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In vivo transfer and expression of a human epidermal growth factor gene accelerates wound repair

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ABSTRACT **This report details the transfer of a human epidermal growth factor (hEGF) expression plasmid to porcine partial-thickness wound keratinocytes by particle-mediated DNA transfer (Accell). After gene transfer an external sealed fluid-filled wound chamber was used to protect the wound, provide containment of the exogenous DNA and expressed peptide, and permit sampling of the wound fluid. Analysis of wound fluid for hEGF and total protein, an indicator of reformation of the epithelial barrier, showed that wounds bombarded with the hEGF plasmid exhibited a 190-fold increase in EGF concentration and healed 20% (2.1 days) earlier than the controls. EGF concentrations in wound fluid persisted over the entire 10-day monitored period, decreasing from 200 pg/ml to 25 pg/ml over the first 5 days. Polymerase chain reaction results showed that plasmid DNA was present in the wound for at least 30 days. These findings demonstrate the possible utility of *in vivo* gene transfer to enhance epidermal repair.**

Epidermal growth factor (EGF) is a potent mitogen originally isolated by Cohen (1) from murine salivary glands. It is present in a number of sources, including platelets, and may thus be an important endogenous regulator of wound healing (2, 3). Exogenous EGF has shown promise as a therapeutic agent (3–9); however optimal delivery of the growth factor to the wound remains a problem (9). The continuous presence of EGF is required during the early stages of repair for optimal healing benefits.

Gene transfer offers an attractive method for delivering growth factors to the healing wound (10), since it promises localized and persistent delivery of the potentially therapeutic polypeptides. Previous retroviral and chemically mediated gene transfer to skin has employed a combination of *in vitro* primary culture, DNA transfection of cultured explants, and subsequent autologous transplantation of recombinant cells (11–14). In contrast, particle-mediated gene transfer utilizes microscopic, DNA-coated projectiles (15) that can be accelerated directly into target tissues *in vivo* (16). Thus, plasmid DNA encoding a particular modulator of repair can be delivered directly to the cells of the healing wound, providing the wound environment with a continuous source of peptide growth factor.

We have developed a porcine wound model that employs an external chamber (17) that provides the wound with a sealed liquid environment. The unique nature of this enclosed system allows us to treat wounds by altering the fluid milieu (i.e., with growth medium, cell transplants, exogenous growth factors, and/or pharmaceuticals) as well as to monitor changes in composition of the wound fluid during the repair process (17, 18). We have used the particle-mediated gene transfer method to deliver an EGF expression plasmid to partial-thickness wounds in pig skin, and the external chamber to monitor presence of the EGF peptide over time, and to determine its effects on healing.

MATERIALS AND METHODS

Wounding. Pigs were maintained in accordance with the Harvard Medical Area Standing Committee on Animals. Surgical procedures were performed under halothane (1–1.5%) anesthesia in a 3:5 mixture of oxygen/nitrous oxide. Partial-thickness excisional wounds (15 × 15 mm, 1.2 mm deep) were created on the dorsum of pigs by using a Padgett dermatome (17). Uniformity of the wounds was ascertained by measuring representative histological sections of the excised skin. After particle-mediated gene transfer, vinyl adhesive chambers (PAM, Columbia, TN) with a basal opening corresponding to the wound size (15 × 15 mm) were sealed to the surrounding skin. Normal saline (1.2 ml; unbuffered, 0.9% NaCl) with penicillin G (100 units/ml) and streptomycin (100 μg/ml) was added to each chamber.

Plasmids. The expression plasmids used in this study were pCMVβ-gal (19) and pWRG1630 (see Fig. 1). The control plasmids, pWRG1637 and pWRG1638, were similar to pWRG1630 except that the former contained the human EGF (hEGF) coding region in an inverted orientation relative to the direction of transcription and the latter contained the coding sequence from an influenza virus hemagglutinin in place of the coding sequence for hEGF. Plasmids were propagated in *Escherichia coli* XL1-Blue MR and supercoiled DNA was prepared on Qiagen (Chatsworth, CA) chromatographic columns as recommended by the manufacturer.

