Genetic modification of cultured skin substitutes by transduction of human keratinocytes and fibroblasts with platelet-derived growth factor-A

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Gene therapy promises the potential for improved treatment of cutaneous wounds. This study evaluated whether genetically modified cultured skin substitutes can act as vehicles for gene therapy in an athymic mouse model of wound healing. Human keratinocytes and fibroblasts were genetically engineered by retroviral transduction to overexpress human platelet-derived growth factor-A chain. Three types of skin substitutes were prepared from collagen-glycosaminoglycan substrates populated with fibroblasts and keratinocytes: HF–/HK–, containing both unmodified fibroblasts and keratinocytes; HF–/HK+, containing unmodified fibroblasts and modified keratinocytes; and HF+/HK–, containing modified fibroblasts and unmodified keratinocytes. Skin substitutes were cultured for two weeks before grafting to full-thickness wounds on athymic mice. The modified skin substitutes secreted significantly elevated levels of platelet-derived growth factor throughout the culture period. Expression of retroviral platelet-derived growth factor-A mRNA was maintained after grafting to mice, and was detected in all HF–/HK+ grafts and one HF+/HK– graft at two weeks after surgery. Although no differences were seen between control and modified grafts, the results suggest that genetically modified cultured skin substitutes can be a feasible mechanism for cutaneous gene therapy. The cultured skin model used for these studies has advantages over other skin analogs containing only epidermal cells; because it contains both fibroblasts and keratinocytes, it therefore offers greater opportunities for genetic modification and potential modulation of wound healing. **(WOUND REP REG 2000;8:26–35)**

Gene therapy promises the potential to improve treatment of acute wounds and accelerate healing of chronic wounds in skin. Because the process of wound healing is well characterized and many of the molecules involved have been identified, improvement of wound healing or correction of genetic defects hypothetically may be achieved through cutaneous gene therapy. Cytokines have been identified that stimulate migration, proliferation, and synthesis of extracellular matrix by skin cells, processes which are critical to the healing of skin wounds. Such cytokines have potential value for use in cutaneous gene therapy approaches. Topical application of exogenous cytokines, such as platelet-derived growth factor (PDGF), insulin-like growth factor-I, and transforming growth factor-α, has been shown to modulate wound healing in both animal studies and human clinical trials.[1–3] However, routine clinical use of topically applied cytokines can be limited due to their inactivation by proteases present in wounds, the need for repeated application, and the high cost of production of pure proteins. Hypothetically, gene therapy can overcome

CSS	Cultured skin substitutes
ELISA	Enzyme-linked immunosorbent assay
GAG	Glycosaminoglycan
PDGF	Platelet-derived growth factor

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these limitations by providing a mechanism for localized production of exogenous gene products at the wound site. One way to accomplish this is by using an in vivo gene therapy approach that delivers the DNA encoding a particular cytokine directly to the wound.[4] For example, particle-mediated gene transfer[5] and adenoviral-mediated gene delivery[6] have been used to overexpress PDGF isoforms in animal wound healing models. Alternatively, ex vivo gene therapy can be used to target cytokine expression to a specific cell type by genetically modifying cells grown from a skin biopsy in culture. These cells can be subsequently transplanted to the wound as epidermal sheet grafts or as components of composite cultured skin replacements.[4] Although time-consuming, the ex vivo approach has several advantages over in vivo gene delivery. For example, if autologous cells are used, grafts can persist for years after transplantation, allowing for extended expression of exogenous genes. In addition, expression of the transfected gene can be assayed prior to transplantation of modified cells and the number and location of modified cells can be more tightly regulated than in in vivo approaches.

PDGF is a cytokine with potential for improving wound healing in both in vivo and ex vivo cutaneous gene therapies. PDGF is mitogenic and chemotactic for cells of mesenchymal origin.[7] It is synthesized by keratinocytes in normal epidermis and during wound healing, and its receptors are expressed in cells of the dermis.[8,9] PDGF is a disulfide-linked homo- or heterodimer of two related subunits, the A chain and the B chain, which are encoded by separate genes.[7] Both PDGF isoforms were found to enhance tensile strength of rat wounds transfected in vivo by particle-mediated gene delivery,[5] and adenoviral-mediated in vivo gene transfer of PDGF-B corrected impaired wound healing in an ischemic rabbit ear model.[6] In other experiments involving ex vivo gene therapy, human keratinocytes modified to overexpress PDGF-A were grafted as epithelial sheets under full-thickness skin flaps of athymic mice.[10] Overexpression of PDGF-A from the keratinocyte grafts led to enhanced cellularity of the subadjacent connective tissue, suggesting that genetic modification of keratinocytes could impact wound healing.[10] In a subsequent study, keratinocytes engineered to overexpress PDGF-A were inoculated on acellular human dermis, and the dermal components of the genetically modified grafts were more cellular after grafting than controls.[11]

Although the cultured skin models used in previous preclinical ex vivo gene therapy studies are reasonable candidates for delivery of gene products to wound sites, they are limited in their performance because they lack cells of the dermis. Cultured skin substitutes (CSS) consisting of biopolymer substrates populated with epidermal keratinocytes and dermal fibroblasts have been shown to more closely resemble native human skin after graft healing.[12] Clinical studies have shown that this cultured skin model is an effective therapy for closure of full-thickness skin wounds.[12–16]

The present study evaluates the feasibility of using this clinically important cultured skin model as a vehicle for gene therapy. In this study, CSS were prepared from syngenic keratinocytes and fibroblasts inoculated onto collagen-glycosaminoglycan (collagen-GAG) substrates. Three types of CSS were prepared: HF–/HK–, containing both unmodified fibroblasts and keratinocytes; HF–/HK+, containing unmodified fibroblasts and PDGF-A-modified keratinocytes; and HF+/HK–, containing PDGF-A-modified fibroblasts and unmodified keratinocytes. Expression of PDGF-A and morphology of the CSS were evaluated during a two week in vitro incubation and for up to two weeks after grafting to athymic mice.

