

Enhanced Vascularization of Cultured Skin Substitutes Genetically Modified to Overexpress Vascular Endothelial Growth Factor¹

Dorothy M. Supp, Andrew P. Supp, Sheila M. Bell,* and Steven T. Boyce

Research Department, Shriners Hospitals for Children, Shriners Burns Hospital, Cincinnati, Ohio, U.S.A.; *Division of Molecular and Developmental Biology, Children's Hospital Research Foundation, Cincinnati, Ohio, U.S.A.

Cultured skin substitutes have been used as adjunctive therapies in the treatment of burns and chronic wounds, but they are limited by lack of a vascular plexus. This deficiency leads to greater time for vascularization compared with native skin autografts and contributes to graft failure. Genetic modification of cultured skin substitutes to enhance vascularization could hypothetically lead to improved wound healing. To address this hypothesis, human keratinocytes were genetically modified by transduction with a replication incompetent retrovirus to overexpress vascular endothelial growth factor, a specific and potent mitogen for endothelial cells. Cultured skin substitutes consisting of collagen-glycosaminoglycan substrates inoculated with human fibroblasts and either vascular endothelial growth factor-modified or control keratinocytes were prepared, and were cultured *in vitro* for 21 d. Northern blot analysis demonstrated enhanced expression of vascular endothelial growth factor mRNA in genetically modified keratinocytes and in cultured skin substitutes prepared with modified cells. Furthermore, the vascular endothelial growth factor-modified cultured skin

substitutes secreted greatly elevated levels of vascular endothelial growth factor protein throughout the entire culture period. The bioactivity of vascular endothelial growth factor protein secreted by the genetically modified cultured skin substitutes was demonstrated using a microvascular endothelial cell growth assay. Vascular endothelial growth factor-modified and control cultured skin substitutes were grafted to full-thickness wounds on athymic mice, and elevated vascular endothelial growth factor mRNA expression was detected in the modified grafts for at least 2 wk after surgery. Vascular endothelial growth factor-modified grafts exhibited increased numbers of dermal blood vessels and decreased time to vascularization compared with controls. These results indicate that genetic modification of keratinocytes in cultured skin substitutes can lead to increased vascular endothelial growth factor expression, which could prospectively improve vascularization of cultured skin substitutes for wound healing applications. *Key words: gene therapy/tissue engineering/wound healing/vascular endothelial growth factor. J Invest Dermatol 114:5-13, 2000*

In extensively burned patients, rapid closure of large skin wounds is critical for recovery. In many of these patients, lack of uninjured donor skin for harvesting of autografts can be a serious problem and has led to the development of alternative materials for wound coverage through tissue engineering (Gallico, 1990). Cultured skin substitutes (CSS) comprised of cultured keratinocytes and fibroblasts in a biopolymer matrix have been successfully used as an adjunctive burn wound therapy (Boyce *et al*, 1993, 1995a, 1999; Harriger *et al*, 1995). After healing, grafted CSS resemble uninjured human skin (Boyce *et al*, 1993; Harriger *et al*, 1995). Although clinical outcome for engrafted

CSS is similar to that for autografts, limitations in anatomy and physiology remain (Boyce *et al*, 1993, 1995a). Notably, CSS lack a vascular plexus, leading to slower vascularization. Split-thickness skin autografts vascularize by a combination of processes, including inosculation and neovascularization. Inosculation is the anastomosis of capillaries in the wound bed to the ends of severed vessels in the dermis of split-thickness grafts, and this is the primary mechanism of early graft vascularization (Young *et al*, 1996). Neovascularization is the growth of new capillaries from the wound bed into the graft, and this process occurs later; grafting of split-thickness human skin on to mice indicated that host endothelial cells did not appear in dermal capillaries of the graft for at least 1 wk (Young *et al*, 1996). Vascularization of CSS takes longer than for autografts because it occurs through the process of neovascularization alone, and this contributes to failure of cultured skin grafts by increasing time for reperfusion, ischemia, and nutrient deprivation of grafted cells (Boyce, 1996). Genetic engineering can hypothetically be used to overcome this limitation inherent to cultured skin. Cultured keratinocytes can be genetically modified to express growth factors that modulate vascularization during the wound healing process.

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Reprint requests to: Dr. Steven T. Boyce, Research Department, Shriners Hospitals for Children, Shriners Burns Hospital, 3229 Burnet Ave., Cincinnati, OH 45229.

Abbreviations: CMV, cytomegalovirus; CSS, cultured skin substitutes; EBm, endothelial cell basal medium; GAG, glycosaminoglycan; HMVEC, human microvascular endothelial cells; LTR, long-terminal repeat; VEGF, vascular endothelial growth factor.

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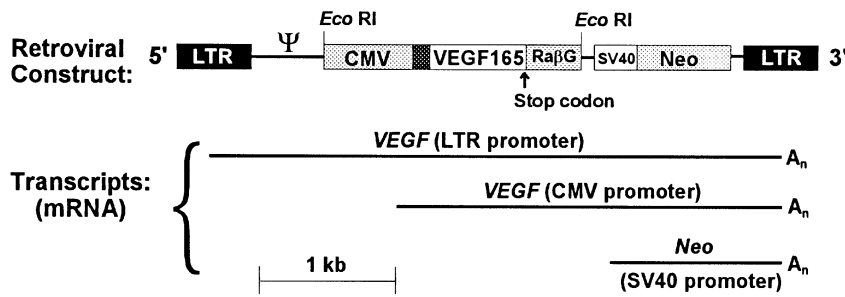


Figure 1. pL-CVEGF-SN retroviral vector. The proviral structure of the recombinant retrovirus is diagrammed (*top*). Predicted VEGF₁₆₅ transcripts originating from both the retroviral LTR promoter and the inserted CMV promoter are shown (*bottom*). Also shown is the neo transcript that permits selection of producer cells with stably integrated viral sequences. LTR, long-terminal repeat; Ψ , retroviral packaging signal; CMV, cytomegalovirus promoter/enhancer; Ra β G, nontranslated sequences from the rabbit β -globin gene. Scale bar: 1 kb.

CSS populated with modified cells can thereby act as vehicles for gene therapy to facilitate and/or accelerate wound healing.

Vascular endothelial growth factor (VEGF) is a growth factor with prospective value for improving the vascularization of CSS. VEGF is a potent and specific mitogen for microvascular endothelial cells (Conn *et al*, 1990; Detmar *et al*, 1995). VEGF was originally named vascular permeability factor because of its ability to increase vascular permeability to plasma proteins (Senger *et al*, 1983). Microvascular hyperpermeability stimulated by VEGF accompanies angiogenesis and results in the leakage of plasma proteins which activate a clotting cascade, leading to deposition of an extracellular matrix to support endothelial cell migration (Dvorak *et al*, 1995). Expression of VEGF is required during embryonic development for the formation of normal blood vessels, and loss of even a single VEGF allele is lethal, suggesting that normal VEGF levels are critical for the regulation of vessel development (Carmeliet *et al*, 1996; Ferrara *et al*, 1996). VEGF is expressed by epidermal keratinocytes, and is upregulated during wound healing, coincident with the period of skin angiogenesis (Brown *et al*, 1992; Frank *et al*, 1995; Weninger *et al*, 1996). VEGF expression also increases in pathologic states that are characterized by angiogenesis, including carcinomas of several tissues and psoriasis, and in normal fetal and adult tissues undergoing physiologic angiogenesis (reviewed by Dvorak *et al*, 1995). One mechanism of VEGF induction is tissue hypoxia, a condition found in both tumors and healing wounds, which has been shown to upregulate VEGF expression in skin cells (Detmar *et al*, 1997). Aberrant regulation of VEGF expression has been associated with defective wound repair, such as the abnormal wound healing seen in diabetic mice (Frank *et al*, 1995; Rivard *et al*, 1999).

