Particle-mediated Gene Transfer with Transforming Growth Factor–β1 cDNAs Enhances Wound Repair in Rat Skin

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Abstract

Based on preliminary but variable results with direct DNA transfer into wounds, we evaluated in vivo gene transfer by particle-mediated DNA delivery to rat skin to determine whether overexpression of TGF-β1 at the site of skin incisions would result in a significant improvement in repair. Optimization of the method with viral promoter–luciferase reporter constructs indicated that expression of luciferase activity persisted up to 5 d and was promoter, pressure, and site dependent (ventral > dorsal). Using cytomegalovirus (CMV)-driven human α1-antitrypsin, transgene expression was immunolocalized within keratinocytes of the stratum granulosum at 24 h. We measured tensile strength of skin incisions at 11–21 d in both normal and diabetic rats transfected with TGF-β1 expression vectors at surgery. Native murine TGF-β1 under an SV40 promoter produced positive effects, while wound strengthening was more pronounced in diabetic animals using a CMV-driven construct. Transfection of rat skin with constitutively active, mutant porcine TGF-β1 under the control of the CMV and Moloney murine leukemia virus promoters significantly increased tensile strength up to 80% for 14–21 d after surgery. Transfection 24 h before surgery was more effective. Particle-mediated gene delivery can be used to deliver viral promoter–cytokine expression constructs into rat skin in a safe, efficient, and reproducible fashion. The extent of wound repair, as evidenced by enhanced tensile strength, can be markedly improved in tissues transfected with TGF-β1 expression constructs. (*J. Clin. Invest.* 1996. 98:2894–2902.) Key words: gene therapy • wound healing • transforming growth factor–β • gene gun • skin

Introduction

Wound repair is a succession of overlapping biochemical and cellular events that result, in the best of circumstances, in the restoration of both the structural and functional integrity of the damaged tissue. This process is mediated, in part, by the appearance of biologically active peptides or cytokines at the site of injury (1). Part of the evidence for growth factor function derives from the observation that supplementation or depletion of several cytokines can influence the rate of wound repair. The exogenous administration of TGF-β1 to incisional wounds has been consistently shown to increase the accumulation of collagen and other matrix components at the site of injury, thereby contributing to increased tensile strength of the healing tissue (1–3). Treatment has been by direct injection in fluid or viscous vehicles, with biological effects being observed with doses of 1–10 μg protein per wound site. Analysis and development of TGF-β isoforms and other growth factors as wound healing agents is hampered by the cost of production and purification of recombinant protein and the necessary reluctance to produce large-scale quantities of structural variants. Expression of growth factors at the wound site by gene therapy could obviate many of these restrictions. Using particle-mediated gene transfer, Andree et al. (4) recently demonstrated that transfection of an excisional wound with an EGF expression construct was effective in influencing the rate of reepithelialization and time to closure, and Nabel et al. (5) have shown that transfection of TGF-β1 into the blood vessel wall elicits a fibrogenic response.

We used luciferase reporter gene expression constructs with several viral promoters to optimize particle-mediated gene delivery as a means of delivering biologically active expression constructs into the skin of rats. Transfection of rat skin with a human α1-antitrypsin expression construct further defined the histological distribution and identification of transfected cells. The major objective was to demonstrate a reproducible biological effect from transient expression of TGF-β1 in a full thickness skin incision. The results show that particle-mediated transfection, using appropriate expression vectors, is a simple and effective means of enhancing the rate of wound repair in an animal model.

Methods

Plasmids. Luciferase expression plasmids containing a variety of viral promoters were used. A simian virus early promotor (SV40)[1]-luciferase construct, pGL2, was obtained from Promega Corp. (Madison, WI). A Rous sarcoma virus promoter (RSV)-luciferase construct was built by subcloning the luciferase cDNA from pGL2 into the Hind-III–BamHI site of the pREP9 plasmid obtained from Invitrogen Corp. (San Diego, CA). A cytomegalovirus promoter (CMV)-luci-

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1. *Abbreviations used in this paper:* CMV, cytomegalovirus; LA, luciferase activity; PVA, polyvinyl alcohol; RSV, Rous sarcoma virus; RT-PCR, reverse transcriptase–PCR; SV40, simian virus 40.

ferase construct was the gift of Dr. K.L. Brigham (Vanderbilt University). The pSV40–TGF-β1 construct contains a full length mouse TGF-β1 cDNA embedded within a globin minigene, under the control of the simian virus early promoter and origin of replication, kindly provided by Dr. H.L. Moses (Vanderbilt University) (6). The pRSV–TGF-β1 construct was made by subcloning the murine TGF-β1 cDNA sequence downstream of the RSV promoter of pREP9. The pCMV–TGF-β1 construct was made by subcloning the murine TGF-β1 cDNA as an EcoRI fragment and placing it under the control of the CMV promoter of the pCEP4 construct obtained from Invitrogen Corp. The pCMV–TGF-$\beta1_{cys/cys}$ plasmid was constructed by subcloning the mutant, constitutively active porcine TGF-$\beta1_{cys/cys}$, pK9a (7) cDNA driven by the metallothionein promoter into the BamHI site of pCEP10, obtained from Invitrogen Corp. The retroviral vector MFG–TGF-β1 was assembled in a two-step process to remove all 5′ and 3′ noncoding sequences of the TGF-β1 cDNA. A fragment from the 5′ end of TGF-β1 (Bgl 1 to BamH1) was isolated, combined with a double-stranded oligonucleotide (5′-CATGCCGCCCTCC-3′ and 5′-GGGCGG-3′) and ligated as a three-part ligation into purified MFG vector, which had been previously cut with Nco1 and BamH1. This intermediate clone, containing the TGF-β1 5′ end, was cut with BamH1, treated with calf intestinal phosphatase, and gel purified. Into this BamH1 site was cloned a PCR product of the remaining 3′ end of the TGF-β1 cDNA. This PCR product, prepared using a forward primer (5′-GAAGTGGATCCACGAGCCCAAGGGCT-3′) and a reverse primer (5′-CTGCTGGATCCAGATCTTCAGCTG-CACTTGCAGGAGCG-3′), was digested with BamH1 and gel purified. The sequence of the final MFG–TGF-β1 vector was verified by DNA sequencing. A CMV (human) α1-antitrypsin construct, pWRG-3107, containing a full length human α1-antitrypsin cDNA, was the gift of Dr. W. Swain of Agracetus, Inc. (Middleton, WI). Recombinant plasmids were propagated in *Escherichia coli* DH1α, and plasmid DNA was isolated and purified in milligram batches by column chromatography using Maxi Prep Columns from Promega Corp. according to the manufacturer's recommendations.