Particle-Mediated Gene Transfer. The procedure for utilizing the Accell (Agracetus) particle-bombardment device has been described elsewhere (16). Briefly, particles were coated by mixing an aliquot of DNA solution, containing the desired amount of DNA, with 10 mg of 1- to 3-μm gold beads and 100 μl of 0.1 M spermidine. One hundred microliters of 2.5 M $CaCl_2$ was then added and left at room temperature for 10 min. A DNA/gold precipitate was formed, which was washed and suspended in absolute ethanol. Three hundred twenty microliters of the ethanolic particle suspension was transferred to a Mylar carrier sheet (18 mm × 18 mm), the ethanol was decanted, and the loaded sheet was allowed to dry. Loaded sheets were maintained in a desiccated atmosphere until used.

At gene transfer, the loaded carrier sheet was accelerated, by an electric arc, against a 100-mesh stainless steel screen that stopped the Mylar carrier sheet but allowed the DNA-coated gold particles to continue and penetrate the target cells. The Accell technique permits adjustment of the velocity of the coated gold particles (by varying the discharge voltage 3–25 kV), the particle size (0.95–15 μm), the amount of plasmid DNA per gold particle, and the amount of DNA-coated gold particles per carrier sheet. Experimental wounds were bom-

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Abbreviations: EGF, epidermal growth factor; hEGF, human EGF; GH, growth hormone; hGH, human GH; bGH, bovine GH.

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barded with gold particles coated with supercoiled plasmid DNA. Control wounds were not bombarded or were bombarded with uncoated gold particles. Wounds bombarded with the hEGF expression plasmid, or pCMVβ-gal, also served as controls for histochemical analysis of gene transfer or analysis of hEGF production and healing time, respectively.

Cell Proliferation Assay. Conditioned medium was prepared by bombarding KB-3-1 cells (kindly provided by M. Gottesman, National Institutes of Health) with the EGF expression vector. The conditioned culture medium (99% Dulbecco's modified Eagle medium, 1% fetal calf serum, L-glutamine, 50 units of penicillin per ml, and 50 μg of streptomycin per ml) was harvested 24 hr after treatment and assayed for EGF biological activity by measuring its effects on [^{3}H]thymidine incorporation by primary human foreskin fibroblasts and Madin–Darby canine kidney (MDCK) cells (kindly provided by V. Hinshaw, University of Wisconsin) (21). Parallel tests were also conducted with conditioned medium from KB-3-1 cells bombarded with the control plasmids, pWRG1637 or pWRG1638, as well as with unconditioned medium. A neutralizing mouse monoclonal antibody specific for human EGF (Ab-2, Oncogene Science) was used for antibody-inhibition assays. The control antibody for these studies was a monoclonal antibody (W6-32, Accurate Chemicals) specific for a human leukocyte antigen (HLA).

Protein Assay. Wound fluid was withdrawn from the chambers every 24 hr. A commercial turbidimetric assay (Stanbio CSF, San Antonio, TX), calibrated by comparison to a bovine serum albumin standard, was used to determine total protein concentration in wound fluid samples (17).

Histological and Histochemical Evaluation. *In vivo* bombarded partial-thickness wounds were biopsied (17 × 10 mm) every third day from day 1, post-bombardment, to day 30. Paraffin-embedded samples were sectioned and stained with hematoxylin/eosin. For histochemical β-galactosidase assays, fresh biopsy specimens were embedded in Tissue Tek O.C.T. compound (Miles), frozen at −70°C, and cut into 8-μm sections. These sections were fixed and stained for β-galactosidase activity with 5-bromo-4-chloro-3-indolyl β-D-galactoside as described by MacGregor *et al.* (22) and then counterstained with hematoxylin.