MATERIALS AND METHODS

Human cadaver skin was obtained from the Ohio Valley Tissue and Skin Center and was screened according to tissue banking standards for absence of transmissible pathogens. Primary cultures of keratinocytes and fibroblasts were isolated from cadaver skin and maintained as described.[17] Keratinocytes and fibroblasts from the same donor were used to inoculate CSS. Nutrient medium[18] was changed every 48 hours, or every 24 hours as cells approached confluence.

Genetic modification of cells

Genetic modification was performed using the replication-incompetent recombinant retrovirus, MFG-PDGF-A, which contains the entire coding sequence of the human PDGF-A gene. Production of the virus-producing cell line was previously described.[10] Briefly, a cDNA encoding the human PDGF-A chain was cloned into the MFG retroviral vector. NIH 3T3-derived Ψ-CRIP packaging cells[19] were transfected with the MFG-PDGF-A construct and a high titer clone was isolated. PDGF-AA secreted by keratinocytes modified with this retroviral vector was shown to be bioactive in a previous study.[10]

For genetic modification of keratinocytes, primary human keratinocytes (1×10^6/75 cm^2 flask) were passaged onto subconfluent (2×10^6/75 cm^2 flask) virus-producing cells pretreated with 15 μg/ml mitomycin C (Life Technologies, Gaithersburg, MD). Cells were co-cultured for five days. Prior to subculturing cells for expansion, co-cultures were vigorously rinsed for 3–5 minutes with 5 mM EDTA to remove producer cells. Keratinocytes were trypsinized, an aliquot was frozen for subsequent DNA isolation for Southern blot analysis, and the keratinocytes were expanded (1×10^6/225 cm^2 flask) and cultured without fibroblasts or producer cells. After 6 days, cells were harvested for CSS inoculation and an aliquot was frozen for RNA isolation. Control unmodified keratinocytes were grown and expanded in the same manner but without co-culture with retrovirus producer cells.

Fibroblasts were transduced by incubation with cell-free supernatant from MFG-PDGF-A producer cells. Conditioned medium containing infectious retroviral particles secreted by producer cells was filtered through a 0.45-μm cellulose acetate filter to remove cells and cell debris. Fibroblasts were inoculated at 3×10^5 cells/75 cm^2 flask, and 24 hours later the medium was changed to retrovirus-containing medium (4 ml/75 cm^2 flask) supplemented with 8 μg/ml polybrene (hexadimethrine bromide; Sigma Chemical Co., St. Louis, MO). After 5 hours at 37 °C, 4 ml of fibroblast growth medium was added to each flask to dilute the polybrene to 4 μg/ml, and medium was removed and changed to fresh growth medium 48 hours later. Fibroblasts were expanded the following day (3×10^5 cells/150 cm^2 flask) and an aliquot was frozen for DNA isolation for Southern blot analysis. After 6 days, cells were harvested for CSS inoculation and an aliquot was frozen for RNA isolation. Control unmodified fibroblasts were grown and expanded in the same manner but without incubation with retrovirus-containing medium.

DNA analysis for PDGF-A gene

Southern blot hybridization was used to assay for the presence of the retroviral PDGF-A gene in modified fibroblasts and keratinocytes. Fibroblast and keratinocyte genomic DNA samples (40 μg/sample) were restriction digested with *Kpn* I (Life Technologies), which releases a fragment containing the entire PDGF-A cDNA from the retrovirus,[10] and were electrophoresed on a 0.8% agarose gel. The equivalents of 2, 1, 0.5, and 0.25 copies/genome of the purified cloned cDNA fragment (26.6 pg, 13.3 pg, 6.7 pg, and 3.3 pg, respectively) were included on the same gel as size and copy number controls. The gel was blotted to a Nytran membrane (Schleicher and Schuell, Keene, NH) and hybridized to a PDGF-A cDNA probe labeled with biotin (BioPrime DNA Labeling System; Life Technologies). The hybridized probe was detected using the PhotoGene Nucleic Acid Detection System Version 2.0 (Life Technologies).

