermally 24 h earlier, no β-galactosidase activity is seen. Both specimens were exposed to X-gal solution to disclose β-galactosidase activity. (c) A non-counter stained histologic section (30×) obtained from (*a*) demonstrates the presence of blue β-galactosidase activity in the lower dermis and subcutis. The most intensely staining cells within these sites are predominately adipocytes. (*d*) Histologic section (300×) of the dermis near a wound margin from a mouse injected intradermally 24 h earlier. The red stained cells containing CAT-antigen, as detected immunohistochemically. Morphologically, these cells are consistent with histiocytes and, less frequently, with fibroblasts. (*e*) Histologic section (300×) of skin from a wound margin from a mouse injected intradermally 24 h earlier with a CMV-luciferase expression plasmid (control) that was concurrently assayed for CAT-antigen expression. CAT-antigen is not detectable.

that DNA doses of 5 μg injected per wound site produced highly significant levels of CAT activity ($p < 0.0005$ *vs* control mice). CAT activity was present at peak levels at injected DNA doses ≥ 25 μg. Although the 5 μg DNA dose produced CAT activity at approximately 75% of maximal levels, unless specifically stated, we used 25 μg plasmid DNA per wound for all subsequent experiments, in order to maximize the levels of gene expression achieved. We also established that the level of transfection in wounded skin was comparable with that produced in normal skin of mice (data not shown), indicating that the presence of a surgical wound site did not noticeably affect the expression of genes injected into mouse skin. Examination of the effect of the DNA/lipid ratio on transfection levels demonstrated that optimal transfection occurs when the DNA is not complexed with lipid; however, complexing with lipid does not reduce transfection levels at ratios up to 2 μg DNA per nmol total lipid. Above this ratio, the presence of lipid progressively suppresses transfection levels (data not shown). This is in direct contrast to the effects of CLDC on the efficiency of gene transfer and expression following intravenous delivery of DNA (Zhu *et al*, 1993; Liu *et al*, 1995); however, it is consistent with the efficient transfection of muscle tissue produced by naked DNA (Wolff *et al*, 1990).

We next determined the time course of CAT gene expression following wound injection with 25 μg per wound of a CMV-CAT expression plasmid, either as DNA alone or as CLDC (DNA/lipid ratio of 2 μg DNA per nmol total lipid). We found that significant levels of CAT activity were present in wounded skin as early as 3 h after local injection (**Fig 1*b***). Peak levels were present by 6 h, and persisted for approximately 1 wk. Significant CAT activity was present for at least 2 wk. All CAT gene-treated groups, killed from 3 h to 2 wk following injection, showed significantly higher levels of CAT activity ($p < 0.0005$) than control animals. At the 5 and 8 wk time points, expression approached baseline levels, although CAT activity in both the DNA alone and CLDC groups was still significantly ($p < 0.05$) above control levels (**Fig 1*b***). The levels of CAT activity produced by DNA alone and by CLDC at a DNA/lipid ratio of 2 μg DNA per nmol of lipid or greater were similar at all time points assayed. Thus, complexation of DNA with cationic lipids did not enhance transfection efficiency in wounded skin at any time point.

We then compared the amount of CAT activity produced in wounded skin and in systemic organs, following either local cutaneous injection with 25 μg per wound of the CMV-CAT expression plasmid, either as DNA alone or as CLDC in a 2:1 DNA/lipid ratio (2 μg DNA per nmol total lipid), or by intravenous injection of 60 μg of CMV-CAT, either alone or complexed to cationic liposomes at either 2:1 or 1:16 DNA/lipid ratios. Local injection of either DNA alone or in CLDC produced high levels of CAT gene expression in the skin (**Fig 1*c***), but did not produce significant levels of CAT activity in extracutaneous tissues (**Fig 1*d***). Intravenous injection of either CLDC (2:1) or DNA alone produced minimal expression in the skin (**Fig 1*c***). Local injection of CLDA at a 1:16 DNA/lipid ratio produced only background levels in extracutaneous tissues (**Fig 1*d***), whereas intravenous injection of the same material produced high levels of CAT gene expression in the heart, lung, and liver (**Fig 1*d***) as well as significant levels of gene expression ($p < 0.005$ *vs* controls) in the skin (**Fig 1*c***).

Using enzyme histochemistry and immunohistochemistry, gene expression was localized within mouse skin following injection of CMV-β-GAL or CMV-CAT expression plasmids directly into wound sites. We found that β-GAL and CAT expression were most intense within discrete areas near the dermal–subdermal interface corresponding to injection sites (**Fig 2*a***). Within these areas, staining of adipocytes was most prominent (**Fig 2*c***), followed

Table I. Skin levels of human G-CSF, 24 h after local injection of CMV-hG-CSF expression plasmid : liposome complexes or CMV-hG-CSF expression plasmids alone *vs* uninjected controls (groups of four mice)

Treatment	Pg hG-CSF per mg tissue[a] (mean ± SD)
hG-CSF gene/liposomes	395[b] ± 274 pg per mg
hG-CSF gene alone	241[b] ± 112 pg per mg
Uninjected control mice	Undetectable[c] (< 10 pg per mg)

[a]hG-CSF levels were measured by ELISA.
[b]$p < 0.025$ when compared to control mice by a two-sided Student's t test.
[c]The ELISA used does not detect mouse G-CSF.

by positive immunostaining of individual of individual cells consistent with histiocytes and occasional fibroblasts near wound margins (**Fig 2*d***). There was no evidence of gene expression in the epidermis.