Enzyme-Linked Immunosorbent Assay (ELISA). A commercially available ELISA kit (Quantikine, R&D Systems) was used to measure hEGF concentration in wound fluid, serum, and culture medium samples. This ELISA does not crossreact with human transforming growth factor α.

Genomic DNA Preparation and Polymerase Chain Reaction (PCR). DNA was prepared from wound biopsies using a Puregene kit (Gentra, Minneapolis). Samples were collected from three wounds for each data collection point. PCR mixtures (23) contained 400 ng of DNA, 0.2 mg of each primer [5′-TCAATAGTGACTCTGAATGTCCCC-3′, nt 3–24 correspond to nt 3347–3368 of GenBank accession no. X04571; and 5′-GGCTGATCAGCGAGCTCTAG-3′, located within the bovine growth hormone (bGH) 3′ region], and 0.5 unit of AmpliTaq DNA polymerase (Perkin–Elmer) in 10 mM Tris·HCl, pH 9.0 (25°C)/50 mM KCl/0.1% Triton X-100/1.5 mM $MgCl_2$/0.2 mM (each) dNTP. After 3 min at 96°C, reactions were subjected to 30 cycles of 60°C for 75 sec, 72°C for 60 sec, and 96°C for 60 sec. Aliquots of the reaction mixtures from triplicate wounds were pooled and 15 μl of each pooled sample was analyzed by agarose gel electrophoresis. PCR products were visualized by staining with ethidium bromide and their identity was confirmed by Southern blots (24) using internal hybridization probes. Preparation of the DNA fragment containing the mature EGF coding region was performed similarly except that reaction mixtures contained 10 ng of λEGF116 DNA, the primers 5′-TCAATAGTGACTCTGAATGTCCCC-3′ and 5′-TCAGCGCAGTTCCCACCACTTCAG-3′, and 10 units of Stoffel fragment DNA polymerase (Perkin–Elmer) in 10 mM Tris·HCl, pH 8.3/10 mM KCl/3 mM $MgCl_2$.

Statistics. To determine healing times, the logarithm of protein concentration was plotted versus time for each wound and then a regression line was calculated through the declining phase. The point at which the regression line crossed the upper 95% confidence interval for values for unwounded skin was defined as the return of epithelial barrier function and the point of healing. Statistical significance between groups was analyzed with the nonparametric Mann–Whitney *U* test (25).

RESULTS

Characterization of the Chimeric EGF Polypeptide Encoded by the EGF Expression Plasmid. The native hEGF gene encodes a 130-kDa transmembrane glycoprotein that contains the mature 6.2-kDa hEGF peptide embedded within the extracellular portion of the protein (26). The EGF expression plasmid used in this study encodes an in-frame fusion of the human growth hormone (hGH) secretory signal peptide and the mature EGF polypeptide (Fig. 1). After signal-peptide cleavage, the polypeptide produced is predicted to contain the 14-amino acid sequence FPTIPLYQASISRV at the amino terminus followed by the mature hEGF sequence.

The production of this variant EGF was confirmed by bombarding KB-3-1 cells with the EGF expression plasmid and, after 24 hr of incubation, analyzing the culture medium by ELISA and Western blot. The Western blot analysis