Construction of CSS

CSS consisting of human keratinocytes and fibroblasts attached to collagen-GAG biopolymer substrates were prepared as previously described[13] with minor modifications. Collagen-GAG substrates (~72 cm^2/substrate) were inoculated with fibroblasts (6×10^5/cm^2); six were inoculated with control unmodified fibroblasts (HF–), and three were inoculated with PDGF-A-modified fibroblasts (HF+). On the following day (CSS culture day 0), keratinocytes (1×10^6/cm^2) were inoculated onto each of the dermal substrates. CSS were prepared ($n = 3$ per group) using control fibroblasts and control keratinocytes (HF–/HK–), control fibroblasts and PDGF-A-modified keratinocytes (HF–/HK +), or PDGF-A-modified fibroblasts and control keratinocytes (HF+/HK–). On culture day 3, CSS were lifted to the air–liquid interface. CSS were cultured for two weeks in identical conditions (37 °C, 5% CO_2) with daily changes of nutrient medium.

Grafting CSS to athymic mice

This study was performed with the approval of the University of Cincinnati Institutional Animal Care and Use Committee following NIH guidelines. Athymic (*nu/nu*; Harlan, Indianapolis, IN) female mice were used, and all surgical procedures were performed in a biological safety hood using aseptic techniques.

Up to four 2 × 2 cm grafts were cut from each CSS; the remaining portions were fixed for histologic analysis or quick frozen in liquid nitrogen for RNA isolation. CSS ($n = 10$ per group) were grafted to 2 × 2 cm full-thickness wounds on the flanks of athymic mice as described elsewhere.[20,21] Ten mice per group were grafted; two mice died during the study period (one each from HF–/HK– and HF+/HK– groups) and were excluded from analysis. Three mice per group were euthanized at 4 and 7 days after grafting, and the remaining mice were euthanized at 14 days after grafting. Grafts were excised from the mice and biopsies were taken for HLA-ABC immunostaining of cryosections.[20] The remainder of each excised graft was bisected and samples were used for histologic analysis and RNA isolation.

Analysis of PDGF-A expression

Expression of retroviral PDGF-A mRNA was analyzed by Northern blot hybridization. Total RNA was isolated from cells or CSS biopsies using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA). RNA was separated on gels using a 50 mM HEPES buffer containing 10 mM EDTA and 0.6% formaldehyde, pH 7.8, and blotted onto Nytran membranes using the Turboblotter system (Schleicher and Schuell). Northern blots were hybridized to a human PDGF-A cDNA probe labeled with ^{32}P using the Random Primers DNA Labeling System (Life Technologies).

PDGF-AA protein levels were measured by enzyme-linked immunosorbent assay (ELISA) (Quantikine Human PDGF-AB Immunoassay, R & D Systems, Minneapolis, MN). This ELISA is directed against PDGF-AB, but has ~10% cross-reactivity with PDGF-AA; differences between controls and modified samples were presumed to be due to retroviral PDGF-A expression. The assay was calibrated with rhPDGF-AA (Boehringer Mannheim, Indianapolis, IN). Samples of culture medium were collected from fibroblasts and keratinocytes at the time of harvest for inoculation of CSS (subconfluence) and from CSS at 7 and 14 days after keratinocyte inoculation. Medium samples were stored frozen at – 20 °C until analyzed by ELISA. Medium samples from flasks of cells (n = 2 per group) or from CSS (n = 3 per group) were measured separately and the means were calculated.

Light microscopy

Biopsies from CSS in vitro were fixed in 2% glutaraldehyde/2% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.4. Samples were processed, embedded in glycol-methacrylate, sectioned, and stained with toluidine blue using standard techniques. Biopsies of CSS excised from mice euthanized at 4, 7, or 14 days after surgery were taken from the dorsal-anterior corners of grafts and were frozen in M1 Embedding Matrix (Lipshaw, Pittsburgh, PA). Cryostat sections (approximately 15–16 μm thick) were stained with hematoxylin and eosin using standard techniques. Sections were viewed and photographed using a Nikon Microphot-FXA microscope (Nikon Corp., Melville, NY).

Statistical analysis

Significant differences ($p < 0.05$) between PDGF-AA protein level groups (mean ± SEM) were determined by one-way analysis of variance to establish group differences and Student's t-test at each time point.

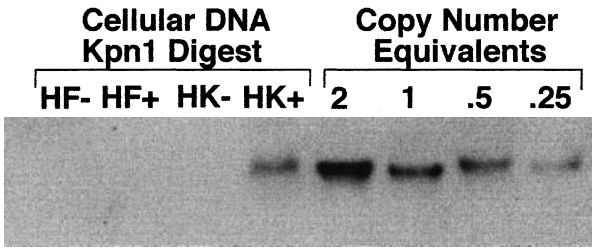

Figure 1. Southern blot analysis of genetically modified cells. Genomic DNA prepared from control and PDGF-A-modified cells was restriction digested with *Kpn* I (left) and probed by Southern blot hybridization with a PDGF-A cDNA fragment. Equivalents of 2, 1, 0.5, and 0.25 copies/genome of the purified retroviral *Kpn* I cDNA fragment (right) were included on the Southern blot to allow a comparison of hybridization intensities and thus an estimation of average transduction efficiency. Abbreviations: HF–, control unmodified human fibroblasts; HF+, PDGF-A-modified human fibroblasts; HK–, control unmodified human keratinocytes; HK+, PDGF-A-modified human keratinocytes.

