Overexpression of Vascular Endothelial Growth Factor Accelerates Early Vascularization and Improves Healing of Genetically Modified Cultured Skin Substitutes

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Cultured skin substitutes (CSS) lack a vascular plexus, leading to slower vascularization after grafting than split-thickness skin autograft. CSS containing keratinocytes genetically modified to overexpress vascular endothelial growth factor (VEGF) were previously shown to exhibit enhanced vascularization up to 2 weeks after grafting to athymic mice. The present study examines whether enhanced vascularization compared with controls persists after stable engraftment is achieved and analyzes VEGF expression, wound contraction, and engraftment. Control and VEGF-modified ($VEGF^+$) CSS were grafted onto full-thickness wounds in athymic mice. VEGF expression was detected in $VEGF^+$ CSS 14 weeks after grafting. Graft contraction was significantly lower in $VEGF^+$ CSS compared with controls, suggesting more stable engraftment and better tissue development. Positive HLA-ABC staining, indicating persistence of human cells, was seen in 86.7% (13/15) of grafted $VEGF^+$ CSS, compared with 58.3% (7/12) of controls. Differences in dermal vascularization between control and $VEGF^+$ grafts were significant 1 week after surgery, but not at later times. However, the distribution of vessels was different, with more vessels in the upper dermis of $VEGF^+$ grafts. These results suggest that VEGF overexpression in genetically modified CSS acts to accelerate early graft vascularization and can contribute to improved healing of full-thickness skin wounds. (J Burn Care Rehabil 2002;23:10–20)

Wound closure is critical for recovery from burn injury. In patients with massive burns, prompt and permanent wound closure is a limiting factor in recovery because of the lack of donor sites for skin autografting. This has led to the development of a number of alternatives for wound closure, including cultured epithelial autograft and allograft,[1–4] cryopreserved human skin allograft,[5] decellularized dermal allograft,[6] acellular collagen-based dermal substitutes,[7,8] keratinocyte-collagen membrane grafts,[9] keratinocyte-fibrin-glue suspensions,[10] and allogeneic or autologous composite cultured skin substitutes.[11–16] Previous studies have shown that cultured skin substitutes (CSS) consisting of autologous cultured human fibroblasts and keratinocytes can provide permanent closure of burn wounds and reduce requirements for split-thickness skin autograft.[17] CSS have similar anatomy to split-thickness skin grafts and can provide permanent replacement of both dermal and epidermal layers. Upon healing, cultured skin performs similarly to native skin autograft. However, because CSS contain only two cell types, they can provide barrier and connective tissue, but cannot replace all of the functions of normal skin. Thus, despite the clinical benefits from CSS, physiological limitations remain that can contribute to clinical morbidity and reduced engraftment. For example, CSS lack a vascular plexus, leading to slower vascularization compared with native skin autograft. Early vascularization of split-thickness skin autograft occurs primarily through inosculation, involving the anastomosis of capillaries in the wound bed to severed vessels in the graft.[18] Because CSS are avascular, they are vascularized by neovascularization, a slower

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process that involves the growth of new vessels from the wound bed into the graft. This increases the time for nutrient deprivation of the cultured skin after grafting and thereby contributes to graft failure.[19] This has been addressed in part through the use antimicrobial and nutrient irrigation solutions after grafting.[14,17,20]