Gene transfer. Particle-mediated gene delivery used the Accell® pulse gun delivery device developed by Agracetus, Inc. Using a protocol also developed by Agracetus, Inc., supercoiled plasmid DNA was precipitated onto gold particles (0.95 μm in diameter) in the presence of 50 μM spermidine and 0.5 M $CaCl_2$ at a final concentration of 2 μg DNA/mg gold. The DNA/gold complexes were washed extensively with absolute ethanol and resuspended in absolute ethanol. The suspension was then used to coat the interior of 1/8′′-o.d. Tefzel® tubing from McMaster-Carr Supply Co., (Elmhurst, IL) in a specially designed loading and drying apparatus designed by Agracetus, Inc. After the particles adhered to the tubing, it was cut into 1/2′′ lengths such that 0.50 mg of DNA-coated gold particles would be delivered with each shot. Prepared tubing segments were stored at −20°C in a sealed, desiccated container. At the time of the experiment, the 1/2′′ DNA/gold-coated tubes were brought to room temperature, loaded into the cylinder of the delivery device, which resembles a revolver, and accelerated into the tissue surface by the rapid release of a pulse of helium with an electrically controlled valve at defined pressures ranging from 200 to 800 psi.

Animals. Male Sprague-Dawley rats (250–300 g; Harlan Sprague Dawley Inc., Indianapolis, IN) were housed in the VA Animal Care Facility maintained according to the American Association for Accreditation for Laboratory Animal Care standards. When required, the animals were made diabetic by a single, intraperitoneal injection of streptozotocin (55 mg/kg) 2 wk before surgery (8). The animals were allowed food and water ad libitum. Serum glucose levels were determined by a colorimetric glucose oxidase enzymatic method, using the One-Touch® device from Johnson & Johnson Co. (Arlington, TX), before the initiation of the studies and at the time of killing. All transfections and surgical procedures were carried out under general anesthesia with ketamine and xylazine. All animal procedures were approved under the guidelines of the local Animal Care and Use Committees.

Cutaneous transfections were carried out with the animal under general anesthesia. The site to be transfected was prepared by clipping the pelt. For some studies, the skin was further prepared by treatment with a depilatory agent, Neet® (Reckitt & Colman Inc., Wayne, NJ), according to the manufacturer's recommendations. The plasmid/gold complexes were then accelerated into the skin at the selected pressure by placing the muzzle of the gene gun in contact with the skin at the target site.

Tensile strength. Two rows of three sites per row distributed transversely across the ventral surface of the rat were transfected with a single dose of either the expression construct or a control DNA. Immediately after transfection, full thickness, transverse incisions measuring 1 cm were made through the center of the target area, and the skin margins were approximated with three 11-mm Michel wound clips. Animals were killed at the specified time points by CO_2 poisoning, after which the pelts were excised and the clips removed. Skin strips bisecting the long axis of the scar were prepared and tensile strength was determined using an Instron tensiometer. The results were normalized for the cross-sectional area of the wound and reported as a ratio between the tensile strength of wound sites transfected with TGF-β1 expression vectors and luciferase vectors (control). The significance of the difference between treatment groups was determined by the Student's *t* test.

Luciferase assay. At intervals from 0 to 7 d after transfection with luciferase vectors, animals were killed, pelts were removed, and tissue was homogenized in a lysis buffer containing 100 mM potassium phosphate, pH 7.8, and 1 mM dithiothreitol, 0.1% Triton X-100, and 10 μg/ml PMSF at 4°C. Luciferase activity was measured in 20-μl aliquots of tissue lysate, with 100 μl luciferase assay buffer consisting of 5 mM ATP, 15 mM $MgCl_2$, and 1 mM D-luciferin as provided by Promega Corp. using a liquid scintillation counter (Packard Instrument Co., Inc., Meriden, CT) set for single photon counting. The total protein concentration was determined by the bicinchoninic acid protein assay from Pierce Chemical Co. (Rockford, IL), with bovine serum albumin as a standard. Luciferase activity (LA) was expressed as counts per minute per microgram total protein.

Sponge implantation and in vivo transfection. In anesthetized rats, four polyvinyl alcohol (PVA) sponges fabricated from blocks of Ivalon made by Unipoint Industries, (High Point, NC) and measuring 10 mm in diameter and 2 mm in depth, were implanted subcutaneously beneath the panniculus carnosus adjacent to the abdominal wall fascia as previously described (9, 10). 4 d after surgery, either 100 μg of supercoiled pSVTGF-β1 or pCH-110 (1 μg/μl) suspended in a 20% sucrose solution was injected into the sponges. At the time of killing, the sponges were recovered and the external capsule and adventitia removed. The sponges were then weighed and analyzed for DNA, protein, and collagen content. DNA content was determined by fluorimetry (11) and protein content by the bichinchonic acid protein assay. Collagen content was determined as an extrapolation from the concentration of hydroxyproline, as measured from its phenylisothiocyanate derivative by reverse-phase high performance liquid chromatography (12).