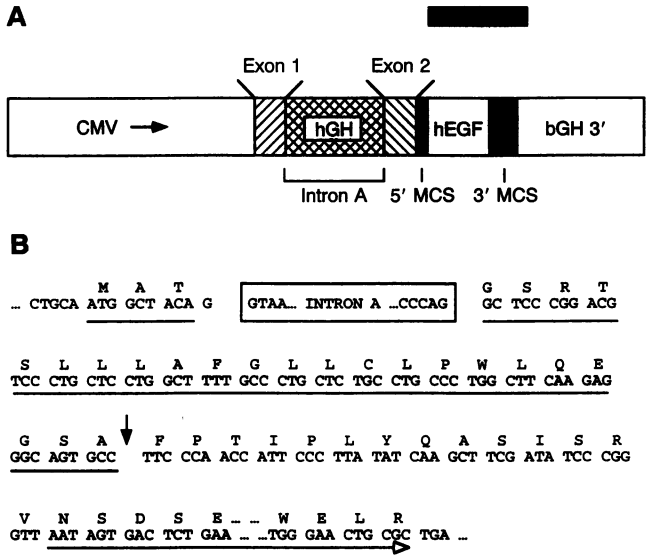

FIG. 1. Structure of the EGF expression plasmid. The plasmid was constructed from pAbP2, an expression/secretion vector (kindly provided by Michael D. Eisenbraun, Agracetus) and a segment of the full-length human EGF cDNA clone, λEGF116 (ref. 27; obtained from ATCC). A DNA segment containing the mature hEGF sequence (nt 3347–3505 of GenBank accession no. X04571) was extracted from λEGF116 by PCR. (*A*) Schematic diagram of the chimeric EGF gene. This gene consists of the cytomegalovirus (CMV) immediate early transcriptional promoter (ref. 28; nt 216–834 of GenBank accession no. K03104), followed by a portion of the hGH gene (ref. 29; nt 275–686 of GenBank accession nos. J00148 and K00612), the mature hEGF coding region, and a segment containing the 3′ untranslated sequence and polyA signal of the bGH gene (from pRc/CMV, Invitrogen). The filled bar above the diagram indicates the region amplified in the PCR analysis shown in Fig. 5. MCS, multiple cloning sites. (*B*) Sequence of the in-frame fusion between hGH and hEGF coding regions. The boxed region indicates the position of hGH intron A. The sequence of the hGH signal peptide is underlined and the expected signal cleavage site is denoted by the filled arrow. The open arrow indicates the coding region for the mature form of hEGF. Ellipses indicate the presence of additional sequence not shown in the figure.

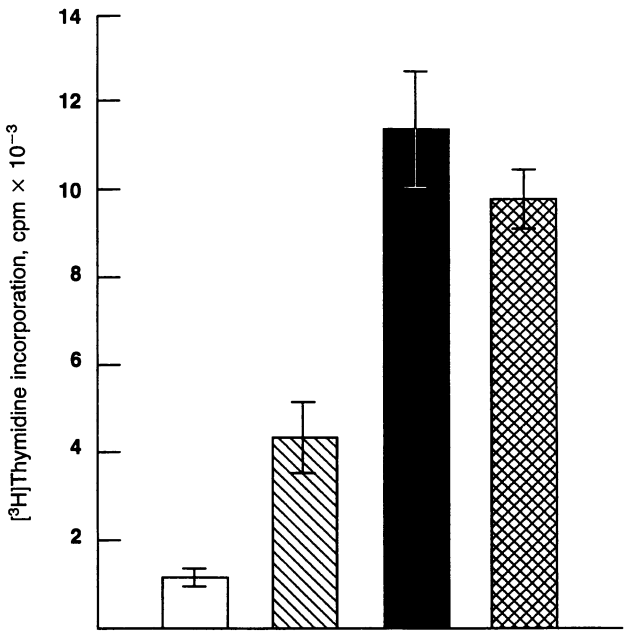

FIG. 2. Mitogenic activity of chimeric hEGF on primary human foreskin fibroblasts: Bars indicate [^{3}H]thymidine incorporation (cpm) for 10^4 cells over 12 hr. Open bar, unconditioned medium; hatched bar, medium from cells bombarded with pWRG1637; solid bar, conditioned medium from cells bombarded with pWRG1637 plus 2 ng of recombinant hEGF per ml; cross-hatched bar, conditioned medium from cells bombarded with the EGF expression plasmid. Data are expressed as mean ± SD.