Modification of CSS to enhance vascularization could lead to increased engraftment and simplified postoperative care regimens. This could hypothetically be achieved through genetic modification of cells used to prepare CSS. Several investigators have demonstrated the ability to express exogenous genes in keratinocytes and have shown that genetically modified cultured keratinocytes retain the capacity to form epidermis.[21–25] Prospectively, the use of cells genetically modified to overexpress an angiogenic cytokine in CSS can improve vascularization and healing after grafting. In a previous study[26] CSS were prepared with keratinocytes engineered to overexpress vascular endothelial growth factor (VEGF), a specific and potent mitogen for endothelial cells.[27,28] After grafting to athymic mice, enhanced vascularization of the upper dermis of VEGF-modified ($VEGF^+$) CSS compared with controls was observed. This difference was seen up to 2 weeks after grafting, the endpoint of that study. Interestingly, no difference in vascularization of the upper dermis was observed between $VEGF^+$ CSS at 1 week after grafting and control CSS at 2 weeks after grafting. In the present study the observation period was extended to evaluate VEGF overexpression in genetically modified CSS at later times, after stable engraftment was achieved. This was necessary to determine whether the stimulation of vascularization seen in the previous study was because of accelerated vascularization of $VEGF^+$ grafts or whether the difference between control and $VEGF^+$ grafts can persist for extended periods. This study also analyzed the duration of VEGF overexpression in $VEGF^+$ CSS after grafting and evaluated wound contraction and engraftment.

MATERIALS AND METHODS

Cell Culture and Genetic Modification

Human cadaver skin, procured from a 35-year-old male donor, 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 and grown as previously described.[29]

The recombinant retroviral vector used to transfer the human $VEGF_{165}$ coding sequence is described in detail elsewhere.[26] Briefly, a 1.89 kilobase (kb) *Eco* RI fragment from the phVEGF165.SR plasmid, containing the $VEGF_{165}$ coding sequence as well as the cytomegalovirus (CMV) promoter/enhancer and partial 3' untranslated rabbit β-globin region, was subcloned into the pLXSN replication-incompetent retroviral vector (Clontech Laboratories, Palo Alto, CA). The resulting pL-CVEGF-SN retroviral plasmid was transfected into the NIH/3T3-based packaging cell line RetroPack PT67 (Clontech Laboratories) and cells were selected with G418 (800 μg/ml; Clontech Laboratories) to yield populations with stably integrated retrovirus. The VEGF protein produced by cells infected with the pL-CVEGF-SN retrovirus was shown in a previous study to be mitogenic to human microvascular endothelial cells.[26] Control virus-producing cell lines were generated using the pLXSN vector without inserted VEGF sequences.

Second-passage keratinocytes were inoculated into flasks containing subconfluent cultures of virus-producing cells pretreated with 15 μg/ml mitomycin C (Sigma, St. Louis, MO). Keratinocytes and virus-producing cells were cocultured for 5 days, after which the flasks were rinsed with 5mM ethylenediamine tetraacetic acid to remove virus-producing cells. The keratinocytes were trypsinized and subcultured to expand cell populations.

Cultured Skin Substitutes

CSS, consisting of control keratinocytes (modified with blank retrovirus) or VEGF-modified keratinocytes and unmodified fibroblasts attached to collagen-glycosaminoglycan (GAG) biopolymer substrates, were prepared as previously described.[30,31] Briefly, collagen-GAG substrates (n = 15) were inoculated with 5×10^5 fibroblasts/cm^2. One day later (culture day 0), dermal substitutes were inoculated with $1 \times 10^6/cm^2$ control (n = 7) or $VEGF^+$ (n = 8) keratinocytes. On culture day 3, CSS were lifted to the air-liquid interface. CSS were cultured for 14 days at 37°C, 5% CO_2, with daily changes of nutrient medium. Grafts were cut to 2 cm × 2 cm squares for transplantation to mice.