In vitro expression of TGF-β1 activity. HeLa cells were cultured in DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and nonessential amino acids, in a 5% CO_2 atmosphere at 37°C to 50% confluence, at which time they were transfected with either 20 μg supercoiled pSV40-TGF-β, pK9a, or pCSF-CAT provided by Dr. L. Matrisian by a modification of the method of calcium phosphate precipitation as described by Parker and Stark (13). Briefly, the supercoiled DNA was added to 0.5 ml of a 0.25 M $CaCl_2$, to which 0.5 ml of a buffer consisting of 280 mM NaCl, 1.5 mM Na_2HPO_4 and 50 mM Hepes, pH 7.1, was added drop-wise while swirling constantly at room temperature. This mixture was gently added to growing HeLa cells at 60% confluency and incubated for 4–6 h at 37°C in a 5% CO_2 atmosphere. At the end of the incubation period, the solution was aspirated and the cells rinsed with 10 ml of PBS, after which 2 ml of 15% glycerol was added to the dish and incubated at room temperature for 15–30 s. The glycerol was aspirated and the cells washed twice with 10 ml PBS, after which 10 ml of cul-

ture medium was added and the cells returned to standard culture conditions. Cells were harvested at 72 h and the conditioned media were clarified by centrifugation. To estimate TGF-β activity, confluent cultures of porcine vocal fold fibroblasts were incubated in the presence of 50% (vol/vol) conditioned medium. In some cases, conditioned medium was acidified according to the following protocol: an aliquot of tissue culture medium (pH 7.8) was dialyzed against 0.1 M HOAc (pH 3) for 16 h, after which it was equilibrated for 48 h with two changes vs. 0.01 M HOAc at 4°C. The final pH of the acidified tissue culture medium was 7.29 when mixed 50:50 (vol/vol) with fresh DMEM. Culture supernatants from vocal fold fibroblasts exposed to conditioned media were harvested and stored at −20°C. The DNA concentration was determined for cell layers and tropoelastin content of the tissue culture medium was determined by indirect ELISA as previously described (14, 15). Data were expressed as molecular equivalents of tropoelastin produced per cell per hour.

RNA isolation and reverse transcriptase-PCR. Total RNA was isolated from transfected tissues by a modification of the guanidine isothiocyanate–acid phenol method (16). Residual plasmid DNA was destroyed by digestion of the RNA preparation with 1 U/ml RNase-free deoxyribonuclease from Promega Corp. in a buffer containing 400 mM Tris-HCl, pH 7.5, 100 mM NaCl, 60 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 U/reaction of RNasin, purchased from Promega Corp., at 37°C for 30 min. RNA was isolated by phenol extraction and ethanol precipitation. First strand cDNA synthesis was primed by the oligonucleotide 5′-AGGACCTTGCTGTACTGAGT, corresponding to nucleotides 1925–1906 of the exon 7 of porcine TGF-β1 (17) in the presence of a buffer containing 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT, 1 mM each dATP, dCTP, dGTP, and dTTP, 100 μg/ml BSA, 1 μg RNA, and 10 U of avian myeloblastosis virus reverse transcriptase (Promega Corp.). The reaction was incubated for 1 h at 42°C, 5 min at 90°C, and chilled on ice. The RNA template was hydrolyzed by digesting the reaction product with RNase H before amplification. 1/10 vol of the first strand synthesis reaction (2 μl) was then amplified by 20 to 30 cycles of PCR in the same buffer used for first strand synthesis with addition of 0.1 μM of the 5′ TGF-β1–specific oligonucleotide 5′-CCTGGATACCAACTACTGCT, which corresponded to nucleotides 1763–1782 of the exon 5 of porcine TGF-β1 (17), and Promega Corp.'s Taq DNA polymerase.

The primers were chosen such that only the resulting amplification product of the endogenous rat TGF-β1 would contain a HaeIII endonuclease site. The amplified products were digested with HaeIII, size fractionated by electrophoresis in a 12% polyacrylamide gel, transferred to a nylon membrane, and subjected to Southern blot hybridization with a random primed, ^{32}P-labeled murine TGF-β1 cDNA.

Histology and immunocytochemistry. Tissue biopsies were fixed in 10% phosphate buffered formalin and embedded in paraffin for routine staining in hematoxylin and eosin. Tissue specimens for immunohistochemistry were fixed in 4% paraformaldehyde in PBS. Tissue sections were deparaffinized, rehydrated in graded alcohols, and blocked in 10% donkey serum for 30 min. Sections (6 μm) were incubated at room temperature for 3 h in 1:200 polyclonal, sheep anti–human α1-antitrypsin, followed by washing in PBS and reaction with a donkey anti–sheep IgG conjugated to alkaline phosphatase at a 1:200 dilution for 30 min. Primary and secondary antibodies were obtained from The Binding Site (Birmingham, UK). Phosphatase activity was detected with an Alkaline Phosphatase Substrate Kit 1 provided by Vector Laboratories, Inc. (Burlingame, CA).

Results

Studies with reporter gene expression in skin

The initial objective of this study was to assess the feasibility of using particle-mediated gene delivery as a method for achieving in vivo transfection in rat skin. To study this DNA delivery process, we accelerated a 0.5-mg bolus of 0.95 μm diameter gold particles containing 1 μg of either a luciferase expression construct driven by a viral promoter or an irrelevant DNA into dorsal and ventral sites. Animals were returned to their housing for intervals of 1–5 d. After killing, an 8-mm punch biopsy of the skin at the site of transfection was analyzed for luciferase activity.