VEGF is a homodimeric glycoprotein which binds to two tyrosine kinase receptors expressed almost exclusively on endothelial cells (Neufeld *et al*, 1999). Four isoforms of VEGF (121, 165, 189, and 206 amino acids) arise from alternate splicing of a single VEGF gene (Houck *et al*, 1991). All four isoforms can elicit mitogenic responses in endothelial cells, but they differ in their binding to extracellular heparin-containing proteoglycans; VEGF₁₈₉ and VEGF₂₀₆ remain cell-associated whereas VEGF₁₂₁ and VEGF₁₆₅ are secreted in soluble form (Park *et al*, 1993). Direct gene transfer of VEGF₁₆₅ by DNA microinjection resulted in therapeutic angiogenesis in ischemic limbs in preclinical and clinical studies (Isner *et al*, 1996; Takeshita *et al*, 1996; Tsurumi *et al*, 1996; Baumgartner *et al*, 1998), and improved perfusion in patients with myocardial ischemia (Losordo *et al*, 1998). Additionally, VEGF injection into ischemic tissues of diabetic mice restored vascularization to normal levels (Rivard *et al*, 1999). These studies demonstrate the effective use of VEGF₁₆₅ in gene therapy applications. Because VEGF secreted by epidermal keratinocytes has a mitogenic effect on dermal microvascular endothelial cells (Detmar *et al*, 1995), modulation of VEGF levels in the epidermis could potentially lead to enhanced skin vascularization. This was shown in K14/VEGF transgenic mice, in which the keratin 14 promoter was used to target VEGF overexpression to basal keratinocytes (Detmar *et al*, 1998). For wound healing purposes, human VEGF₁₆₅ could hypothetically be used to improve vascularization of CSS by genetically engineering epidermal cells within the CSS to overexpress VEGF₁₆₅.

In this study, retroviral-mediated gene transfer was used to generate a population of human keratinocytes overexpressing the gene encoding VEGF₁₆₅. Cultured skin substitutes were made from fibroblasts and either VEGF-modified or control (sham-modified) keratinocytes. Expression of VEGF was assayed in CSS during a 21 d *in vitro* incubation and for up to 14 d after grafting to athymic mice. Bioactivity of VEGF secreted by genetically modified CSS was measured using a human microvascular endothelial cell growth assay. Vascularization of VEGF-modified CSS grafted to athymic mice was evaluated to determine whether the genetic modification impacted wound healing with CSS.

MATERIALS AND METHODS

VEGF₁₆₅ retroviral vector construction A plasmid containing the cDNA encoding the 165 amino acid isoform of human VEGF (phVEGF165.SR) was generously provided by Jeffrey M. Isner, MD (St Elizabeth's Medical Center, Boston, MA). In addition to the coding sequence for VEGF₁₆₅, this clone contained the cytomegalovirus (CMV) promoter/enhancer to drive VEGF₁₆₅ mRNA expression, and sequences from the rabbit β -globin gene (second intron and poly(A) addition signal). A 1.89 kb *Eco*RI restriction fragment containing the CMV promoter/enhancer, VEGF₁₆₅ coding sequence, and part of the rabbit β -globin sequence (not including poly(A) addition signal) was subcloned into the *Eco*RI site in the replication-incompetent retroviral vector pLXSN (Clontech Laboratories, Palo Alto, CA) to create the VEGF₁₆₅ retroviral vector pL-CVEGF-SN (**Fig 1**). This vector contains the viral packaging signal Ψ , the VEGF₁₆₅ coding sequence, and the G418-selectable neomycin resistance gene, but lacks the retroviral structural genes required for replication and particle formation. Restriction enzyme digestion and DNA sequencing were used to confirm the correct orientation of the inserted VEGF₁₆₅ sequences relative to the 5' and 3' long-terminal repeat (LTR) elements in the retroviral vector. Because the pL-CVEGF-SN retroviral construct contains the CMV promoter in addition to the 5' LTR from the pLXSN vector, two VEGF₁₆₅ transcripts with different initiation sites, but identical open-reading frames, were predicted. Poly(A) addition for both transcripts was predicted to occur using a cryptic poly(A) addition signal in the 3' LTR.

Generation of replication-incompetent retroviral producer cell lines

pLXSN and pL-CVEGF-SN plasmid DNAs were prepared using the QIAprep Spin Miniprep Kit (QIAGEN, Santa Clarita, CA), and were sterilized by ethanol precipitation. The VEGF₁₆₅ retroviral producer cell line was generated by transfecting the pL-CVEGF-SN retroviral plasmid into the NIH/3T3-based packaging cell line RetroPack PT67 (Clontech Laboratories), which has stable integration of the structural genes necessary for viral particle formation and replication (gag, pol, and env). Control producer cells were generated by transfecting the parent pLXSN plasmid, without inserted VEGF₁₆₅ sequences, into a separate population of PT67 cells. For transfection, 8×10^5 PT67 cells per well were inoculated into a 6 well multiwell plate and were transfected the following day with 2 μ g plasmid DNA/well using the CalPhos Maximizer and Transfection Kit (Clontech Laboratories). Selection with G418 was used to generate lines of producer cells containing stably integrated vector sequences. Cells were selected for 10 d in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Gibco/Life Technologies, Gaithersburg, MD) and 800 μ g per ml G418 (Clontech Laboratories). This medium was found to be lethal for nontransfected cells.

Cell culture and genetic modification 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 (Boyce and Ham, 1985).

Keratinocytes and fibroblasts from the same donor were used to inoculate CSS.

Cultures of human keratinocytes (second passage) were inoculated (1×10^6 per T75 flask) on to subconfluent cultures of either pL-CVEGF-SN or pLXSN (control) retroviral producer cells pretreated for 2 h with $15 \mu\text{g}$ per ml mitomycin C (Gibco/Life Technologies). Genetic modification was achieved by coculturing keratinocytes and growth-arrested producer cells for 5 d. After a brief 5 mM ethylenediamine tetraacetic acid rinse to remove producer cells, the keratinocytes were trypsinized and subcultured to expand the cell populations.

DNA was prepared from both pLXSN-modified (control) and pL-CVEGF-SN-modified keratinocytes for Southern blot analysis. Hybridization of *Eco*RI-digested DNA to a VEGF cDNA probe was used to confirm the presence of retroviral DNA in pL-CVEGF-SN-modified keratinocytes (data not shown). Similarly, DNA prepared from pLXSN-modified control keratinocytes was digested with *Hind*III plus *Xba*I and Southern blot hybridized to linearized pLXSN plasmid probe to confirm the presence of retroviral DNA. In both experiments, comparison of the hybridization intensities with copy-number controls suggested an average of 0.5 retroviral copies per cellular genome (data not shown).