Grafting to Athymic Mice

All animal studies were performed with the approval of the University of Cincinnati Institutional Animal Care and Use Committee following National Institutes of Health guidelines. VEGF-modified (n = 33) and control (n = 29) CSS were grafted to 2 cm × 2 cm full-thickness wounds on the flanks of athymic mice as previously described,[26,30] with minor modifications. Briefly, each wound was prepared leaving the panniculus carnosus intact. Grafts with overlying

N-terface® nonadherent dressing (Winfield Laboratories, Richardson, TX) were sutured to wounds. Grafts were dressed with gauze pads coated with antibiotic ointment, the dressings were covered with OpSite (Smith & Nephew Medical, Hull, UK), and the grafted areas were bandaged with Coban (3M Medical Division, St. Paul, MN). At 2 weeks after surgery, stitches were removed and dressings were replaced with dry gauze and Coban bandages. All dressings were removed at 3 weeks after surgery. Mice were killed at 1 week (n = 3 control; n = 4 $VEGF^+$), 2 weeks (n = 3 per group), 4 weeks (n = 3 per group), 6 weeks (n = 4 per group), 8 weeks (n = 4 per group), and 14 weeks (n = 12 control, n = 15 $VEGF^+$) after surgery. Grafts were excised, and biopsies were taken for histological analysis, immunohistochemistry, and RNA isolation. Serum was collected from mice at 1, 2, and 4 weeks after surgeries for use in an enzyme-linked immunosorbent assay (ELISA) for human VEGF protein.

Analysis of VEGF RNA expression

Expression of retroviral VEGF RNA was analyzed using nonisotopic Northern blot hybridization. Cells or CSS biopsies used for RNA isolation were snap-frozen in liquid nitrogen and stored at −70°C until needed. Total cellular RNA was isolated using the RNeasy Mini Kit (QIAGEN, Santa Clarita, CA) and was electrophoretically separated on 1% agarose gels in buffer containing 0.6% formaldehyde, 50 mM HEPES pH 7.8, 1 mM ethylenediamine tetraacetic acid, with 0.2 ng/ml ethidium bromide. Gels were transferred overnight to BrightStar™-Plus Membranes (Ambion, Inc., Austin, TX) using the Turboblotter system (Schleicher & Schuell, Keene, NH). The $VEGF_{165}$ cDNA probe was a 1.89 kb *Eco* RI fragment from the pL-CVEGF-SN plasmid[26] that was biotin-labeled using the BrightStar™ Psoralen-Biotin kit (Ambion). Hybridizations were done overnight at 68°C using PerfectHyb Plus buffer (Sigma, St. Louis, MO), and detection of bound probe was performed using the BrightStar™ BioDetect™ kit (Ambion).

Analysis of VEGF Protein Secretion

Aliquots of culture medium from sub-confluent control and VEGF-modified keratinocytes (n = 8 per group) at the time of inoculation of CSS, and from CSS (n = 7 control, n = 8 $VEGF^+$) at 1 and 2 weeks of in vitro incubation, were collected and stored at −20°C until assayed for VEGF protein concentration. These samples were taken before media changes, thus they were conditioned by cells or CSS for 24 hours at the time of collection. Blood was collected from mice at 1, 2, and 4 weeks after surgery; samples were centrifuged and serum was stored at −20°C until assayed. Human $VEGF_{165}$ protein levels were determined using ELISA (Quantikine Human VEGF Immunoassay, R&D Systems, Minneapolis, MN). Mean values and standard error of the mean (SEM) are presented.

Statistical analyses of VEGF concentrations in culture media were performed using SigmaStat statistical analysis software (SPSS Science, Chicago, IL). Pairwise comparisons were done by *t*-test to determine significant differences ($P < 0.001$) between control and $VEGF^+$ samples at each time point.

Measurements of Wound Contraction

Wound perimeters were directly traced onto sterile frosted Mylar sheets at the time of surgery (original area) and at 2, 3, 4, 5, 6, 8, 10, 12, and 14 weeks after surgery. Wound areas were determined by computer planimetry using the Image-1 image analysis system (Universal Imaging Corp., Downingtown, PA). Data were expressed as percent original wound area calculated individually for each animal at each time point. Mean values ± SEM are presented. Statistical analysis was performed using one-between, one-within repeated-measures analysis of variance (RM ANOVA). The between factor was the group (control vs $VEGF^+$); the within, or repeated, factor was time (0–14 weeks). Univariate ANOVAs were done at each time point to determine significant differences ($P < 0.05$) between control and $VEGF^+$ groups.