Role of pressure and site. To examine the effect of the driving force of pressurized helium on the level of luciferase activity in rat skin, the ventral and dorsal surfaces were transfected with luciferase expression constructs at pressures of 400–800 psi and assayed for LA at 24 h after transfection. The results indicted a clear relationship between the force at which the particles were driven into the skin and the measured luciferase activity on both the ventral and dorsal surfaces (Fig. 1, *A* and *B*). Maximal luciferase activity in the abdominal surface of the animal was achieved with 400–500 psi, after which there was a sharp drop in activity. In contrast, the greatest level of LA in the back was seen at 800 psi; however, LA was seven times higher on the abdominal surface as compared with the dorsal surface. We did not observe a preferential topographic expression of LA at different sites on either the ventral or dorsal surface (data not shown). The difference in efficiency of LA expression between the dorsal and ventral surfaces may be explained, in part, by the relative thickness of the epidermis, and especially the stratum corneum. When the dorsal skin was pretreated with a depilatory agent before transfection, luciferase activity was similar to that achieved by transfection of the ventral skin surface (Fig. 2). Maximal expression was correlated with a driving pressure that caused particles to accumulate just above the dermal–epidermal junction with a minor

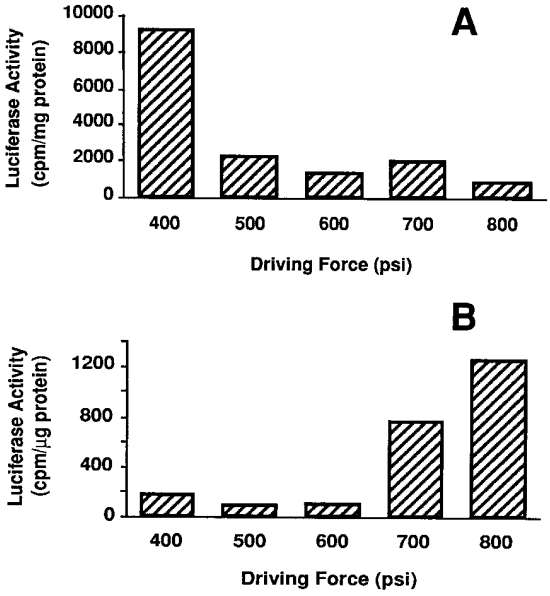

Figure 1. Efficiency of transgene expression as a function of pressure and site. The skin of rats was transfected with pCMV-luc at the pressures indicated, as described in the text. The animals were killed at 24 h after transfection and luciferase activity was determined in homogenized skin extracts. Data are expressed as counts per minute luciferase activity per milligram protein vs. driving pressure in pounds per square inch. Background in this assay with irrelevant plasmids was < 100 cpm/μg protein. (*A*) Pressure-dependent response in the ventral skin of the rat. (*B*) Pressure-dependent response in the dorsal skin of the rat. Tissues were assayed as described above.

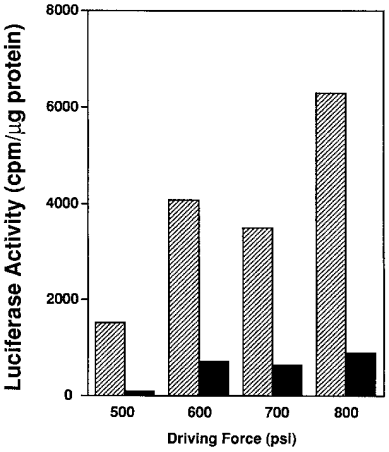

Figure 2. Effects of pretreatment with a depilatory agent on the luciferase activity in dorsal rat skin after transfection with pCMV-luc. The dorsal pelts of rats were shaved with mechanical clippers, after which one half of the back was treated with a depilatory agent. Rats were transfected with pCMV-luc at the indicated pressures. The animals were killed 24 h after transfection and luciferase activity (counts per minute per milligram protein) was determined in homogenized skin extracts by liquid scintillation spectrometry. ▨, depilated skin; ■, skin that was only shaved.

population of particles seen within the dermis (data not shown).

Localization of expression. The ventral skin of rats was transfected with a human α1-antitrypsin expression vector driven by the CMV promoter. Transfected tissue was removed from killed animals after 24 h, and protein expression was detected immunohistochemically. Despite initial accumulations of gold particles at the dermal–epidermal junction (data not shown), only a relatively small number of basal or suprabasal cells expressed the mature protein after 24 h (Fig. 3, *a* and *b*). Keratinocytes within the stratum granulosum represented the majority of the transfected cells, with a small number of positive cells noted in the dermis. There was no evidence of an inflammatory response.

Effect of viral promoter. To examine the effect of the viral promoter, we compared LA in the skin of rats transfected with luciferase expression constructs driven by SV40, RSV, and CMV promoters. Ventral skin was transfected at 500 psi and LA was determined at 24 h after transfection. Luciferase activity was detected in all transfected target areas (Fig. 4), and there was a clear hierarchy of activity with the CMV construct inducing the highest level of LA, followed by the RSV and SV40 constructs. Differences approached one order of magnitude.

Duration of luciferase activity. We evaluated the decay kinetics of luciferase activity in the ventral and dorsal skin of rats transfected with either the RSV or the CMV expression constructs at days 1, 3, 5, and 7 after transfection. The results of these studies (Fig. 5) confirmed the superior LA levels in tissues transfected with the CMV constructs as compared with the RSV constructs and the influence of epidermal thickness. The ventral skin showed 10-fold higher LA for both CMV and RSV constructs as compared with the dorsally transfected counterparts. LA was sustained at elevated levels for the CMV constructs for at least 5 d while tissues transfected with RSV constructs had demonstrable LA for ~ 3 d after transfection.

Duration of TGF-β1 transgene expression. An expression construct encoding murine TGF-β1 under the control of a CMV promoter was transfected into ventral skin at multiple sites. Total RNA was isolated and subjected to reverse transcriptase (RT)–PCR using TGF-β1–specific oligonucleotide primers