Cultured skin substitutes CSS consisting of human keratinocytes and fibroblasts attached to collagen-glycosaminoglycan (GAG) biopolymer substrates were prepared as previously described (Boyce *et al*, 1991) with minor modifications. CSS ($n=3$ per group) were prepared using either pL-CVEGF-SN-modified (VEGF⁺) or pLXSN-modified (control) keratinocytes. Collagen-GAG substrates ($\approx 75 \text{ cm}^2$ per substrate) were each inoculated with 4.0×10^7 fibroblasts, and on the following day were inoculated with 8×10^7 keratinocytes (culture day 0). On culture day 3, CSS were lifted to the air-liquid interface. CSS were cultured for 21 d in identical conditions (37°C , 5% CO_2) with daily changes of nutrient medium.

Grafting to athymic mice All animal studies were performed with the approval of the University of Cincinnati Institutional Animal Care and Use Committee following NIH guidelines. After the 21 d *in vitro* culture period, VEGF⁺ and control CSS (4 cm^2 , $n=12$ per group) were grafted to $2 \text{ cm} \times 2 \text{ cm}$ full thickness wounds on the flanks of athymic mice (nu/nu; Harlan; Indianapolis, IN) as previously described (Boyce *et al*, 1991) with minor modifications. Briefly, each wound was prepared such that the panniculus carnosus was left intact and the graft was sutured, along with an overlying piece of nonadherent dressing (N-Terface; Winfield Laboratories, Richardson, TX), to the wound margins and wound bed. Grafts were dressed with sterile gauze coated with antibiotic ointment (equal parts, Bactroban/Neosporin/Nystatin); sutures were tied over the dressings, the grafted area was covered with OpSite (Smith & Nephew Medical, Hull, UK), and the mice were wrapped with Coban bandages (3M Medical Division, St Paul, MN). Dressings were left undisturbed until the mice were killed. Each CSS preparation was used to graft four mice. Three mice died during the course of the study (two from the VEGF⁺ group and one from the control group); these animals were therefore excluded from the analysis. Three mice from each group were killed at 3 d and 7 d after grafting, and the remaining mice (four VEGF⁺ and five controls) were killed at 14 d after grafting. Grafts were excised from the mice and biopsies were taken for HLA-ABC staining (Boyce *et al*, 1991). The remainder of each graft was used for RNA isolation, histologic analysis, and immunohistochemistry.

Analysis of VEGF₁₆₅ RNA expression Expression of retroviral VEGF₁₆₅ mRNA was analyzed by northern blot hybridization. Total RNA was isolated from cells or CSS biopsies using the RNeasy Mini Kit (QIAGEN) and was electrophoresed on 1% agarose gels in buffer containing 0.6% formaldehyde in 50 mM HEPES pH 7.8 and 1 mM ethylenediamine tetraacetic acid. Gels were soaked in water to remove formaldehyde and RNA was transferred to Nytran membranes using the TurboBlotter system (Schleicher & Schuell, Keene, NH) according to the manufacturer's protocol. Northern blots were hybridized to a human VEGF cDNA probe labeled with ³²P using the Random Primers DNA Labeling System (Gibco Life Technologies).

Analysis of VEGF protein secretion Aliquots of culture medium were taken from T75 flasks of keratinocytes ($n=6$ per group) at near-confluence (day of CSS inoculation, culture day 0), and from CSS ($n=3$ per group) at culture days 7, 14, and 21. Medium was sampled immediately before being changed, thus samples were conditioned by cells or CSS for approximately 24 h. Medium samples were supplemented with 2% fetal bovine serum to stabilize soluble VEGF protein, and samples were stored frozen at -20°C

until assayed. VEGF protein levels in culture medium were measured by enzyme-linked immunosorbent assay (Quantikine Human VEGF Immunoassay, R&D Systems, Minneapolis, MN). Overall group differences were determined by two-way analysis of variance (ANOVA). Subsequent pairwise comparisons were done by Tukey's test to determine significant differences ($p < 0.05$) between control and VEGF⁺ samples at each time point.

Endothelial cell growth assay Medium samples from one control and one VEGF⁺ CSS were collected at culture day 10, supplemented with 2% fetal bovine serum, and stored frozen at -70°C . Initially, keratinocyte growth medium was tested, but this medium itself was found to stimulate human microvascular endothelial cell (HMVEC) growth, without conditioning or addition of rhVEGF. To isolate the mitogenic effect of VEGF from effects due to medium composition, media from CSS cultures were used.

Human microvascular endothelial cells (Cascade Biologics, Portland, OR) were plated at 3×10^3 cells per cm^2 in six-well multiwell dishes coated with Attachment Factor (Cascade Biologics) and were grown in endothelial basal medium (EBM; MCDB 131 + 2% fetal bovine serum). After 48 h, medium was changed to fresh EBM (group 1), or 90% EBM + 10% test medium (groups 2–8). The different test media were as follows: group 2, CSS medium (unconditioned); group 3, control CSS-conditioned medium; group 4, control CSS-conditioned medium + 1:500 anti-VEGF antibody; group 5, VEGF⁺ CSS-conditioned medium; group 6, VEGF⁺ CSS-conditioned medium + 1:500 anti-VEGF antibody; group 7, 10 ng per ml recombinant human VEGF (rhVEGF; R&D Systems); group 8, 10 ng per ml rhVEGF + 1:500 Anti-VEGF antibody. For groups 4, 6, and 8, the test medium was preincubated for 1 h with a neutralizing antibody to human VEGF₁₆₅ (R&D Systems). Medium was changed after 48 h, and cells were trypsinized and counted with a hemacytometer 48 h later. Three wells per group were counted in duplicate. For statistical analysis, overall group differences were determined by one-way ANOVA. Subsequent pairwise comparisons were done by Tukey's test to determine significant differences ($p < 0.05$) between groups.

Light microscopy Biopsies from CSS *in vitro* and from the ventral-anterior corner of each graft excised from mice 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. Sections were viewed and photographed using a Nikon Microphot-FXA microscope (Nikon, Melville, NY).

Immunohistochemical staining Biopsies from the dorsal-anterior corners of grafts excised from mice killed at 3, 7, or 14 d after surgeries were frozen in M1 Embedding Matrix (Lipshaw, Pittsburgh, PA). Cryostat sections (approximately 15–16 μm thick) were dehydrated in methanol and fixed in acetone at -20°C . After air-drying, sections were rehydrated in phosphate-buffered saline (PBS) pH 7.6.

For immunohistochemical staining used to quantitate murine dermal endothelial cells, sections were incubated in 0.3% H_2O_2 in PBS for 30 min to quench endogenous peroxidases. Immunohistochemical staining was performed using the TSA Direct (Green) Kit (NEN Life Science Products, Boston, MA) according to manufacturer's instructions, except that PBS buffer was used in place of the Tris/NaCl buffer. The primary antibody, rat anti-mouse CD31/PECAM-1, and secondary antibody, biotin-conjugated mouse anti-rat IgG2a, were purchased from Pharmingen (San Diego, CA). Both antibodies were diluted to $12.5 \mu\text{g}$ per ml (1:40) in PBS/0.1% bovine serum albumin pH 8.0. Incubations with blocking buffer, primary and secondary antibodies, and streptavidin-horseradish peroxidase were done for 30 min each in a humidified chamber at room temperature. After the final wash step of the TSA Direct protocol, sections were incubated for 5 min in $0.5 \mu\text{g}$ per ml propidium iodide nuclear stain (Sigma, St Louis, MO) to permit visual identification of the dermal/epidermal junction. Slides were then washed twice in PBS/bovine serum albumin and twice in water, and were coverslipped using Fluoromount-G mounting media (Southern Biotechnology Associates, Birmingham, AL).