Light Microscopy

Biopsies of CSS were fixed in 2% glutaraldehyde/2% paraformaldehyde for a minimum of 24 hours. Samples were processed, embedded in glycol-methacrylate, sectioned, and stained with toluidine blue using standard techniques. Sections were examined using a Nikon Microphot-FXA microscope (Nikon, Melville, NY) and were photographed using a Spot Jr. Digital Camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

Immunohistochemistry

Biopsies of CSS excised from mice were frozen in M-1 Embedding Matrix (Lipshaw, Pittsburgh, PA). A two-step procedure was used to ensure that each biopsy was sectioned at a 90° angle to the surface of the epidermis. Briefly, each tissue sample was frozen flat in a small volume of M-1 matrix in a small steel base mold held on dry ice. This embedded sample was removed from the steel base and the M-1 matrix was trimmed on one side to the level of the graft; the biopsy was rotated 90° to an upright position and

placed in an embedding ring that was then filled with M-1 matrix and frozen. Cryostat sections were dehydrated in methanol and fixed in acetone at −20°C. After air-drying, sections were rehydrated in phosphate-buffered saline at pH 7.6.

Human keratinocytes in grafted CSS were stained using direct immunofluorescence of frozen sections with a fluorescein-labeled anti-human HLA-ABC antibody (Accurate Chemical and Scientific Corp., Westbury, NY), and sections were counter-stained with Propidium Iodide (Sigma).

Murine endothelial cells in grafted CSS were stained using direct immunofluorescence of frozen sections with a fluorescein-labeled anti-mouse PECAM-1/CD31 antibody (BD Pharmingen, San Diego, CA). Additionally, collagen IV staining was performed using Rabbit Anti-Mouse Collagen Type IV Antibody (Biodesign International, Kennebunkport, ME), followed by visualization with Goat Anti-Rabbit Ig Texas Red-labeled antibody (Southern Biotechnology Associates, Inc., Birmingham, AL).

Quantitation of Endothelial Cell Densities in Grafted CSS

Sections were double-labeled as described for both PECAM-1/CD31 and collagen IV. Sections were viewed using a Nikon Microphot-FXA microscope equipped with an epifluorescence illumination system (Nikon), and digital images were captured using a Spot Jr. Digital Camera. An average of five nonoverlapping images per mouse were captured at ×31.2 magnification. The number of fields analyzed per mouse varied because of variations in section width; regions of mouse skin bordering the cultured skin grafts were not analyzed. PECAM-1/CD31 staining in the dermal regions of cultured skin grafts was quantified using the Metamorph Imaging System (Universal Imaging Corp.). Dermal regions corresponding to 1-mm lengths of epidermis were analyzed. The upper and lower limits of the epidermis were identified using collagen IV staining, which permitted localization of the dermal/epidermal basement membrane and visualization of the panniculus carnosus layer. Dermal regions were manually traced using the freehand region tool in the Metamorph software program, and the percent area of the region staining positive for PECAM-1/CD31 was measured using a defined constant threshold value. Staining in the upper dermis was similarly quantified, but the region analyzed corresponded to a depth of 0.1 mm below the dermal/epidermal junction. Control or $VEGF^+$ values at each time point (1, 2, 4, 6, 8, and 14 weeks after grafting) were grouped for analysis, and mean values and SEM are presented. Pairwise comparisons were done by *t*-test using SigmaStat statistical analysis software (SPSS Science) to determine significant differences between groups.