RESULTS

Human keratinocytes were transduced by co-culture with growth-arrested virus-producing cells. Because retroviral producer cell lines are derived from fibroblastic cell cultures, a different approach was used to transduce human fibroblasts. To avoid contamination of dermal fibroblasts with virus-producing cells, fibroblasts were modified by incubation with cell-free retrovirus-containing producer cell supernatants. Southern blot hybridization analysis revealed the presence of the PDGF-A retrovirus in DNA of modified, but not control, keratinocytes (Figure 1). Picogram quantities of the purified *Kpn* I retroviral cDNA fragment were included on the same Southern blot as copy number controls, allowing a rough approximation of the efficiency of gene transfer to be made.[22,23] Comparison of hybridization intensities (Figure 1) indicated that an average of 0.5 retroviral genomes were present per modified keratinocyte genome, suggesting that the efficiency of transduction of the keratinocyte population was approximately 50%. The retrovirus was not detected in DNA of either control or modified fibroblasts (Figure 1), suggesting that the average gene transfer to modified fibroblasts was below 0.25 copies/genome.

Despite the relatively low transduction efficiency in modified fibroblasts, Northern blot hybridization indicated elevated PDGF-A mRNA expression compared to control fibroblasts (Figure 2). In transduced keratinocytes, greatly enhanced expression of PDGF-

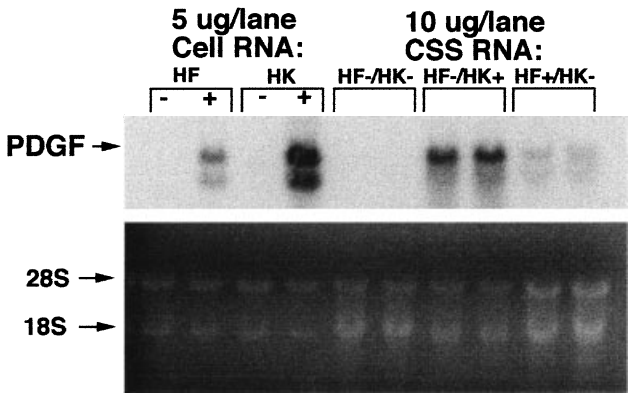

Figure 2. Enhanced expression of PDGF-A mRNA in genetically modified skin cells and CSS in vitro. A northern blot containing total RNA isolated from control (–) and PDGF-A-modified (+) cultured cells (5 μg/lane), and control and PDGF-A-modified CSS (10 μg/lane), was hybridized to a ^{32}P-labeled PDGF-A cDNA probe (top). Representative samples are shown; exposure time, 5 hours. The ethidium bromide-stained gel prior to blotting (bottom) shows the presence of RNA in each lane. Abbreviations: HF, human fibroblasts; HK, human keratinocytes; CSS, cultured skin substitutes; HF–/HK–, CSS containing unmodified (control) cells; HF–/HK+, CSS containing modified keratinocytes; HF+/HK–, CSS containing modified fibroblasts.

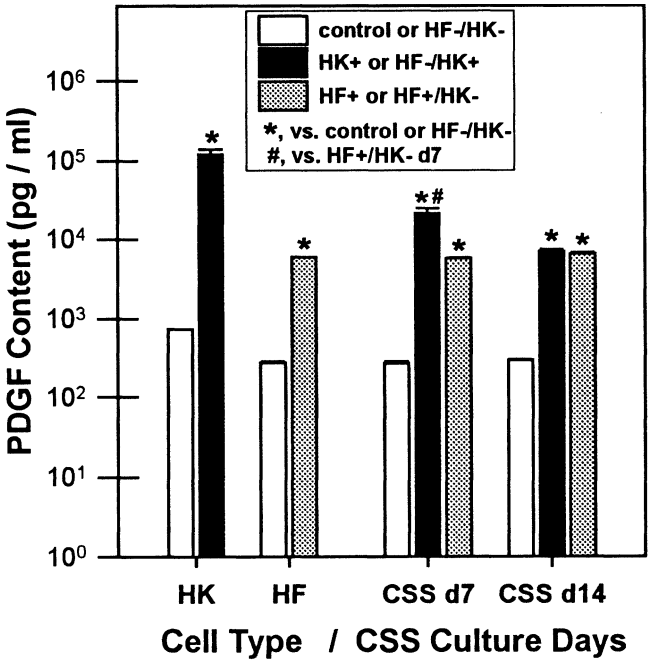

Figure 3. Increased secretion of PDGF-AA protein from genetically modified cultured skin cells and CSS. PDGF-AA levels in media samples taken from subconfluent cultures of human keratinocytes (HK) and fibroblasts (HF) at the time of CSS inoculation (left), and from CSS at culture days 7 and 14 (right), were determined by ELISA. Values plotted (pg/ml) are means; error bars = SEM. *, #, significant differences as indicated in legend ($p < 0.05$).

A was observed compared to controls (Figure 2), with greater relative expression in modified keratinocytes than in modified fibroblasts. The two bands seen by Northern blot hybridization may represent spliced and unspliced PDGF-A transcripts from the MFG-PDGF-A retrovirus.[10]

Secretion of PDGF-AA protein by genetically modified fibroblasts and keratinocytes, measured by ELISA, was found to be significantly elevated compared to control unmodified cells. Fibroblasts and keratinocytes were grown to near-confluence for inoculation of CSS, and culture media were sampled at the time of harvest of each cell population. Medium conditioned for 24 hours by PDGF-A-modified fibroblasts (HF +) contained an average of 5.9×10^3 pg/ml PDGF-AA, compared to 2.8×10^2 pg/ml in conditioned medium from control fibroblasts (HF–), an increase of over 20 fold due to genetic modification (Figure 3). Conditioned medium from PDGF-A-modified keratinocytes (HK +) was found to contain 1.2×10^5 pg/ml PDGF-AA, compared to 7.3×10^2 pg/ml in medium conditioned by control keratinocytes (HK +) (Figure 3). Thus, genetic modification resulted in a greater than 160 fold increase in PDGF-AA secretion in keratinocytes.