Immunostained sections were viewed and photographed using a Nikon Microphot-FXA microscope equipped with an epifluorescence illumination system.

Quantitation of endothelial cell densities in grafted CSS Sections were stained as described with anti-CD31/PECAM-1, an antibody specific for mouse endothelial cells (Vecchi *et al*, 1994). Sections were analyzed from CSS of all mice killed at 7 d after grafting, and from four controls and three VEGF⁺ mice killed at 14 d after grafting. Six nonoverlapping

photographs per section were taken using fluorescent illumination at $62.5\times$ magnification. The area (μm^2) staining positive for CD31/PECAM-1 was quantitated using Image-1 image analysis software (Universal Image, Media, PA) and was expressed as a percentage of the total area analyzed. The fields analyzed spanned to a depth of approximately $100\mu\text{m}$ below the dermal/epidermal junction and were either $490\mu\text{m}$ wide (day 7 after grafting) or $367\mu\text{m}$ wide (day 14 after grafting). The $100\mu\text{m}$ depth was chosen to eliminate inclusion of staining blood vessels in the panniculus carnosus and to focus on endothelial cells present in the upper part of the dermis. Significant differences ($p < 0.05$) between groups (mean \pm SEM; $n = 18$ for day 7 control and days 7 and 14 VEGF⁺; $n = 24$ for day 14 control) were determined by one-way ANOVA to establish group differences and Student's *t* test to determine pairwise differences.

RESULTS

Genetic modification of keratinocytes to increase VEGF expression The replication-incompetent retroviral vector, pL-CVEGF-SN, used for genetic modification of keratinocytes is shown in **Fig 1**. Northern blot analysis showed that keratinocytes genetically modified with the pL-CVEGF-SN retrovirus (VEGF⁺) expressed high levels of VEGF mRNA (**Fig 2A, lane 2**) compared with control keratinocytes (**Fig 2A, lane 1**). Cultured skin substitutes prepared with VEGF⁺ keratinocytes also expressed high levels of VEGF mRNA (**Fig 2A, lanes 6–8**), even after 21 d of *in vitro* culture. Note that two transcripts of approximately 4.2 kb and 2.9 kb, representing retroviral VEGF₁₆₅ transcripts originating from both the LTR and CMV promoters, respectively, hybridized in VEGF⁺ keratinocyte and CSS RNA. These transcripts both differ in size from the endogenous 3.7 kb human VEGF message (**Fig 2A, lane 1**). With longer autoradiograph times, a 1.8 kb human VEGF mRNA was also seen (data not shown).

VEGF secretion is elevated by genetic modification with pL-CVEGF-SN Medium conditioned for 24 h. by VEGF⁺-modified keratinocytes contained 36.9 ng per ml VEGF, compared with 2.0 ng per ml in control keratinocyte-conditioned medium (**Fig 2B**). CSS prepared with fibroblasts and VEGF⁺-modified keratinocytes secreted significantly more VEGF into the culture medium than controls (**Fig 2B**), and this high level of secretion was sustained throughout 21 d of *in vitro* CSS incubation. In contrast, note the declining secretion of VEGF in control CSS. After 21 d in culture, VEGF⁺-modified CSS secreted 28.8 ng per ml VEGF, compared with only 0.2 ng per ml for control CSS, an increase of over 140-fold due to genetic modification.

Morphology of CSS *in vitro* Light microscopy of control and VEGF⁺ CSS biopsies taken at the time of grafting, 21 d after keratinocyte inoculation, are shown in **Fig 3**. Retroviral transduction had no effect on the morphogenesis of the CSS. Further, the VEGF⁺ CSS were indistinguishable from control CSS. Both control and VEGF⁺ CSS were organized into epidermal and dermal tissue compartments similar to native human skin. The dermal compartments are densely packed with fibroblasts, and darkly staining reticulations of the collagen-GAG substrate can be seen. The epidermal components of the CSS consist of stratified epithelia: the basal, nucleated cells are attached to the collagen-GAG substrate, and well-keratinized stratum corneum layers are seen at the epithelial surfaces. The total thickness of the CSS at the end of the culture period ranged from approximately 200 to 400 μm .

Bioactivity of VEGF secreted by genetically modified CSS To demonstrate the bioactivity of VEGF secreted by cells modified with the pL-CVEGF-SN vector, CSS-conditioned medium was tested for the ability to stimulate the growth of HMVEC. Mitogenicity was assayed by comparing the number of HMVEC present after growth in either EBM (basal medium) or EBM supplemented with 10% CSS conditioned medium collected on day 10 of *in vitro* CSS incubation. Enzyme-linked immunosorbent assay indicated that the control CSS-conditioned medium contained 0.3 ng per ml VEGF, and the VEGF⁺-modified

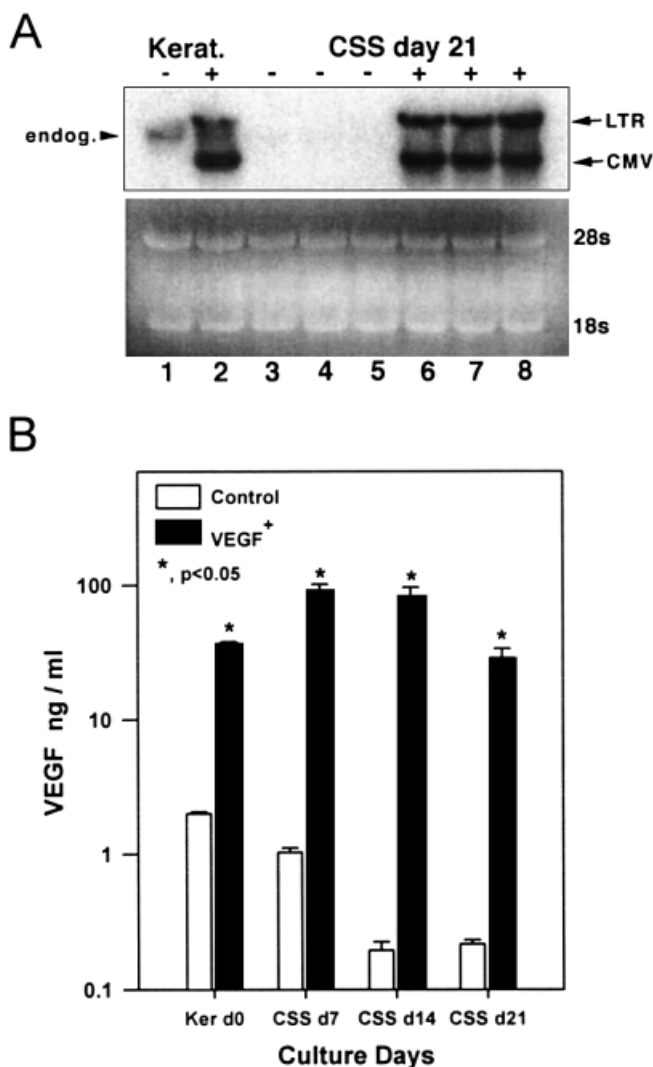


Figure 2. Genetic modification with pL-CVEGF-SN increases VEGF₁₆₅ mRNA expression and protein secretion *in vitro*. (A) A northern blot containing 5 μg total RNA per lane was hybridized to a ³²P labeled VEGF cDNA probe (top). Lanes 1–2, RNA prepared from control (–) and VEGF⁺ (+) keratinocytes at the time of CSS inoculation. Lanes 3–8, RNA prepared from control (–) and VEGF⁺ (+) CSS after 21 d of *in vitro* culture, at the time of grafting to mice. Arrowhead (left) indicates the position of the endogenous VEGF₁₆₅ transcript (approximately 3.7 kb); arrows (right) indicate positions of retroviral VEGF₁₆₅ transcripts (approximately 4.2 kb and 2.9 kb). A 4 h. autoradiograph exposure is shown. The ethidium bromide stained gel is shown (bottom) to demonstrate equivalence of RNA loading. (B) VEGF secretion from keratinocytes (day of CSS inoculation; Ker day 0) and CSS (7, 14, and 21 d after keratinocyte inoculation) is expressed as ng VEGF per ml culture medium. Plotted are mean VEGF concentrations ($n = 6$ per group, Ker day 0; $n = 3$ per group, CSS days 7–21). VEGF protein levels from VEGF⁺-modified keratinocytes and CSS (filled bars) were significantly different (*) from controls (open bars) at each time point. Error bars: SEM.