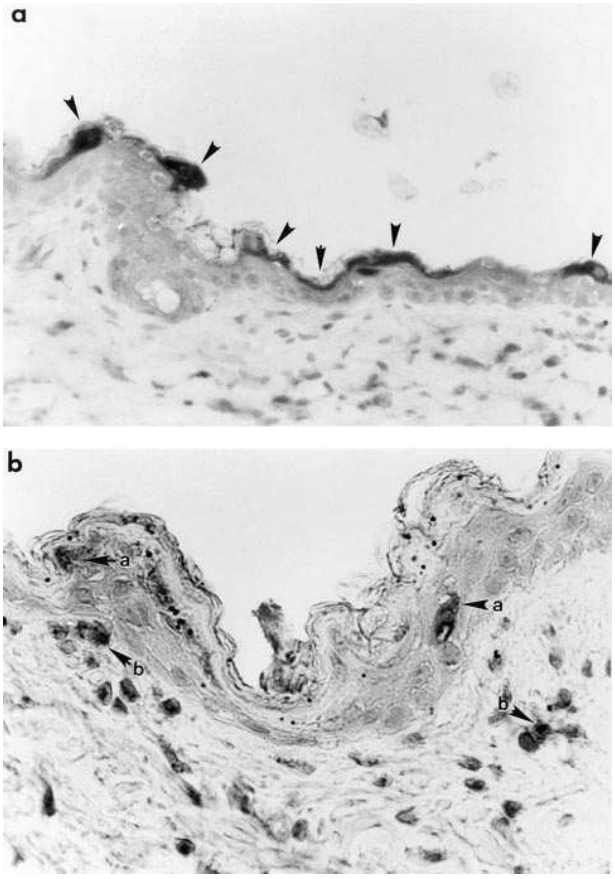

Figure 3. Immunolocalization of human α1-antitrypsin in the abdominal skin of rats transfected with pCMV-α1 antitrypsin (human) 24 h after transfection. (*a*) Low power magnification (35×) of transfected skin shows numerous intensely stained epidermal cells (*arrowheads*) localized within the stratum granulosum. Note the conspicuous absence of inflammatory cells. (*b*) High power magnification (71×) of transfected skin showing numerous gold particles in the stratum granulosum and stratum corneum with expression in the epidermis (*a*, *arrowheads*) and a few transfected cells (*b*, *arrowheads*) noted in the dermis.

that flanked a HaeIII restriction site unique to the endogenous rat TGF-β1 (Fig. 6 *A*). Restriction digest of PCR products with HaeIII, size fractionation by PAGE and Southern blot analysis with a radiolabeled cDNA (Fig. 6 *B*) demonstrated a strong

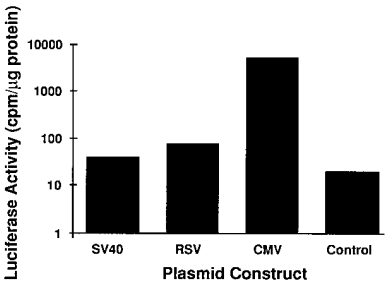

Figure 4. Comparison of luciferase activities in abdominal rat skin transfected with luciferase expression constructs under the control of SV40, RSV, and CMV promoters. The ventral skin of rats was transfected with either pGL2 (*SV40* promoter), pRSV-luc (*RSV* promoter), pCMV-luc (*CMV* promoter), or pCH110 (*Control*) at 400 psi as described in Methods. 24 h after transfection, the rats were killed and luciferase activity was determined in aliquots of homogenized skin extracts. Data are expressed as counts per minute LA per microgram protein on a logarithmic scale.

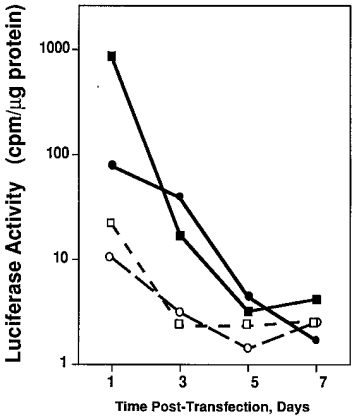

Figure 5. Decay kinetics of luciferase activity in the ventral and dorsal surfaces of rats transfected with either pRSV-luc or pCMV-luc. The ventral (■, □) and dorsal (●, ○) skin of rats were transfected with pCMV-luc (■, ●) and pRSV-luc (□, ○) expression constructs at 500 or 800 psi, respectively. Skin was homogenized at 1, 3, 5, and 7 d after transfection, and aliquots were assayed for luciferase activity. Luciferase activity in ventral rat skin (■) and in dorsal rat skin (●) transfected with pCMV-luc are represented by the solid lines. Luciferase activity in ventral rat skin (□) and in dorsal rat skin (○) transfected with pRSV-luc are represented by the broken lines. Luciferase activity is expressed in counts per minute per microgram protein on a logarithmic scale.

185-bp band, the signature of the transgene, in RNA isolated from tissue transfected with the pCMV-TGF-β1 at all time points tested. A faint band, indicating minor cross-contamination of the RNA samples, was present in RNA isolated from tissues transfected with a luciferase expression construct. Since identical primers and low PCR cycle number were used to detect both rat and mouse transcripts, we estimated the rate of decay of transgene expression under these semiquantitative conditions (Fig. 6 *C*). Reaction mixtures that omitted reverse transcriptase were negative (data not shown), ruling out plasmid DNA as a source of the murine specific signal.

In vitro activities of the TGF-β1 constructs

To verify biological activity of the constructs, HeLa calls were transfected with pSVTGF-β1, a plasmid expressing latent, murine TGF-β1 (6) or pK9a, a porcine TGF-β1 construct driven by the Zn^{++}-inducible metallothionein promoter and containing two cys→ser mutations that render the expressed product constitutively active (17). HeLa supernatants were added to vocal fold fibroblast cultures, and elastin production was measured. Supernatants of pSVTGF-β1 transplants caused a threefold induction of elastin in vocal fold fibroblasts only after acidification, while supernatants of Zn^{++}-treated pK9a transfectants had greater than fivefold induction of elastin compared with controls (Fig. 7).

In vivo activity of TGF-β1 constructs

Transfection of granulation tissue with TGF-β expression construct in surgically implanted PVA sponges. In preliminary studies, we tested granulation tissue as a site for in vivo transfection by injecting either pSVTGF-β1 or pCH110 (a β-galactosidase expression vector) suspended in 20% sucrose into surgically implanted PVA sponges in the rat, and we analyzed the sponges for protein, collagen, and DNA content on days 8 and 11 after transfection. β-Galactosidase was detected in sponge tissue (data not shown). There was a twofold increase in the collagen content of pSVTGF-β1–injected sponges, as compared with the pCH110-injected sponge controls on day 11 (data not shown). Collagen content increased both in absolute and relative concentration, while cellularity was unchanged, consistent with an increase in fibroblast collagen production rather than fibroblast numbers.