Genetically modified cultured skin substitutes

Media samples were collected for analysis during in vitro incubation of CSS, at days 7 and 14 after keratinocyte inoculation. After 7 days in culture, both CSS generated with PDGF-A-modified keratinocytes (HF–/HK +) or modified fibroblasts (HF+/HK–) secreted significantly more PDGF-AA into the medium than control CSS (HF–/HK–) (Figure 3). In addition, HF–/HK+ CSS secreted significantly more PDGF-AA than HF+/HK– CSS at 7 days after inoculation. The difference in PDGF-AA secretion between HF–/HK+and HF+/HK– CSS was not significant at 14 days after inoculation, although both types of CSS containing modified cells secreted significantly more PDGF-A than controls at this time point.

Northern blot hybridization analysis showed elevated PDGF-A mRNA expression in genetically modified CSS compared to controls after two weeks of in vitro culture, with relatively higher expression in HF–/HK+ CSS compared to HF+/HK– CSS (Figure 2). Expression of PDGF-A in control CSS (HF–/HK–) was below the level of detection in this assay.

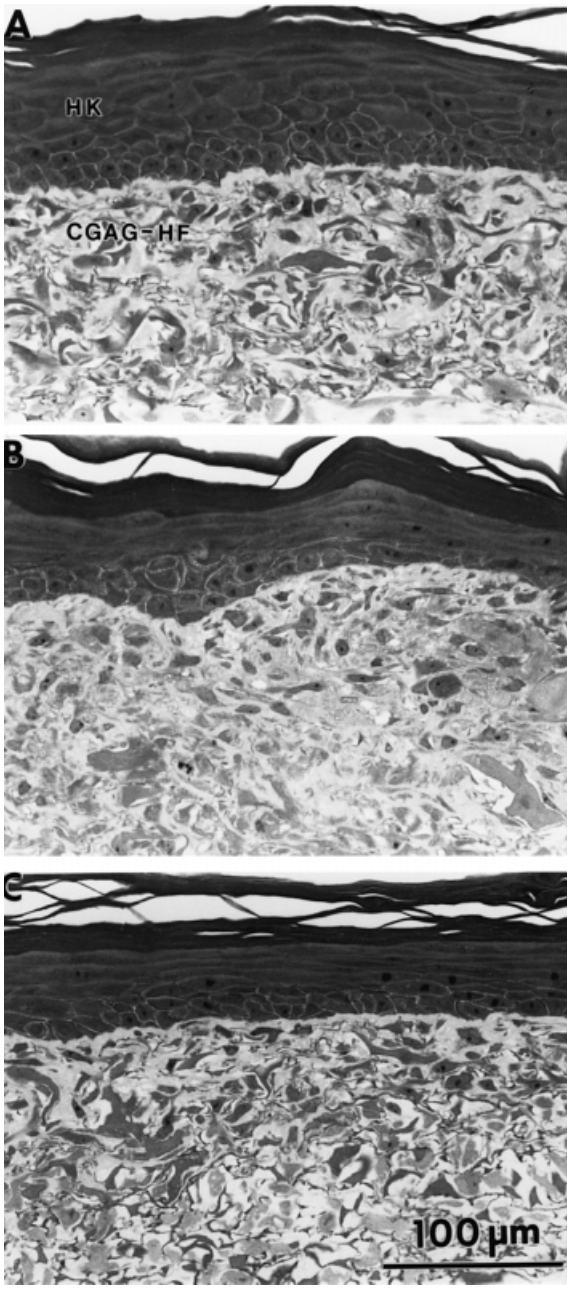

Figure 4. Histological sections from CSS in vitro. Shown are representative toluidine blue-stained plastic-embedded sections of CSS taken at the time of grafting to mice, two weeks after keratinocyte inoculation. (A) HF–/HK–; control CSS. (B) HF–/HK+; CSS populated with control fibroblasts and PDGF-A-modified keratinocytes. (C) HF+/HK–; CSS populated with PDGF-A-modified fibroblasts and control keratinocytes. Scale bar in (C) is for all sections in this figure. Abbreviations: HK, human keratinocytes; CGAG-HF, collagen-glycosaminoglycan substrate populated with human fibroblasts.

Light microscopy was used to assess the morphogenesis of the CSS after two weeks of in vitro culture, at the time of grafting to mice. Representative sections are shown in Figure 4. No significant differences in morphology were observed between control CSS (Figure 4A), HF–/HK+ CSS (Figure 4B), and HF+/HK– CSS (Figure 4C). The dermal compartments of all grafts were densely packed with fibroblasts. The epidermal components of the CSS consisted of stratified epithelia: the basal, nucleated cells were attached to the collagen-GAG substrate, and well-keratinized layers analogous to stratum corneum were seen at the epithelial surfaces. The total thickness of the CSS at the end of the culture period ranged from approximately 200–400 µm, with no differences in thickness between the groups.