CSS-conditioned medium contained 100 ng per ml VEGF. As illustrated in **Fig 4**, no mitogenic effect was observed when HMVEC were cultured in EBM supplemented with either unconditioned CSS medium (group 2) or control CSS-conditioned medium (group 3). Medium conditioned by VEGF⁺-modified CSS, however, was mitogenic to HMVEC. The numbers of HMVEC counted after growth in 10% VEGF⁺-modified CSS-conditioned medium (**Fig 4, group 5**) were significantly higher than controls, and were not statistically different from counts obtained using an equivalent amount (10 ng per ml) of rhVEGF (**Fig 4, group 7**). As shown in **Fig 4**, the growth response elicited by VEGF⁺ CSS-conditioned medium was

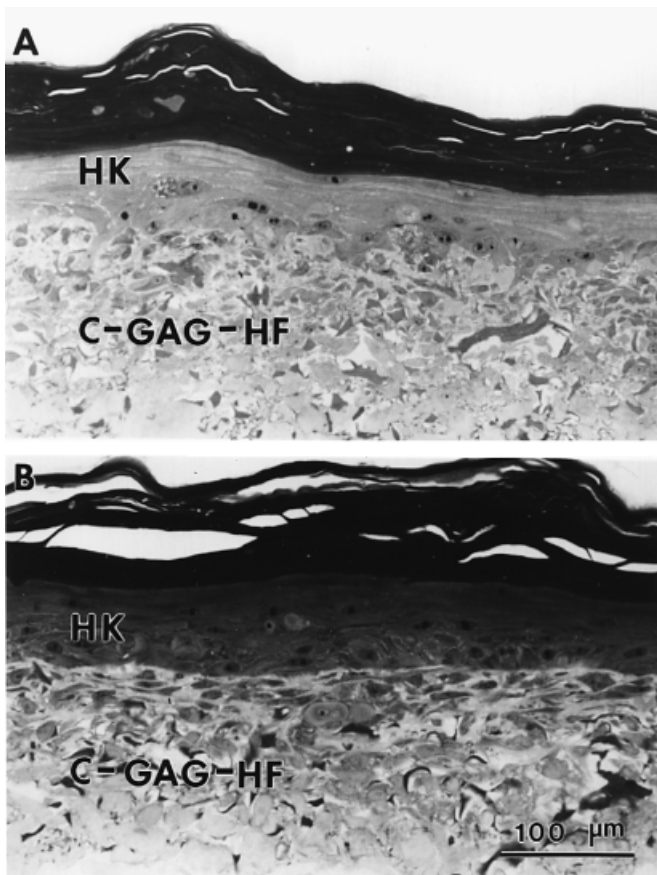


Figure 3. Morphogenesis of CSS *in vitro* is unaffected by genetic modification. (A) Control CSS at 21 d; (B) VEGF⁺ CSS at 21 d. Both control-modified and VEGF⁺-modified CSS develop a stratified epidermal layer and a dermal compartment densely packed with fibroblasts during the 21 day *in vitro* culture period. Representative sections are shown; differences in staining intensity are not due to differences in cell density. HK, human keratinocytes; C-GAG-HF, collagen-GAG substrate populated with human fibroblasts. Scale bar: 100 μ m.

neutralized by an antibody specific for human VEGF (group 6). This neutralizing effect was also seen when medium containing rhVEGF was preincubated with anti-VEGF antibody (Fig 4, group 8).

Elevated VEGF mRNA expression in VEGF⁺-modified CSS after grafting to athymic mice Healing CSS excised from mice at 3, 7, and 14 d after surgery showed positive immunostaining for HLA-ABC antigens, confirming the persistence of human keratinocytes from the CSS in all animals (data not shown). Northern blot analysis indicated that transcription of the retroviral VEGF₁₆₅ gene persisted for at least 14 d after grafting to mice (Fig 5). Although VEGF mRNA was detected to varying degrees in all graft biopsies excised from mice at 3, 7, and 14 d after grafting, greatly elevated levels were detected in VEGF⁺-modified grafts, demonstrating strong expression of retroviral VEGF mRNA. In fact, the level of VEGF expression at 14 d after grafting did not appear to be greatly diminished from the level measured at 3 d after grafting.

Improved vascularization in VEGF⁺-modified CSS after grafting to mice Upon excision from mice killed at 3, 7, and 14 d after grafting, it was noted that VEGF⁺ CSS were better adhered to the wound beds and bled much more than control CSS. Examination of histologic sections of CSS by light microscopy suggested the presence of greater numbers of blood vessels in the dermis of VEGF⁺ CSS. Representative sections are shown in Fig 6(A-F), and these correspond to the same CSS shown *in vitro*

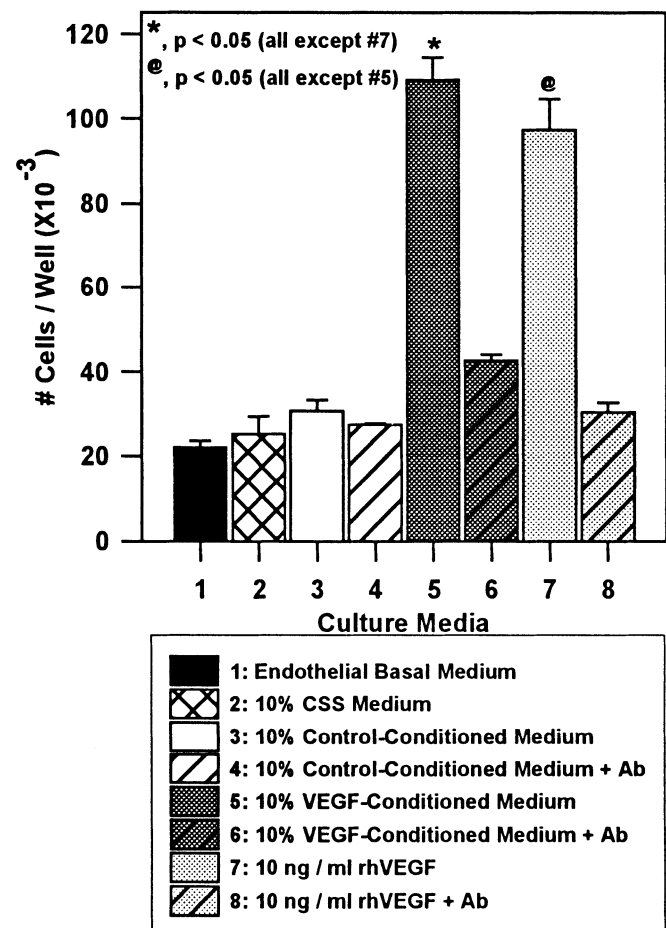


Figure 4. VEGF secreted by genetically modified CSS is bioactive. Culture medium was tested for mitogenic activity in a microvascular endothelial cell growth assay. Culture conditions are specified in the key; details are given in *Materials and Methods* section. Briefly, endothelial cells were grown in basal medium (EBM) alone (1), or EBM supplemented with 10% unconditioned CSS medium (2) or CSS medium conditioned by control (3, 4) or VEGF⁺-modified (5, 6) CSS. As a positive control, cells were grown in EBM supplemented with 10 ng per ml recombinant human VEGF (7, 8). Preincubation of media with a VEGF-neutralizing antibody (4, 6, 8) was used to show that the mitogenic response is due to VEGF. Plotted are mean cell numbers (in thousands) for each condition (n = 3 per group). Values for conditions 5 and 7 were significantly different (*, @) than all other values. Error bars: SEM.