RESULTS

VEGF mRNA and Protein Expression

The replication-incompetent retroviral vector L-CVEGF-SN is diagrammed in Figure 1A. Note that two VEGF transcripts of different sizes are predicted from the L-CVEGF-SN retrovirus: a larger transcript from the retroviral long terminal repeat (LTR) promoter, and a smaller transcript from the CMV promoter/enhancer (Figure 1A). Both retroviral transcripts encode identical $VEGF_{165}$ proteins and differ in size from the endogenous human and murine $VEGF_{165}$ messages. Keratinocytes genetically modified by transduction with this vector expressed high levels of both retroviral VEGF transcripts (Figure 1A). The level of expression is greatly elevated compared with expression of the endogenous VEGF gene. Relatively high levels of retroviral VEGF mRNA expression are maintained in CSS prepared with $VEGF^+$ keratinocytes (Figure 1A).

Similarly, VEGF protein secretion was significantly elevated in $VEGF^+$ keratinocytes and CSS (Figure 1B). Culture media conditioned by control keratinocytes contained an average of 0.9 ng/ml VEGF. Media conditioned by VEGF-modified keratinocytes contained an average of 4.7 ng/ml VEGF, a somewhat modest five-fold increase because of genetic modification. Interestingly, the difference between control and VEGF-modified CSS was more dramatic, particularly at culture day 14 when CSS were grafted to mice. Control CSS-conditioned media contained an average of 0.19 ng/ml VEGF, compared with 7.2 ng/ml in $VEGF^+$ CSS-conditioned media, an approximately 38-fold increase.

Morphology of CSS

Light microscopy was used to assess the development of the CSS before grafting to mice. Representative histological sections from culture day 10 are shown in Figure 2. No differences were seen between control and $VEGF^+$ CSS. Both groups displayed a stratified epidermal layer, with nucleated basal cells attached to the collagen-GAG substrate and development of a cornified layer analogous to stratum corneum at the air-exposed epidermal surface. The dermal compartments were filled with fibroblasts. The total thickness of the CSS ranged from 200 to 500 μm, with no differences observed between groups.