PDGF-A expression and morphology of CSS after grafting to mice

Positive immunostaining for HLA-ABC histocompatibility antigens was seen in all CSS excised from mice (data not shown). This indicates the persistence of human keratinocytes from the CSS and thus documents the absence of graft rejection.

Elevated PDGF-A mRNA expression was observed in all HF–/HK+ CSS at 4, 7, and 14 days after grafting (Figure 5). In contrast only one HF+/HK– CSS, excised at 14 days after surgery, was found that expressed PDGF-A mRNA above controls levels (Figure 5).

Despite these differences in PDGF-A expression between groups, no significant differences in graft morphology were found at any of the time points examined. Representative sections of CSS excised from mice at 4, 7, and 14 days after surgery are shown in Figure 6. The dermal components of the CSS from all three groups contained numerous fibroblasts, and the cell density appeared to increase with time after grafting. No differences in cell number or distribution of fibroblasts were observed between groups, either in hematoxylin and eosin-stained sections (Figure 6) or in sections stained with propidium iodide as a nuclear label (data not shown). Darkly staining reticulations of implanted collagen were evident in sections of CSS at 4 and 7 days after surgery, but not at 14 days after surgery (Figure 6). This suggests that most or all of the implanted collagen is replaced by newly synthesized collagen within two weeks after grafting to mice. Consistent with this hypothesis, immunohistochemistry with an antibody specific for human type I collagen showed a high degree of staining in the dermal compartments of the CSS grafts, with no differences observed between groups (data not shown). To examine vascularization of CSS grafted to mice, immunohistochemistry was performed with antibodies against

murine collagen type IV, which is found in the basement membranes of blood vessels, and murine CD31/PECAM-1, an endothelial cell marker. Evidence of vascularization was observed in CSS from all groups excised at 7 and 14 days after grafting (data not shown), and the degree of vascularization was similar to that previously reported for grafted control CSS.[24] CSS generated with control or PDGF-A–modified cells also displayed similar epidermal organization and morphology. All CSS exhibited a stratified epidermal layer that increased in thickness and organization with time after grafting (Figure 6).

DISCUSSION

The present study investigated the feasibility of using a cultured skin substitute comprised of fibroblasts and keratinocytes cultured in a collagen-GAG biopolymer substrate as a vehicle for therapeutic gene delivery. Cultured human keratinocytes and fibroblasts were genetically modified by transduction with a replication-incompetent retrovirus, resulting in significantly elevated levels of PDGF-A mRNA expression and PDGF-AA protein secretion in vitro. Interestingly, after two weeks in culture, CSS containing modified keratinocytes (HF–/HK+) displayed a higher level of PDGF-A mRNA expression than CSS containing modified fibroblasts (HF+/HK–). However, there was no significant difference in PDGF-AA secretion at this time point between HF–/HK+ and HF+/HK– CSS. This suggests the possibility of post-transcriptional regulation of PDGF-A expression, at the level of translation or secretion.

After grafting to athymic mice, elevated PDGF-A expression was detected in all CSS containing modified keratinocytes. PDGF-A mRNA expression was highest at 4 days after grafting, but was detected above control levels in all HF–/HK+ CSS up to weeks after surgery. By Northern blot analysis, only one HF+/HK–graft expressed PDGF-A mRNA significantly above control levels. The level of PDGF-A expression, both in vitro and after grafting, appears to be related to the efficiency of transduction of the different cell populations. Thus the relatively low level of PDGF-A expression in modified fibroblasts compared to modified keratinocytes, and in HF+/HK– CSS compared to HF–/HK+ CSS, probably reflects a lower relative efficiency of transduction of the fibroblasts used for these experiments. Despite elevated PDGF-A mRNA levels in modified fibroblasts the presence of retroviral DNA was below the level of detection by Southern blot analysis, though it was readily detected in keratinocyte DNA. This suggests a higher proportion of PDGF-A-modified cells in the keratinocyte population, compared with the fibroblast population. The higher efficiency of keratinocyte transduction probably resulted from their coculture with virus producer cells. The keratinocytes were not only exposed to retrovirus for 5 days of culture, but they were actually in contact with cells secreting retrovirus. In contrast, the fibroblasts were exposed to producer cell supernatant containing infectious retrovirus for only 48 hours, with no contact with producer cells, probably leading to less efficient transduction. Prospectively, improving the efficiency of genetic modification of fibroblasts should lead to increased expression of ex-