Fig 3. At 3 d after grafting, blood vessels were found only in the panniculus carnosus underlying control CSS grafts (Fig 6A). In contrast, blood vessels were evident in the dermal layer of VEGF⁺ CSS grafts, above the level of the panniculus (Fig 6D). By 7 d after grafting, small vessels were seen in the dermal layer of control grafts (Fig 6B). At this time point, the VEGF⁺-modified grafts (Fig 6E) contained numerous blood vessels scattered throughout the entire dermal compartment, including many in proximity to the dermal/epidermal junction, and these appeared larger than in control grafts. At 14 d after grafting, both the control (Fig 6C) and VEGF⁺ CSS grafts (Fig 6F) had evidence of blood vessels near the dermal/epidermal junction, but these were apparently larger and more numerous in the VEGF⁺ grafts.

Except for differences in the observed number and size of vessels, histologic analyses revealed no significant morphologic differences between grafted VEGF⁺ and control CSS. At 3 d after grafting, darkly staining reticulations of the implanted collagen-GAG were present in both control and VEGF⁺ CSS (Fig 6A, middle left of panel, and Fig 6D, middle right of panel). These were not seen at 14 d after grafting, suggesting replacement by newly synthesized collagen. Both control and VEGF⁺ CSS exhibited stratified

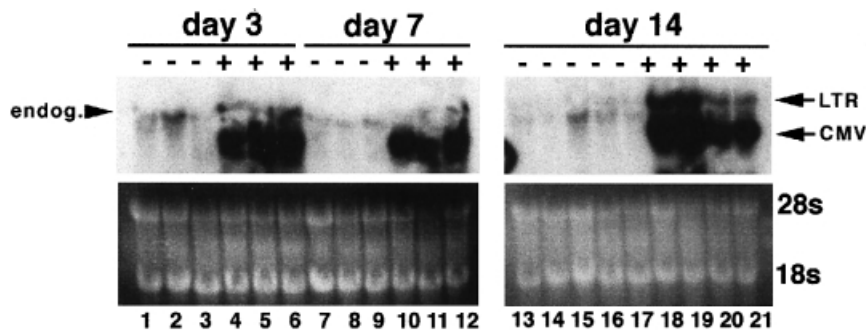


Figure 5. Enhanced VEGF₁₆₅ mRNA expression in VEGF⁺ CSS *in vivo*. Northern blots containing total RNA (10 μg per lane) were hybridized to a ³²P labeled VEGF cDNA probe (top). Arrowhead (left) indicates the position of the endogenous 3.7 kb human VEGF₁₆₅ transcript; arrows (right) indicate retroviral VEGF₁₆₅ transcripts. A 7 h. autoradiograph exposure is shown. The ethidium bromide stained gels are shown (bottom) to demonstrate equivalence of RNA loading.