Skin transfection and wound studies. To examine the biological effects of transient, local expression of a TGF-β1 transgene on tissue repair, we used particle-mediated gene delivery

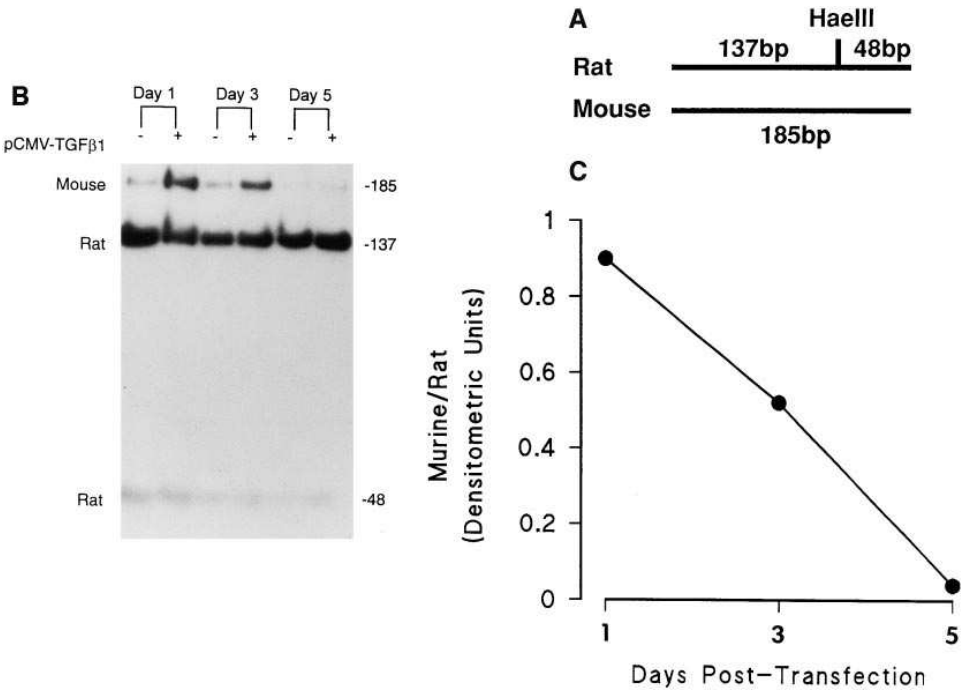

Figure 6. Detection of transgene expression in vivo. The ventral pelts of rats were transfected with either pCMV-TGF-β1 (+) or pCMV-luc (−), and the rats were killed on 1, 3, and 5 d after transfection. Total RNA was isolated from the targeted tissue and amplified with specific primers by RT-PCR. (*A*) The predicted results from the RT-PCR amplification of TGF-β1 mRNA, depending on the species of origin. The PCR product of both species is 185 bp. In the rat, this product is further cleaved by HaeIII into 137- and 48-bp products. The digestion products of the RT-PCR reaction were analyzed by Southern blotting with a radiolabeled, porcine TGF-β1 cDNA as described in Methods, and they are displayed in *B*. The right margin indicates fragment size in base pairs. The endogenous rat TGF-β1 reaction products migrate at 137 and 48 bp, while the uncleaved mouse transgene is detected at 185 bp. There is a small amount of undigested product present in the luciferase-transfected (−) samples, probably representing RNA carryover in the homogenizer probe used for tissue disruption. In *C*, the intensity of the mouse-specific transgene signal, as quantified by scanning densitometry, is represented as a function of days after transfection.

to transfect the skin of both healthy and diabetic rats with expression constructs designed to express latent or active TGF-β1. An initial study with the relatively weak SV40 promoter and latent TGF-β1 indicated that, while not statistically significant, there was a modest increase of tensile strength in the majority, but not all, of the wounds transfected with pSVTGF-β1 as compared with control lesions (Fig. 8 *A*). Since normal rat skin may be relatively less responsive to the biological effects of exogenous TGF-β1, full thickness skin incisions in the streptozotocin-induced diabetic rat were used as a more sensitive test environment. As reporter assays confirmed the higher potency of the CMV promoter when compared with either SV40 or RSV, we further modified the experiment by transfecting the skin with a CMV–TGF-β1 construct. This study included an analysis of both diabetic rats (Fig. 8 *B*, filled circles) and four rats that failed to become hyperglycemic after the injection of streptozotocin (Fig. 8 *B*, open triangles). Pretreatment of wounds in both normal and diabetic rats with a CMV-driven murine cDNA encoding latent TGF-β1 increased the tensile strength of transfected incisional wounds for all (diabetic plus normal) rats between 50 and 75% over that of control wounds at 14 and 21 d.

We next employed the transgene expressing constitutively active TGF-$\beta1_{cys/cys}$ mutant cDNA under the control of the CMV promoter in nondiabetic rats. The results of this study (Fig. 8 *C*) demonstrated a highly significant increase in the tensile strength of the healed wounds at both 14 and 20 d after transfection. Average wound strength was increased by 35% ($P = 0.007$) at 14 d and 75% ($P = 0.04$) at 20 d. Thus, the transient expression of a constitutively active TGF-β1 peptide exerted a reproducible and physiologically significant increase in the extent of wound healing. In further studies, a constitutively active construct driven by the murine sarcoma virus LTR produced an 80% ($P = 0.021$) increase in the average response of incisions at 14 d (Fig. 9) under conditions where the CMV construct produced a 30% increase and TGF-β itself (2 μg) also produced an ~40% increase. To determine the optimal timing for treatment, animals were transfected 0, 24, 48, or 72 h before surgery with the CMV–TGF-β1 construct (Fig. 10). A clear optimum was observed when sites were treated 24 h before surgery (48±13% increase, $P = 0.005$).