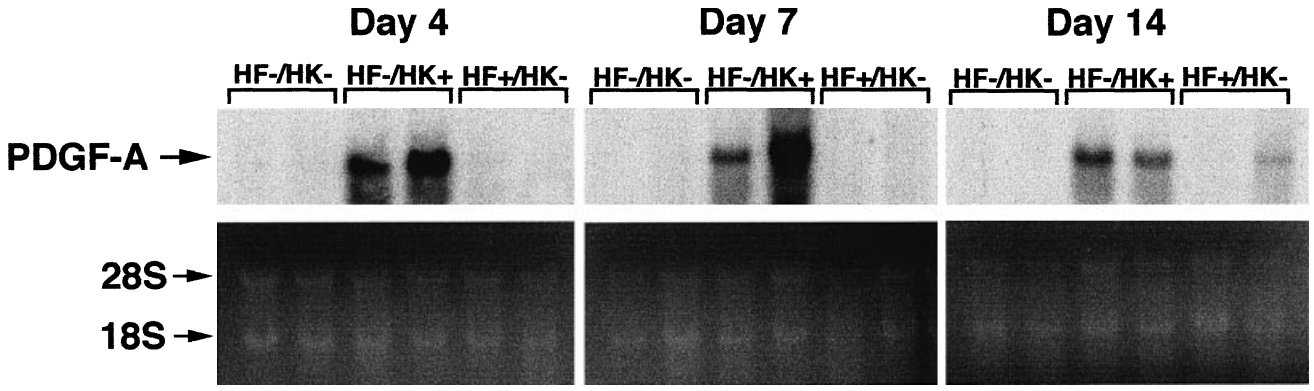

Figure 5. Enhanced expression of PDGF-A mRNA in genetically modified CSS after grafting to athymic mice. A Northern blot containing 10 µg/lane total RNA isolated from HF-/HK-, HF-/HK+, and HF+/HK- CSS excised from mice at 4, 7, and 14 days after grafting was hybridized to a PDGF-A cDNA probe (top). Representative samples are shown; exposure time, 5 hours. The ethidium bromide-stained gel prior to blotting (bottom) shows the presence of RNA in each lane.