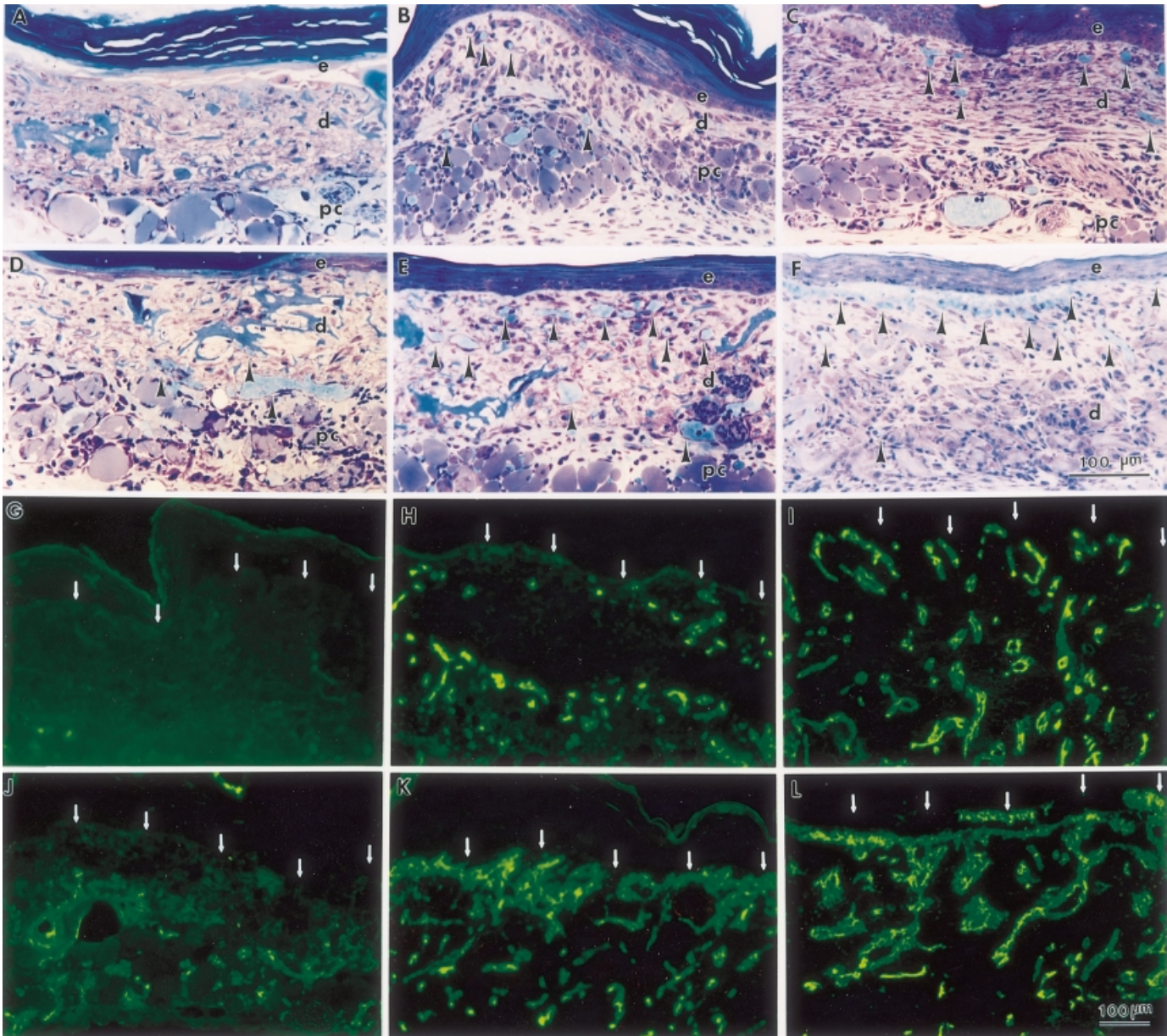


Figure 6. Enhanced vascularization in VEGF⁺ CSS after grafting to athymic mice. Biopsies for light microscopy and immunohistochemistry were taken from control (A–C, G–I) and VEGF⁺ (D–F, J–L) grafts excised at 3 d (A, D, G, J), 7 d (B, E, H, K), and 14 d (C, F, I, L) after surgeries. (A–F) Light microscopy of histologic sections. e, epidermis; d, dermis; pc, panniculus carnosus. Arrowheads point to examples of blood vessels in the dermis. Note light blue-stained erythrocytes within blood vessels. Excised grafts shown here correspond to the same grafts shown *in vitro* in Fig 3. (G–L) Immunostaining with an antibody against CD31/PECAM-1 to identify mouse endothelial cells. Control immunohistochemistry without primary antibody showed no specific staining (data not shown). The dermal/epidermal junction at the top of each panel is indicated by white arrows. Scale bars: 100 μm (bar in F is for A–F; bar in L is for G–L).

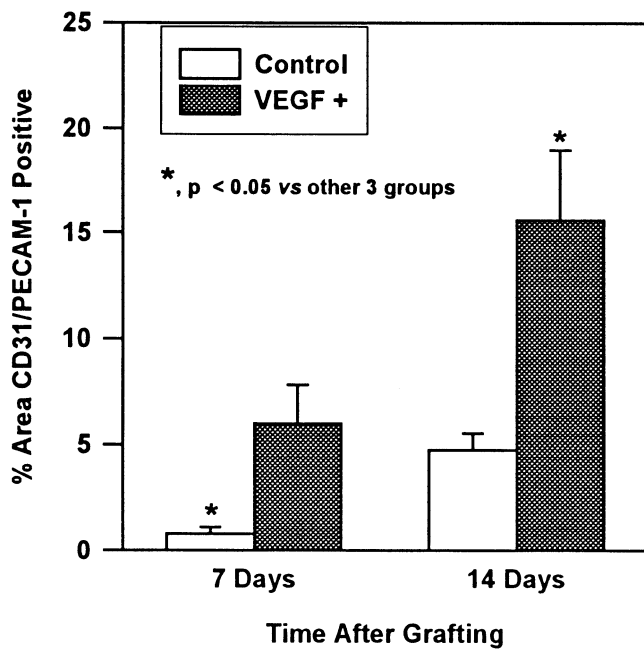


Figure 7. Quantitative analysis of endothelial cell densities in control and VEGF⁺ CSS after grafting to athymic mice. The total area staining positive for the endothelial cell-specific marker CD31/PECAM-1 in the uppermost 100 μ m of the dermis was quantitated and expressed as a percent of area analyzed. Plotted are mean percent areas at 7 and 14 d after grafting. Error bars: SEM.

epidermal layers, with nucleated basal cells and cornified outer layers. Low to moderate leukocyte infiltration was observed at all time points, and variations were seen between CSS grafted to different mice and between different regions of individual CSS (data not shown). No consistent differences in leukocyte infiltration were seen between groups, indicating that variations were not due to VEGF overexpression.

Immunohistochemical staining with an antibody directed against CD31/PECAM-1, a mouse endothelial cell-specific antigen, revealed a greater number of endothelial cells in the dermal layers of VEGF⁺ CSS grafts (Fig 6G–L). The results were similar to those seen by histologic analysis. At 3 d after grafting, CD31/PECAM-1-positive cells were found only in the panniculus layer under control grafts (Fig 6G), whereas immunopositive cells were visible in the lower part of the dermal compartments of VEGF⁺ grafts (Fig 6J). At 7 d after surgery, CD31/PECAM-1 staining was observed throughout the dermal layer of VEGF⁺ grafts (Fig 6K), but only in the lower part of the dermis of control grafts (Fig 6H). By 14 d after grafting, both control (Fig 6I) and VEGF⁺ CSS grafts (Fig 6L) had numerous CD31/PECAM-1-positive cells throughout the dermis. The endothelial cell density in the upper part of the dermis, however, appeared higher in VEGF⁺ grafts compared with control grafts, and large vessels beneath the dermal/epidermal junction were seen (Fig 6L).

A quantitative analysis of endothelial cell densities in the uppermost 100 μ m of the dermis of control and VEGF⁺ CSS is illustrated in Fig 7. At both 7 and 14 d after grafting, the areas staining positive for CD31/PECAM-1 were significantly greater in VEGF⁺ CSS than control CSS. The mean CD31/PECAM-1-positive area in VEGF⁺ grafts at 7 d after grafting was not statistically different from control CSS 14 d after grafting.

DISCUSSION

This study describes the inclusion of genetically modified keratinocytes in CSS to enhance vascularization after grafting. Transduction with a replication-incompetent retrovirus was used to introduce a CMV promoter/enhancer-driven VEGF₁₆₅ gene into cultured human keratinocytes. Control keratinocytes were trans-

duced with a blank retrovirus, with no inserted VEGF gene; thus, any observed differences between control and VEGF⁺-modified cells are presumed to be due to inserted VEGF sequences and not simply the result of retroviral transduction. Genetic modification of keratinocytes with pL-CVEGF-SN led to greatly increased expression of VEGF₁₆₅ mRNA and elevated VEGF protein secretion compared with controls. The VEGF⁺-modified CSS secreted elevated levels of VEGF into the culture media throughout the 21 d *in vitro* culture period.

The pL-CVEGF-SN retroviral construct included the CMV promoter/enhancer upstream of the VEGF₁₆₅ coding sequence. Two VEGF transcripts were detected by northern blot hybridization after this construct was introduced into keratinocytes. The transcript sizes are consistent with the initiation of transcription from both the retroviral 5' LTR and the CMV promoter. A comparison of the relative hybridization intensities of the two retroviral VEGF transcripts suggests a higher level of transcription from the CMV promoter. Both retroviral transcripts differ in size from the endogenous 3.7 kb human VEGF message seen in control keratinocytes, which has also been previously reported by others in human epidermis (Weninger *et al*, 1996). With longer autoradiograph exposure times, the 1.8 kb human VEGF mRNA observed by others in human epidermis (Weninger *et al*, 1996) was also seen (data not shown). Note that at 14 d after surgery, two VEGF transcripts are observed in control CSS. The additional higher molecular weight transcript may represent an alternate transcript of the human VEGF gene, or it may indicate expression of murine VEGF, because the size of this transcript (approximately 4.2 kb) corresponds to the major VEGF transcript observed by others in murine skin (Larcher *et al*, 1996).

High levels of *in vivo* retroviral VEGF gene expression were detected in genetically modified CSS from all mice, up to 14 d after grafting, with higher relative expression of the smaller retroviral transcript. In a previous study from this laboratory,¹ genetically modified grafts were prepared with keratinocytes transduced with the MFG-PDGF-A retrovirus (Eming *et al*, 1995). In that study, although high levels of LTR-driven PDGF-A expression were detected in keratinocytes and CSS *in vitro*, relatively low expression was detected after grafting. The inclusion of the CMV promoter in the retroviral construct used in this study may have contributed to the extended strong expression of the retroviral VEGF gene. Future experiments with additional animals and later time points will be required to measure the duration of retroviral VEGF₁₆₅ expression *in vivo*.