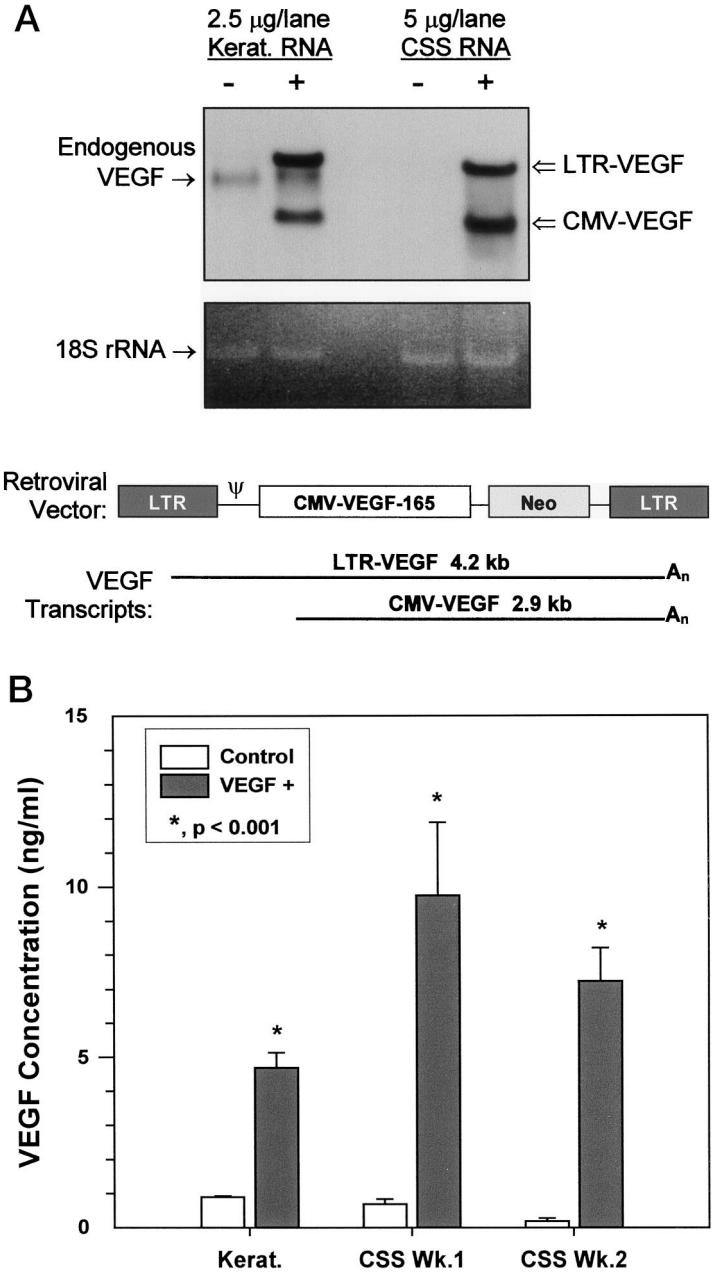

Figure 1. Increased VEGF expression in VEGF-modified keratinocytes and CSS in vitro. A. Northern blot hybridization analysis (top) of VEGF RNA expression in control (−) and VEGF-modified (+) keratinocytes and CSS. The arrow at left indicates the position of the ~3.7 kb endogenous human VEGF transcript; the open arrows at right indicate the positions of the retroviral transcripts from the LTR and CMV promoters. A photograph of the ethidium bromide-stained gel showing the 18S ribosomal RNA band is presented to demonstrate equivalence of RNA loading between control and VEGF-modified samples. The structure of the L-CVEGF-SN retroviral vector, previously described in detail elsewhere,[26] is also shown (bottom). B. Increased secretion of VEGF protein from VEGF-modified keratinocytes, at the time of CSS inoculation, and CSS at 1 and 2 weeks of in vitro incubation. Concentrations of VEGF are presented as ng/ml culture media; error bars, ± SEM. Differences between groups were statistically significant ($P < 0.001$). *Kerat.*, keratinocytes; *CSS*, cultured skin substitutes; *VEGF*, vascular endothelial growth factor; *LTR*, long terminal repeat; *VEGF*$^+$, VEGF-modified; *ψ*, packaging signal sequence; *Neo*, neomycin resistance gene; *Wk.*, week.

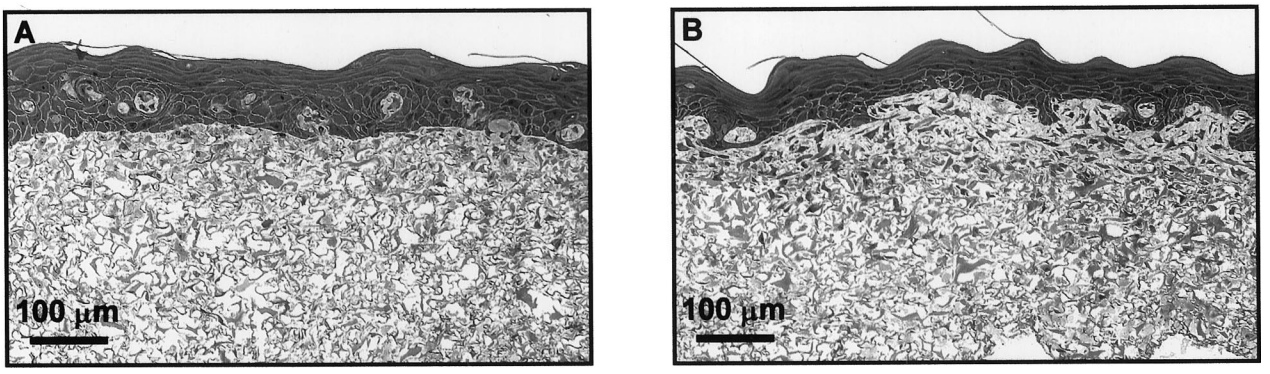

Figure 2. Morphology of cultured skin substitutes (CSS) in vitro. Shown are histological sections of biopsies taken at culture day 10 stained with toluidine blue. A. Control CSS. B. Vascular endothelial growth factor-modified CSS. In both panels the epidermis is at the top of the section.