revealed a band that reacted with antibodies to hEGF and whose electrophoretic mobility was consistent with the predicted size (data not shown). The culture medium was also assayed for mitogenic activity on primary human skin fibroblasts (Fig. 2) and MDCK cells to confirm the biological activity of the peptide. Both cell types showed similar responses. The conditioned medium from nonbombarded KB-3-1 cells showed significant mitogenic activity in this assay as did the medium from cells bombarded with the control plasmids, pWRG1637 and pWRG1638. However, the conditioned medium from KB-3-1 cells bombarded with the EGF expression plasmid (EGF concentration = 7 ng/ml) exhibited ≈2-fold greater mitogenic activity than the control medium, similar to that observed with a saturating concentration (2 ng/ml) of recombinant EGF (Clontech) added to conditioned medium from cells bombarded with the control plasmids. The increase in mitogenic stimulation was unaffected by preincubation with an anti-HLA monoclonal antibody but was substantially inhibited by preincubation with a monoclonal antibody specific for hEGF (data not shown). Preincubation of the antibodies with control conditioned medium plus added recombinant EGF gave similar results.

Gene Transfer to Partial-Thickness Wounds in Pig Skin. Optimal DNA transfer conditions for gene transfer were found to be 1- to 3-μm (diameter) gold particles, 300 μg of gold particles per cm^2, 2.5 μg of plasmid DNA per mg of particles (1.7 μg per wound), and 25 kV. DNA-coated gold particles introduced under these conditions were diffusely and uniformly distributed throughout the wound bed at depths up to 60 μm (Fig. 3) and resulted in reliable levels of transgene expression.

In wounds bombarded with pCMVβ-gal, the presence of β-galactosidase was observed only in keratinocytes in the epidermis and the hair follicles (Fig. 3). No stained cells were observed in either uncoated-particle or nonbombarded control wounds or in wounds bombarded with the EGF expression plasmid. Stained cells were detected up to 6 days postbombardment and at the later time points were located in more superficial layers, suggesting that bombarded cells mature as normal keratinocytes and ultimately exfoliate from the skin. The observation of staining only in keratinocytes could imply a cell-specific expression of the plasmid by keratinocytes but not mesenchymal cells; however, we have demonstrated successful bombardment of primary pig fibroblasts as well as keratinocytes *in vitro* (unpublished data).

EGF Gene Expression in Bombarded Wounds and Effects on Healing. Partial-thickness wounds were bombarded with the EGF expression plasmid by particle-mediated gene transfer using the optimal bombardment parameters. Expression of the EGF transgene was monitored daily by measuring EGF concentration in wound fluid. We found a 193 ± 13-fold increase in EGF concentration 24 hr after bombardment in wounds bombarded with the EGF expression plasmid (n = 26) versus nonbombarded (n = 16), uncoated-particle-bombarded (n = 10), or pCMVβ-gal-bombarded control