Finally, we injected the gene coding for the therapeutically relevant hG-CSF protein directly into wounded skin. We chose to use the hG-CSF gene because recombinant hG-CSF protein is now commonly administered to immunosuppressed human patients (Kearns *et al*, 1993) and because we use an hG-CSF enzyme-linked immunosorbent assay that does not detect murine G-CSF activity (Liu *et al*, 1993), thus avoiding background interference. An hG-CSF expression plasmid was injected either as DNA alone or as CLDC at a 2:1 ratio and the wound site harvested 24 h later. Injection of the hG-CSF plasmid as CLDC produced 395 ± 274 pg (SD) or hg-CSF protein per mg of tissue. Injection of the hG-CSF plasmid alone produced 241 ± 112 pg G-CSF per mg tissue, whereas neither uninjected nor mock-injected control mice showed detectable (< 10 pg per mg tissue) hG-CSF activity. The hG-CSF gene-treated groups showed significantly higher levels of hG-CSF protein in the skin ($p < 0.025$) than did the control groups.

Thus, we have shown that wounded skin can be transfected either by local injection of naked DNA or CLDC or by intravenous injection of optimized CLDC. The former produces high levels of gene expression localized to the dermal and subdermal layers of the skin, whereas the latter produces both lower levels of gene expression within wounded skin as well as generalized gene expression in extracutaneous tissues. The favorable time course of gene expression (peak levels lasting from 6 h to 1 wk postinjection) as well as the high level expression of the therapeutically relevant hG-CSF gene produced by local injection of plasmid DNA or CLDC suggest that this approach may become clinically useful for the treatment of dysfunctional wound healing, as well as for other genetic and acquired cutaneous diseases.

Delayed wound healing has represented a major clinical problem for some patients receiving radiotherapy, chemotherapy, steroids, and/or ultraviolet radiation, as well as in patients with microvascular disorders, such as diabetes. Among the many factors thought to interfere with wound healing in these patients, neutropenia, and neutrophil dysfunction, leading to severe or chronic bacterial infection, are considered to be important predisposing factors. Several strategies to modulate skin wound healing have already been reported (Young, 1988; LaVan and Hunt, 1990; Violaris and Bridger, 1990; Miller and Rudolph, 1990, Vogt *et al*, 1994; Andree *et al*, 1994; Benn *et al*, 1996), with positive but limited effects. To date, poor wound healing remains a difficult clinical problem. Therefore, further investigations are required to accelerate wound healing in these patients.

One way to accelerate problematic wound healing is to strengthen host defense systems, in order to prevent and/or treat bacterial infection. G-CSF, the principal hematopoietic growth factor that stimulates PMN production, has been proved to be efficient in treating neutropenia in a number of clinical settings. Recent research has shown that, in addition to increasing the number of PMN, G-CSF may also play an important part in multiple aspects of enhancing PMN function. These include: (i) enhancing CD11b (Hakanson *et al*, 1997) and FcγRi expression (Gericke *et al*, 1995); (ii) enhancing PMN adhesion (Hakanson *et al*, 1997); (iii) phagocytosis (Hoglund *et al*, 1997); (iv) degranulation (Xu *et al*, 1996); and (v) chemokinesis (Yong 1996). Furthermore, G-CSF may also modulate peripheral mature PMN activation (Metcalf *et al*, 1996) and regulate PMN chemokine responsiveness (Betsuyacu *et al*, 1999). Subcutaneous application of G-CSF has been found to be effective in reducing bacterial translocation due to burn wound sepsis (Yalcin *et al*, 1997). Finally, G-CSF has been used clinically to promote wound healing successfully in a neutropenic patient after head and neck surgery (Cody *et al*, 1999).

In this study, we achieved high-level G-CSF expression following local cutaneous injection of either plasmid DNA coding for G-CSF alone or in CLDC. In addition, we have recently developed a novel, Epstein–Barr virus-based long-term expression vector that produces therapeutic levels of hG-CSF gene expression for at least 2 mo after a single injection in mice of CLDC containing the hG-CSF gene into mice (data not shown). This vector may allow us to investigate further skin gene therapy for poor wound healing, targeting other genes that have potential applications for skin disorders. In addition, the costs involved in upscaling the use of cutaneous delivery of DNA plasmids for potential therapeutic approaches in human patients with cutaneous disorders may be substantial. The use of novel expression vectors that produce more durable and/or higher levels of expression of delivered genes may make nonviral, plasmid-based approaches more cost-effective, when compared with the significant costs involved in preparing either recombinant viral vectors or recombinant proteins for cutaneous therapy studies. Combining expression plasmids that produce longer durations of gene expression together with lower injected doses of DNA that still produce significant levels of cutaneous gene expression (see **Fig 1*a***), may make this approach feasible from a cost basis.

In summary, we have demonstrated that direct intradermal injection of multiple plasmid DNAs into wound sites consistently results in site-specific gene expression that is targeted to dermal and subdermal layers as well as a number of different cell types. We have also shown that this approach resulted in high levels of therapeutically relevant hG-CSF gene expression, following local injection of either plasmid DNA alone or CLDC. In contrast to other gene transfer modalities, which are potentially infectious and/or immunogenic as viral vectors are used, or which require sophisticated and expensive technical support, the injection of naked DNA is not only efficient, but simple, safe, and inexpensive. These properties are also largely consistent with the requirements for a clinically applicable cutaneous gene transfer system. Given the experience with existing wound repair models, it is therefore conceivable that effective cutaneous gene therapy approaches for the many clinical problems associated with abnormal wound healing are feasible.

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