Discussion

A key stumbling block in the investigation and clinical success of growth factor therapy is drug delivery. Virtually all animal experiments and clinical trials have required large, often repeated, doses of growth factor (1, 18), suggesting that exogenous presentation can lead to sequestration, clearance, or destruction of otherwise remarkably potent molecules. Gene therapy at the wound site represents a clinically relevant appli-

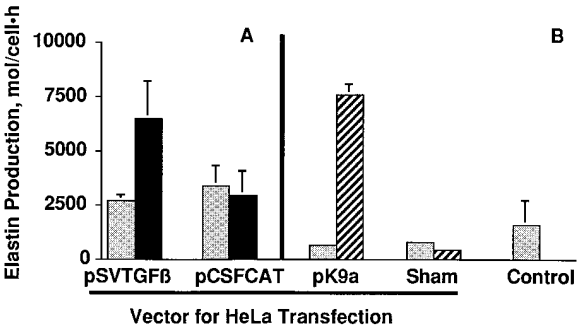

Figure 7. Production of biologically active TGF-β by in vitro transfection. HeLa cells were transfected with pSVTGF-β1, pK9a, pCSF-CAT (CAT), or nothing (sham) by the calcium phosphate precipitation method. The conditioned media were harvested at 48 h and stored at −20°C before or after being acid treated to activate latent TGF-β1. Porcine vocal fold fibroblasts were cultured in the presence of the native or acid-treated conditioned media at a final concentration of 50% (vol/vol) for 48 h. Tropoelastin production was assayed in the culture media by indirect ELISA and expressed as tropoelastin equivalents produced per cell per hour. HeLa cells did not produce significant levels of tropoelastin. The data from each experiment are displayed as the means±SEM. (*A*) Production of latent TGF-β activity by cells transfected with pSVTGF-β1. Elastogenic activity of supernatants from HeLa cells transfected with control and TGF-β1 vectors was assayed on porcine vocal fold fibroblasts with (■) or without (□) prior acid activation. (*B*) Constitutively active TGF-β1 expression regulated by the metallothionein promoter. Zinc was used to stimulate the expression of a TGF-β1 activity that did not require acid activation. HeLa cells were transfected with the indicated constructs, and conditioned media were harvested after incubation of transfectants in the presence (▨) or absence (□) of Zn^{++}. Media were added to confluent vocal fold fibroblast cultures, and tropoelastin production was measured in the culture supernatants. Basal tropoelastin production was lower in this batch of fibroblasts.

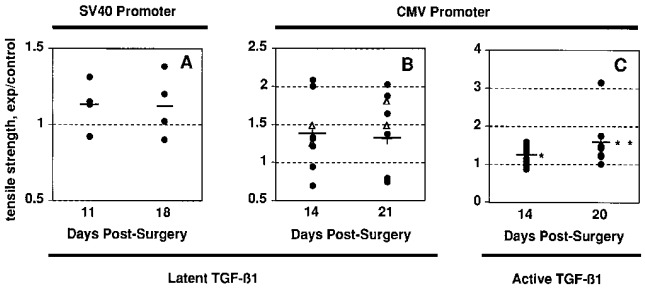

Figure 8. Tensile strength of full thickness abdominal incisions in rat skin transfected with TGF-β1 expression constructs by particle-mediated gene delivery. The abdominal skin of adult Sprague-Dawley rats was transfected with both TGF-β1 (experimental) and luciferase (control) expression constructs, after which four full thickness incisional wounds were made across the targeted tissue sites. The animals were killed on the day indicated, the pelts excised, and tissue strips cut perpendicular to the long axes of the incisions. Tensile strengths (grams per millimeter squared) were determined as described in Methods, and the results are reported as ratios of tensile strengths measured in the wounds transfected with TGF-β1 expression constructs to those in incisional wounds transfected with the luciferase construct for each animal (experimental/control). The horizontal bars represent the means of the experimental/control within each group of animals. (*A*) Experimental wound sites were transfected with pSVTGF-β1, in which a murine latent TGF-β1 cDNA is under the control of the SV40 promoter. Two groups of animals ($n = 4$ animals per group) were transfected and wounded. Experimental:control ratios are represented by filled circles. (*B*) Rats were made diabetic with streptozotocin. Experimental wound sites were transfected with pCMV–TGF-β1. Ratios are represented by filled circles, and ratios in four animals that did not become hyperglycemic are shown as triangles. The means are calculated only for the diabetic rats. (*C*) Experimental wound sites were transfected with pCMV–TGF-$\beta1_{cys/cys}$, a constitutively active form of the peptide. Student's *t* test was used to calculate significant differences of paired values. $*P < 0.01$, $**P < 0.05$.

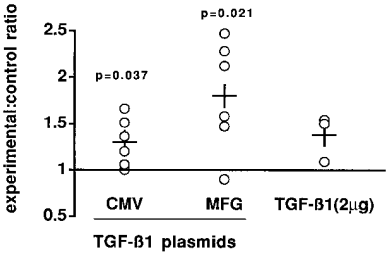

Figure 9. Comparison of transfection and direct injection of TGF-β. Wound sites were transfected with constitutively active TGF-β1 vectors driven by either the murine sarcoma virus LTR (MFG) or the CMV promoter. For comparative purposes, data from a previous study (54) is included. Data are expressed at the experimental:control ratio of each of six paired wound sites in three different animals. Means are shown by crosses. Significance was determined by Student's *t* test.