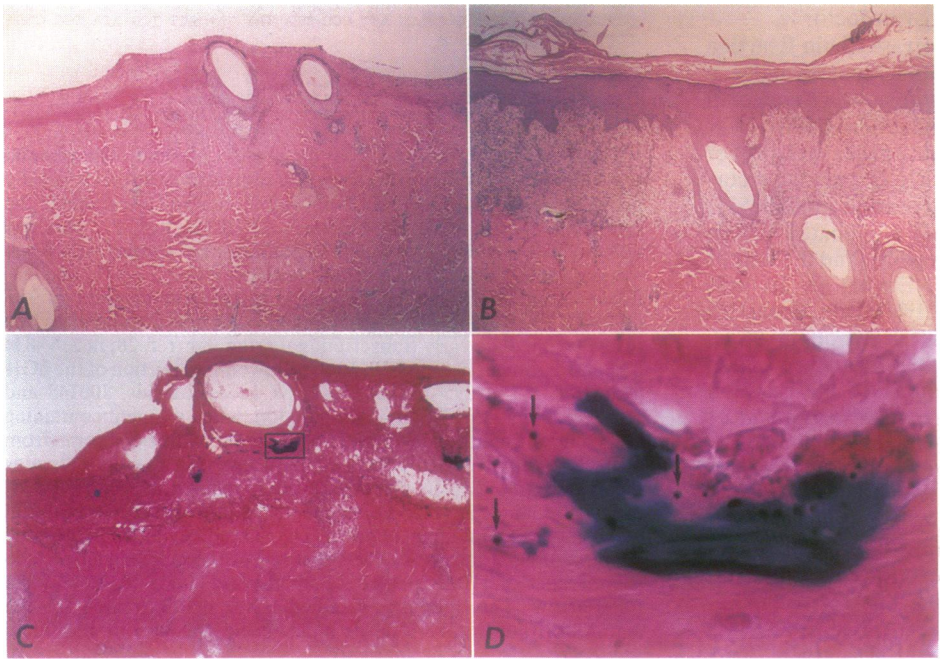

FIG. 3. (*A* and *B*) Histologic sections of wounds after bombardment with the EGF expression plasmid. Cross sections of wounds 1 day (*A*) and 9 days (*B*) after particle-mediated gene transfer are shown. (×38.) Sections confirm regeneration of epidermis (*B*) as well as minimal tissue trauma and inflammatory reaction. (Note that the gold beads are not seen at this magnification.) (*C* and *D*) *In situ* cytochemical staining of wounds after bombardment with pCMVβ-gal. (*C*) Cross section of a wound 3 days after particle-mediated gene transfer. (×75.) (*D*) Enlarged rectangle from *C* shows transgenic cells (blue) and gold beads (arrows). (×900.)

wounds (Fig. 4). Detectable EGF concentrations were maintained over the entire 10-day monitored period but declined rapidly over the first 5 days, decreasing from ≈200 pg/ml at day 1 posttreatment to ≈25 pg/ml at 5 days posttreatment.

Healing times were determined from daily measurements of total protein in the wound fluid. Decrease of protein efflux into the wound fluid provides a precise noninvasive indicator