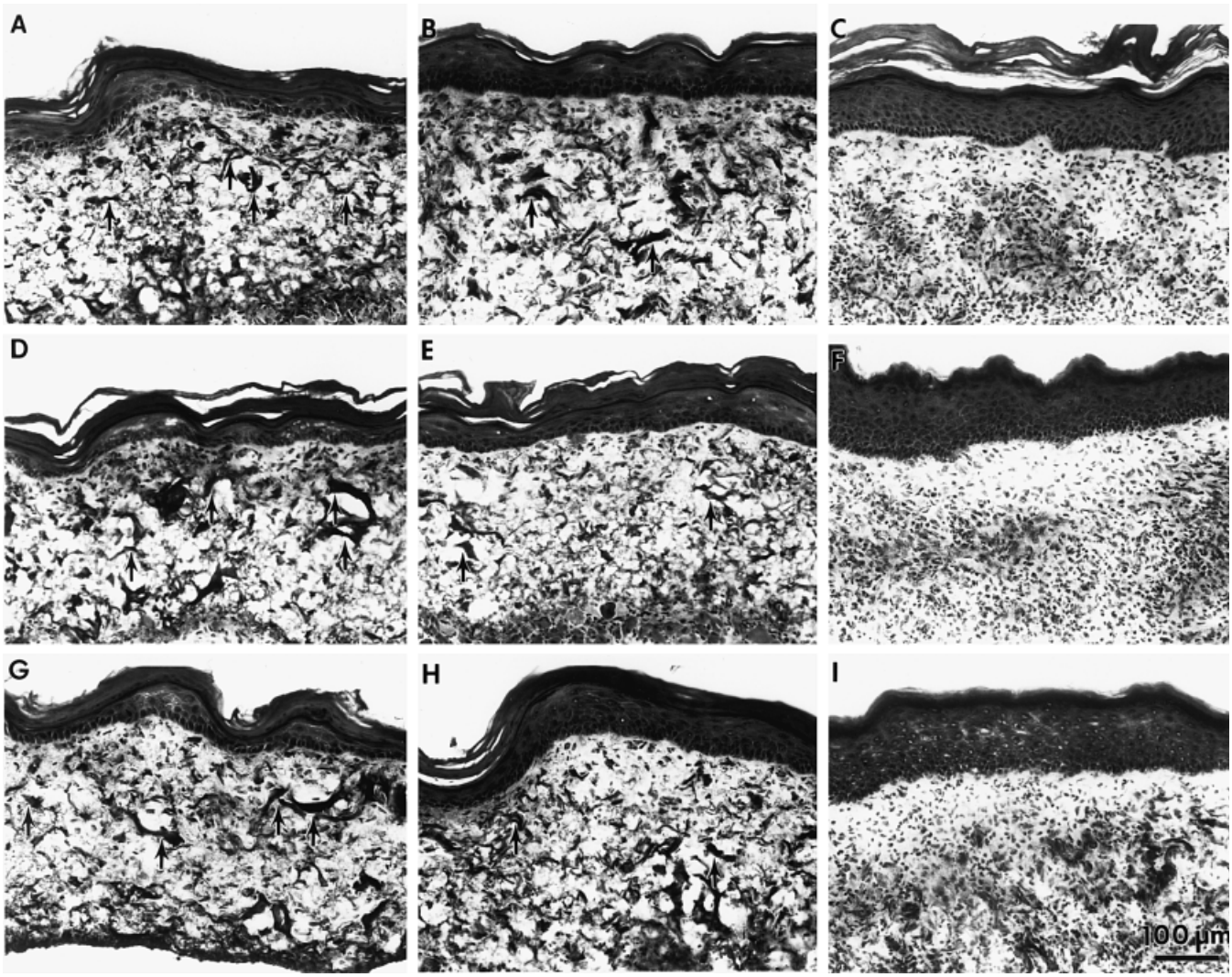

Figure 6. Histological sections from CSS after grafting to athymic mice. Shown are representative hematoxylin and eosin-stained cryosections of CSS excised from mice at 4, 7, and 14 days after grafting. The epidermal layer of the grafted skin substitute is at the top of each panel. (A–C), HF–/HK– CSS. (D–F), HF–/HK+ CSS. (G–I), HF+/HK– CSS. A, D, and G show CSS excised at 4 days after grafting. B, E, and H show CSS excised at 7 days after grafting. C, F, and I, show CSS excised at 14 days after grafting. Arrows point to examples of implanted collagen at 4 and 7 days after grafting. Scale bar in I is for all sections shown in this figure.

ogenous transcripts, allowing them to be expressed more reliably after grafting of modified CSS.

The experiments described here show that this cultured skin model can be genetically modified, and that modification can result in expression of transduced sequences after grafting to athymic mice. Interestingly, the inclusion of PDGF-A-modified cells had no demonstrable effect, either negative or positive, on the morphogenesis of the CSS. The absence of a negative effect indicates that retroviral transduction did not adversely affect the maturation or viability of the CSS. However, none of the positive effects that had been observed in previous ex vivo gene therapy studies with PDGF-A overexpression[10,11] were seen. For example, Eming and colleagues[10] grafted keratinocytes modified with the PDGF-A retrovirus as epithelial sheets under dorsal skin flaps on athymic mice. In that study, the connective tissue subadjacent to the modified grafts was thicker, more cellular, and had more blood vessels at one week after grafting than control keratinocyte grafts.[10] In a subsequent study[11] PDGF-A-modified keratinocytes seeded on an acellular dermis were grafted to full thickness wounds on athymic mice. The performance of those grafts was improved by inclusion of the genetically modified cells: the PDGF-A-modified grafts exhibited increased der-

mal cellularity, vascularization, type I collagen deposition, and decreased wound contraction one week after grafting.[11] The results of that study suggested that elevated PDGF-A expression might have enhanced "graft take" during the first week after grafting. However, most of the differences between modified and control grafts were not evident at later time points, indicating that the genetic modification may have simply accelerated the normal process by which host cells repopulate the dermis of the composite graft.[11]

In the present study, no significant differences between control and PDGF-A-modified grafts were observed, either during the in vitro culture period or after grafting to athymic mice. This apparent discrepancy with previous published reports may be attributed to differences in the cultured skin models used. The models employed in the previous studies contained only keratinocytes; fibroblasts present in the host wound bed were required to infiltrate the region beneath epidermal sheet grafts[10] or into acellular dermal substitutes to populate the dermis.[11] These processes were enhanced by overexpression of PDGF-A in transplanted keratinocytes. Thus, genetic modification of keratinocytes with the PDGF-A retrovirus served to overcome the inherent dermal deficiencies of these cultured skin models. The cultured skin substitute model employed in the present study contains both keratinocytes and fibroblasts prior to grafting. Fibroblasts are simultaneously cultured from the same skin biopsy used to isolate keratinocytes, and are inoculated onto the collagen-GAG substrate one day prior to keratinocyte inoculation. A dermal layer densely populated with fibroblasts is observed after a relatively short culture period, and continued fibroblast growth is observed after grafting to mice. In the experiments described here, graft take was observed in both control and PDGF-A-modified CSS, and no differences in cell density or graft morphology were observed. These results indicate that, although overexpression of PDGF-A can have a beneficial effect on grafts that contain only keratinocytes,[10,11] it is not necessary for satisfactory performance of a cultured skin model containing both keratinocytes and fibroblasts. This suggests that the levels of PDGF-A and/or other growth factors present in unmodified cells used in these experiments are sufficient for the maturation of CSS in vitro and for the healing of grafted CSS.

While the CSS performed well in the studies described here, they are not without their limitations. For example, CSS lack a vascular plexus, leading to slower vascularization compared with native skin autograft. This limitation can hypothetically be overcome by genetic modifications that lead to overexpression of angiogenic cytokines. For example, genetically modified CSS overexpressing vascular endothelial growth factor showed enhanced vascularization compared to control CSS after grafting to athymic mice.[24] Clearly, the selection of cytokines is of major importance, and must be designed to meet specific requirements based on the cultured skin model used, the intended application, and the desired outcome.

Despite results indicating that PDGF-A overexpression does not improve the outcome of grafted CSS containing fibroblasts in addition to keratinocytes, the present study shows that cultured skin substitutes can be used prospectively as a vehicle for gene therapy. Overexpression of the transduced gene was readily observed two weeks after grafting modified CSS to mice. Further, because the CSS used here contains cells of both the epidermis and dermis, the opportunities for genetic modification and therefore modulation of wound healing are greater than for the cultured epidermal grafts used in previous experiments. Future studies could examine the effects of overexpressing multiple cytokines, or the impact of expressing a cytokine in the epidermis and its receptor in the dermis. Cutaneous gene therapy provides broad and numerous opportunities for molecular regulation of wound healing, and cultured skin substitutes will be a valuable model for investigation of these approaches.

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