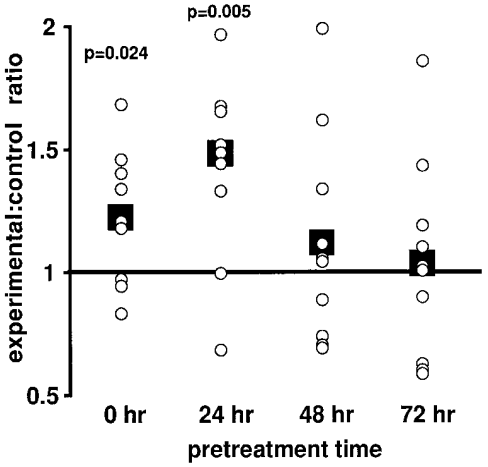

Figure 10. Effect of transfection timing on wound healing. Surgical sites were transfected by particle-medicated acceleration at 0, 24, 48, and 72 h before the time of surgery. Incisional strength was measured on tissue harvested 14 d after surgery. Data are expressed as the experimental:control ratio of each of nine paired wound sites in five different animals. Means are shown by filled squares. Significance was determined by Student's *t* test. *P* values ≤ 0.05 are illustrated.

cation of the technology of particle-mediated gene delivery. It also has practical value at the experimental level, since advancement of our understanding of the role of cytokines in tissue repair is limited by the availability of the molecules in pharmacological quantities. Use of recombinant DNA directly allows the investigator to alter and evaluate the biological consequences of changes in peptide structure rapidly. The successful introduction of naked, supercoiled DNA expression constructs into tissue within the intact animal has been reported in many systems (19–23). The present studies were initiated with protocols that had been used for successful transfection of reporter genes into skeletal muscle (22, 23) and vascular endothelium (5, 24, 25). Although we were able to achieve statistically significant increases in collagen content of experimental granulation tissue with an SV40-driven vector expressing native, latent, murine TGF-β1 (26), the biological effects were inconsistent. Similar studies were carried out with PVA sponge implants using liposome-mediated transfer with a commercial reagent, Transfectam®, obtained from Promega Corp.; however, reproducibility and magnitude of the effect (increased collagen concentration) were variable and costly to achieve (data not shown). Successful transduction of mouse skin and tumors (27) with the particle delivery technology (28, 29) offered the advantages of low cost, an appropriate design for skin transfection, and the guarantee of transient, epidermal expression by the high turnover rate of keratinocytes.

Key parameters in obtaining biologically significant expression of transgenes using the gene gun included promoter selection and accelerating pressure. As observed by Cheng et al. (29), site-specific variation in epidermal thickness, largely attributable to the stratum corneum, was an important variable. Depilation with a commercial preparation appeared to equalize regional transfection efficiencies, which is significant since our current wound healing studies use the more conventional, dorsal surface of the animal. Kinetic analysis of luciferase expression confirmed the transient nature of peptide expression, and immunolocalization suggested that the vast majority of expression was in maturing strata of the epidermis, at least at intervals greater than 24 h after particle gene delivery. Subsequent evaluation of expression of the TGF-β1 transgene confirmed the transient nature of transduction.

TGF-β is a pleiotropic cytokine family having three mammalian isoforms that interact with a heterodimeric, serine-threonine kinase receptor to initiate an intracellular signal cascade (30, 31). The fibrogenic properties of TGF-β1 suggested that modulation of TGF-β1 activity could alter the process of tissue repair and fibrosis (32), a thesis that has been borne out by numerous investigations in early wound healing models or fibrotic models (5, 33, 34). TGF-β1 is present as a complex in plasma, and significant quantities can be mobilized at sites of injury by platelet degranulation. In addition, many epithelial cells, including keratinocytes and bronchial epithelium, can release and secrete TGF-β isoforms if injured (35). The bioavailability of TGF-β is regulated in a complex fashion. The molecule adheres strongly to several components of the extracellular matrix, including thrombospondin, the small proteoglycans decorin and biglycan, and fibronectin. TGF-β is secreted as a precursor that can be activated by acidification or proteolysis, but the physiologic mechanism appears to involve a multistep, surface reaction in which plasminogen activator, transglutaminase, and the mannose-6-phosphate receptor are involved (36). Activation does not necessarily dissociate the propeptide (latency-associated peptide) from the active, COOH-terminal portion. Latency is also controlled by the presence of latent TGF-β binding proteins, a family of at least three proteins whose structure is represented in the central domain of fibrillin, a component of elastic fiber microfibrils (37–39). The ability of TGF-β administration to increase wound strength appears to be correlated, at least in part, with chemotactic activity for monocytes, stimulation of matrix gene expression, repression of protease expression, stimulation of protease inhibitor expression, and stimulation of fibroblast contractility. TGF-β can promote the differentiation of myofibroblasts, a characteristic element of wound repair. At high levels, TGF-β can repress epithelial cell proliferation, although it has the capacity to stimulate division of many cell types through induction of mitogens such as PDGF. Matrix effects may also be affected by the induction in connective tissue cells of the novel cytokine, connective tissue growth factor (40). TGF-β1 also plays a key role in immunomodulation, as evidenced by the perinatal lethality of a targeted deletion (41). Many studies have noted the persistent biological effects of single-dose or short-term administration of TGF-β1, presumably due to its autoinduction (42, 43) and transactivation of other isotypes.

As was the case of EGF vectors in porcine skin wounds (4), particle-mediated gene delivery with TGF-β expression vectors produced significant biological effects. TGF-β1 has also been shown activity as a transgene in a vascular wound model (5). Key factors that shifted our results from trends to consistency were the use of a strong CMV or Moloney murine leukemia virus promoter, a constitutively active form of the growth factor, and particle-mediated transfection. Timing of introduction was also an important consideration. There appeared to be a priming effect, since transfection was more effective 24 h before surgery. Before possible use of DNA as a therapeutic agent for skin, assessment of the antigenicity of modified, human proteins will be essential. Although RT-PCR failed to detect transgene expression after 5 d, others have reported sustained, low level expression in the dermis (29) when the dermis was surgically exposed before transfection. We feel that it will be essential to develop data showing insignificant persistence of growth factor expression to override concerns that prolonged expression of such molecules might disrupt normal skin physiology.

In wounds, growth factor expression is complex and transient. Defects in wound healing could arise from growth factor, receptor, or signal transduction deficiencies. Strategies such as virally-mediated transfection (44–48) or the introduction of ex vivo transfected cells (45, 49–53) could certainly have a role in delivery of exogenous growth factors. Particle-mediated gene delivery is a simple and effective method of stimulating the autogenous, transient expression of molecules that improve the healing of wounds. Further studies with combined growth factor treatment in healing-impaired models should help to validate further the principles outlined in this report.

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