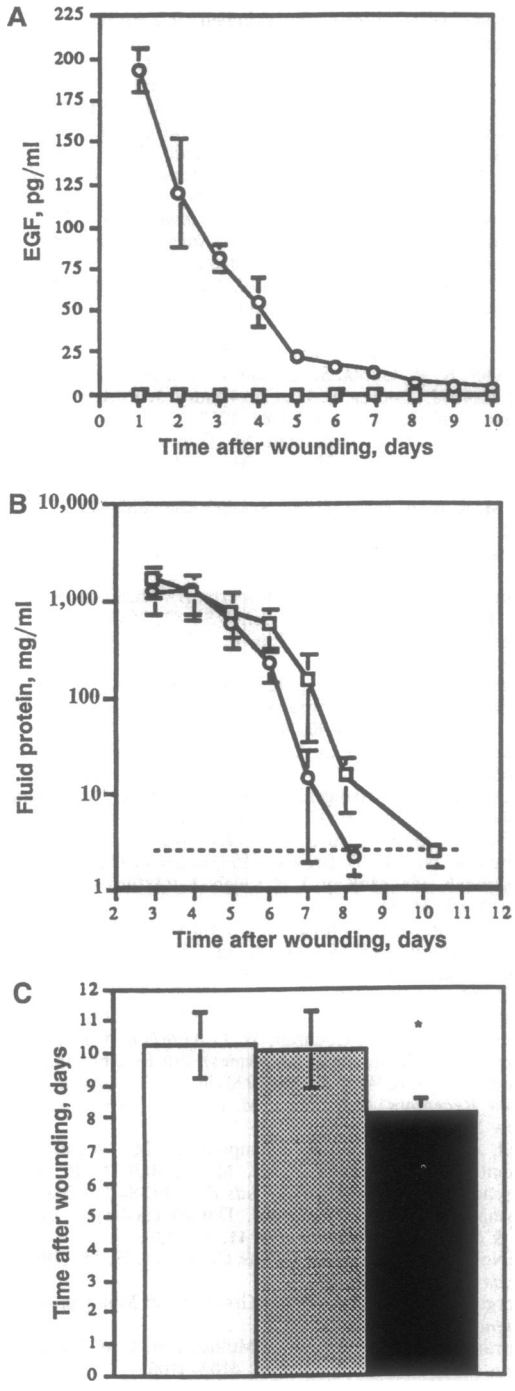

FIG. 4. EGF and total protein concentration and healing time measurements after particle-mediated gene transfer (means ± SD). (*A*) EGF concentrations in wound fluid. ○, Fluid from wounds bombarded with the EGF expression plasmid; □, fluid from control wounds and serum from bombarded animals. (*B*) Protein concentration in wound fluid. ○, EGF bombarded wounds; □, control wounds; - - - line, protein efflux from unwounded skin (baseline). (*C*) Healing times (return of the epithelial barrier function) calculated from *B* (17). Open bar, control wounds; stippled bar, wounds bombarded with uncoated particles; solid bar, wounds bombarded with the EGF expression plasmid. *, $P < 0.0001$.

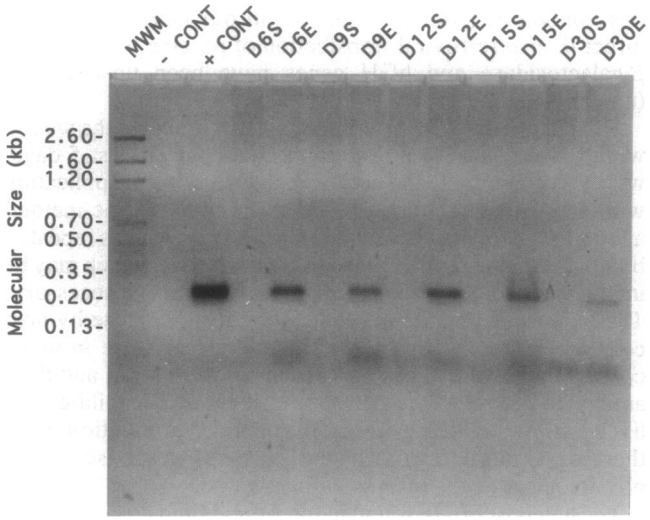

FIG. 5. PCR analysis of wound biopsies. Lanes are labeled according to the templates for the PCR reactions. The negative control sample (− CONT) is from a reaction using lymphocyte DNA from an untreated pig; the positive control sample (+ CONT) used the negative control DNA spiked with 0.22 pg of the EGF expression plasmid. Experimental sample lanes are labeled as follows: numbers indicate the number of days after treatment that biopsies were collected, lanes designated E indicate samples from wounds bombarded with the EGF expression plasmid, lanes designated S indicate samples from wounds that were not bombarded. Molecular weight markers (MWM), and their sizes, are indicated.

of reformation of the epithelial barrier (17). EGF bombarded wounds required 8.2 ± 0.4 days to reepithelialize as compared to 10.3 ± 1.0 days for controls (Fig. 4), showing a significant ($P < 0.0001$) acceleration of healing in the wounds producing high levels of EGF. Wounds bombarded with uncoated particles healed at the same rate as nonbombarded controls, as did wounds bombarded with pCMVβ-gal, indicating that bombardment, under optimal conditions, does not negatively affect healing. Tissue trauma and impaired healing were observed in wounds bombarded at 10-fold higher particle rates (data not shown).

Persistence of Plasmid DNA in Bombarded Wounds. Biopsy specimens from wounds bombarded with the EGF expression plasmid, and nonbombarded control wounds, were collected 1, 3, 6, 9, 15, and 30 days after bombardment and analyzed by PCR to evaluate the presence of plasmid DNA in the wound bed. The amplified DNA fragment has a predicted size of 253 bp and spans the junction between the EGF coding region and the bGH 3′ region in the EGF expression plasmid as shown in Fig. 1 and is thus unique to this plasmid. The results showed that plasmid DNA was detectable at the wound site for at least 30 days (Fig. 5).