CSS generated with fibroblasts and VEGF⁺-modified keratinocytes in a collagen-GAG substrate developed a stratified appearance and were morphologically indistinguishable from control (sham-modified) CSS (Fig 3) or unmodified CSS (data not shown). This demonstrates that genetic modification and subsequent overexpression of VEGF do not have a negative effect on the morphogenesis of cultured skin.

Endothelial cell proliferation assays have been used by others to demonstrate the mitogenic activity of purified VEGF (Conn *et al*, 1990) and VEGF secreted by keratinocytes (Detmar *et al*, 1995). The bioactivity of VEGF secreted by genetically modified CSS in this study was demonstrated by its ability to stimulate the growth of human dermal microvascular endothelial cells *in vitro*. The neutralization of this activity by an antibody specific for human VEGF confirmed that the observed stimulatory effect was due to VEGF in the medium. VEGF bioactivity was further demonstrated *in vivo* after grafting to athymic mice. A qualitative analysis of graft morphology and a quantitative analysis of endothelial cell density revealed increased vascularization in VEGF⁺ CSS compared with controls. This indicates that VEGF produced by genetically modified keratinocytes in the CSS was able to penetrate the

¹Supp DM, Supp AP, Bell SM, Swope VB, Morgan JR, Boyce ST: Genetic modification of cultured skin substitutes by transduction of human keratinocytes with platelet derived growth factor A. *Wound Repair Regen* 6:A265, 1998 (abstr.)

epidermal/dermal basement membrane, as has been reported for VEGF expression targeted to basal keratinocytes in native skin of K14/VEGF transgenic mice (Detmar *et al*, 1998), and also penetrate the dermis to promote vascularization from mouse endothelial cells present in the wound bed.

Vascularization was quantitated by measuring the percent area of the upper dermis staining positive for the endothelial-specific marker CD31/PECAM-1. Both the control and VEGF⁺ CSS had significantly more CD31/PECAM-1 staining at 14 d after grafting than at 7 d after grafting. This indicates that graft vascularization is incomplete at 7 d and continues until at least 14 d after grafting. Inclusion of keratinocytes overexpressing VEGF₁₆₅ led to enhanced vascularization of CSS grafts, with VEGF⁺ CSS showing significantly more CD31/PECAM-1 staining at both 7 and 14 d after grafting as compared with control CSS. Interestingly, the percent area staining positive for CD31/PECAM-1 in VEGF⁺ CSS at 7 d after surgery was not statistically different from control grafts at 14 d after surgery. This suggests that, in addition to an increase in the number of blood vessels, the time to vascularization of VEGF⁺-modified grafts was reduced by up to 1 wk.

These results suggest that inclusion of VEGF⁺-modified cells could hypothetically result in significant improvement in the healing of large wounds treated with CSS. Lack of a vascular plexus is an important limitation of CSS, contributing to graft failure by extending time for reperfusion, ischemia, and nutrient deprivation of grafted cells (Boyce, 1996). This limitation has been addressed by the application of topical antimicrobials (Boyce *et al*, 1997) and nutrient solutions (Boyce *et al*, 1995b) to grafted CSS to improve the wound environment and nourish the cells until adequate vascularization is attained. In the experiments described here, topical nutrients were not applied to dressings after surgery; instead, a single application of antimicrobial ointment was administered. When grafts were excised, it was observed that the VEGF⁺ CSS grafts were better adhered to the wound beds than control grafts. Though anecdotal, this observation suggests that, by reducing the time required for vascularization, VEGF⁺ overexpression could hypothetically reduce the need for postoperative topical nutrient application. This would represent an important advance in the care of large wounds treated with CSS, such as burns. Further studies will be required to assess the outcome if wounds representing a large percentage of the total body surface area are treated with genetically modified CSS. Because the possibility of systemic increases in circulating protein exists if a large area of the body is treated, the effects of VEGF overexpression in these situations will need to be carefully monitored. Initial clinical studies would treat small areas (<5% of the body surface) with extensive safety monitoring before large areas of the body would be treated.

Enhanced expression of VEGF in CSS could prospectively increase their usefulness in impaired wound healing environments, such as chronic ulcers, which are often associated with vascular deficiencies, or in diabetic wound healing, which is associated with reduced VEGF expression (Frank *et al*, 1995). Others have reported that VEGF treatment of ischemic dermal ulcers improved healing by counteracting the deficit in wound healing produced by ischemia (Corral *et al*, 1999). The effect of VEGF on wound healing is likely indirect, resulting from increased angiogenesis and vascular hyperpermeability which enhance the delivery of plasma proteins and migration of cells to the site of injury (Corral *et al*, 1999).

The ease with which keratinocytes can be genetically modified using retroviral transduction makes CSS a feasible mechanism for cutaneous gene therapy to improve wound healing. In the experiments described in this study, second-passage human keratinocytes were cocultured with a layer of mitomycin-C-treated producer cells. The keratinocytes were then expanded to increase cell number and to eliminate carry-over of virus-producing cells. This expansion step increases the time required from skin biopsy to graft preparation, which would be undesirable in a clinical setting where quick wound coverage is essential. Hypothetically, primary

cultures of keratinocytes could be inoculated directly on to growth-inactivated retroviral producer cells in place of the fibroblast feeder cell layer that is typically used, eliminating any increase in time to graft preparation due to genetic modification.

Gene therapy with VEGF has been shown to promote therapeutic angiogenesis in preclinical models of tissue ischemia (Asahara *et al*, 1996; Takeshita *et al*, 1996; Tsurumi *et al*, 1996; Rivard *et al*, 1999) and in human clinical trials (Isner *et al*, 1996; Baumgartner *et al*, 1998; Losordo *et al*, 1998). The present results indicate that VEGF is a promising candidate for gene therapy in wound healing applications. Caution must be exercised, however, before using VEGF overexpression in clinical applications. VEGF expression is found in transformed cells of various tumors (reviewed by Dvorak *et al*, 1995), including tumors of the skin (Larcher *et al*, 1996; Weninger *et al*, 1996). Further, overexpression of VEGF in cultured melanoma cells (Claffey *et al*, 1996) or in the skin of transgenic mice carrying a v-H-ras transgene (Larcher *et al*, 1998) resulted in accelerated tumor development and experimental metastasis. Its role in tumor development appears to be related primarily to the requirement for blood supply to the tumor. Hypothetically, VEGF could increase the growth of an existing tumor, but because the skin can be readily monitored and tumors easily removed, cutaneous gene therapy with VEGF has significant advantages over other forms of gene delivery. Additionally, therapeutic benefits can be obtained for healing wounds with relatively short durations of VEGF expression, thereby avoiding potential negative effects resulting from chronic VEGF exposure. To increase the safety of VEGF in cutaneous gene therapy applications, nonviral gene delivery methods could be used to eliminate the possibility of unintentional spread of vector and, if necessary, specific or inducible promoters could be used to more specifically regulate or limit VEGF expression.

In summary, transduction of human keratinocytes with a replication-incompetent retrovirus was used to generate a population of cells overexpressing bioactive VEGF₁₆₅. The level and duration of VEGF expression in CSS prepared with genetically modified cells was sufficient to measurably increase microvascular endothelial cell number and reduce the time required for vascularization of CSS after grafting. These results suggest that gene therapy with VEGF, combined with tissue engineering, can be used therapeutically to improve healing of cutaneous wounds.

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