DISCUSSION

Clinical use of recombinant growth factors to enhance wound healing has been limited, despite reports of potential benefits, primarily by lack of practical delivery systems. Our results show that gene transfer offers an effective method for delivering polypeptide growth factors to wounds. Furthermore, the plasmid DNA used is inexpensive and stable even at ambient temperatures. Use of the wound chamber complements the gene transfer approach by furnishing containment of the exogenous DNA and the expressed peptide and permitting sampling of the wound fluid. In addition, the chamber provides protection and a route of delivery to the wound.

Several groups, including ours, have successfully transplanted retrovirally transfected keratinocytes into wounds as either single cell suspensions or sheet grafts (30–34). However, the use of retroviruses for gene therapy is laborious and

has limitations (35, 36). Attempts in our laboratory to use direct *in vivo* retroviral transfection of porcine wounds with β-galactosidase and hGH genes have been unsuccessful (unpublished data).

Particle-mediated *in vivo* gene transfer provides the wound with a significant level of growth factor for at least 5 days, which may eliminate the need for frequent topical application with accompanying disturbance of the wound. The method allows for facile delivery of multiple genes as well as repeated bombardment with the same or different genes, which may be important if combinations of growth factors prove necessary. The expressed peptide is produced in wound tissue by host cells and is presented to the target cells in more intimate contact than is the case with topical application. In addition, anticipated developments in gene expression capabilities will likely allow tighter control of peptide production and, thereby, a more regulated and balanced response to the physiological needs of the healing wound.

The levels of hEGF found in the wound fluid are lower than the doses typically used in topical applications of the peptide. It is important to recognize that the hEGF levels measured in wound fluid reflect the sum of production, receptor binding and uptake, and diffusion gradients between wound tissue and wound fluid. Partitioning of the EGF produced among various compartments may be complicated and, thus, the levels observed in wound fluid may be much lower than the effective concentration at the site of action.

Particle-mediated *in vivo* gene transfer yields gene expression for a limited time interval during early wound repair. The concentration of hEGF in wound fluid decreased sharply over the first 5–6 days following gene transfer. Results from the histological β-galactosidase assays are consistent with this time course, indicating loss of transgene expression after 5–6 days. This decrease in gene product is due, at least in part, to exfoliation of the bombarded keratinocytes from the skin. At the same time, the PCR results show the plasmid DNA to be present for at least 30 days. Other reports of engraftment of stably transfected keratinocytes into wounds have found temporary expression of the exogenous genes (30–34). The mechanism of this loss of gene expression is unknown. In our system, gene expression could be extinguished by loss of the introduced gene, by repression of gene expression, or by exfoliation of cells that express the gene.

Permanent gene incorporation, in the context of EGF and other potentially oncogenic growth factors, could result in permanent or sporadic ectopic gene expression, which may be undesirable. None of the wounds bombarded with the EGF expression plasmid have thus far shown abnormal hyperplasia, tissue disorganization, or other indications of pathological transformation during 30 days of observation. The observation that particle-mediated gene transfer targets skin keratinocytes, which follow a normal pattern of differentiation and eventually exfoliate from the body, further mitigates the risk of this procedure.

The 20% reduction in healing time we observe compares favorably with the 10–20% healing time reductions previously obtained by topical application of EGF (6, 20). In the pig model that we used, the wounds repair relatively rapidly without augmentation from exogenously applied factors and thus provide a rigorous test for demonstration of any beneficial effects from the expressed peptide. Moreover, the wound chamber itself provides a significant therapeutic benefit over gauze dressings (17). More dramatic therapeutic benefits may be realized from the treatment of wounds where repair is delayed or deficient. Theoretically, a variety of cutaneous conditions including chronic nonhealing ulcers, keloids and hypertrophic scars, as well as malignant and nonmalignant epidermal diseases might be treated with particle-mediated gene therapy.

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