Engraftment, Contraction, and VEGF Expression After Grafting to Mice

Immunohistological evaluation of grafted epidermal regions for the presence of human cells was used to confirm engraftment at 14 weeks after surgery. An example of positive staining for the human leukocyte antigen complex HLA-ABC is shown in Figure 3A. Positive HLA-ABC staining was seen in 86.7% (13/15) of $VEGF^+$ grafts at 14 weeks after surgery. By comparison, 58.3% (7/12) of control grafts showed positive staining for HLA-ABC.

Figure 4 shows light microscopy of sections of healed cultured skin. In both control and $VEGF^+$ grafts a well-stratified epidermis is present, and evidence of vascularization is observed. The total thickness of the healed grafts ranged from approximately 300 to 600 μm, with no significant group differences between control and $VEGF^+$ CSS.

A quantitative analysis of wound area was performed to assess graft contraction (Figure 5). Values are presented as percent original wound area; greater values indicate lower levels of contraction, which reflect greater graft stability. Significantly higher percent original wound area values were measured in the $VEGF^+$ grafts compared with controls from 5 to 14 weeks after surgery (Figure 5).

Retroviral VEGF mRNA expression was detectable in all $VEGF^+$ grafts excised from mice (data not shown). Even up to 14 weeks after surgery, retroviral VEGF mRNA expression was detected in HLA-ABC-positive $VEGF^+$ CSS examined by Northern blot hybridization (Figure 6). Although the levels of expres-

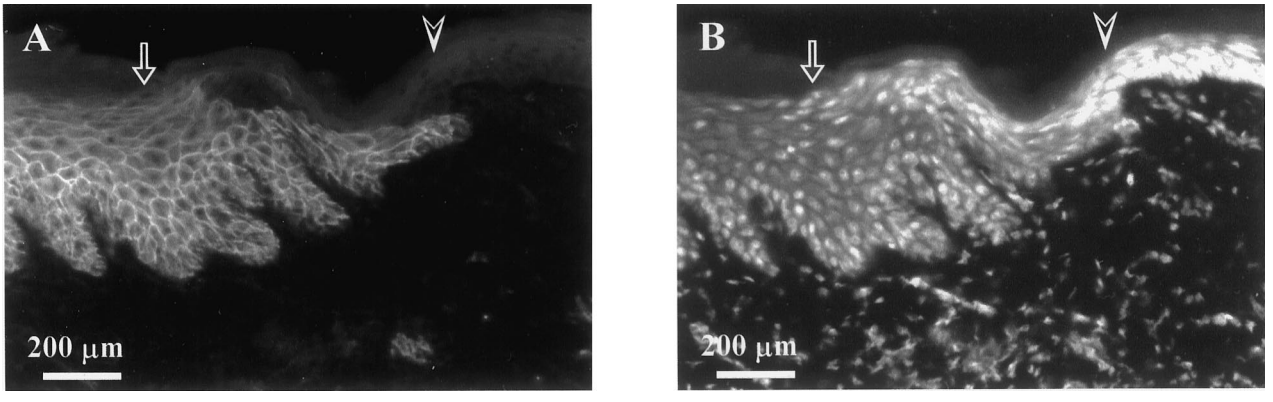

Figure 3. Confirmation of engraftment by identification of human keratinocytes in grafted cultured skin substitutes. A. HLA-ABC immunostaining of a vascular endothelial growth factor-modified graft excised at 14 weeks after surgery. Note positive epidermal staining (arrow) on the left side of the field; bordering mouse skin (arrowhead) is found on the right side. B. Propidium iodide staining of cell nuclei in the same section allows visualization of epidermal and dermal cells of both human and mouse origin.

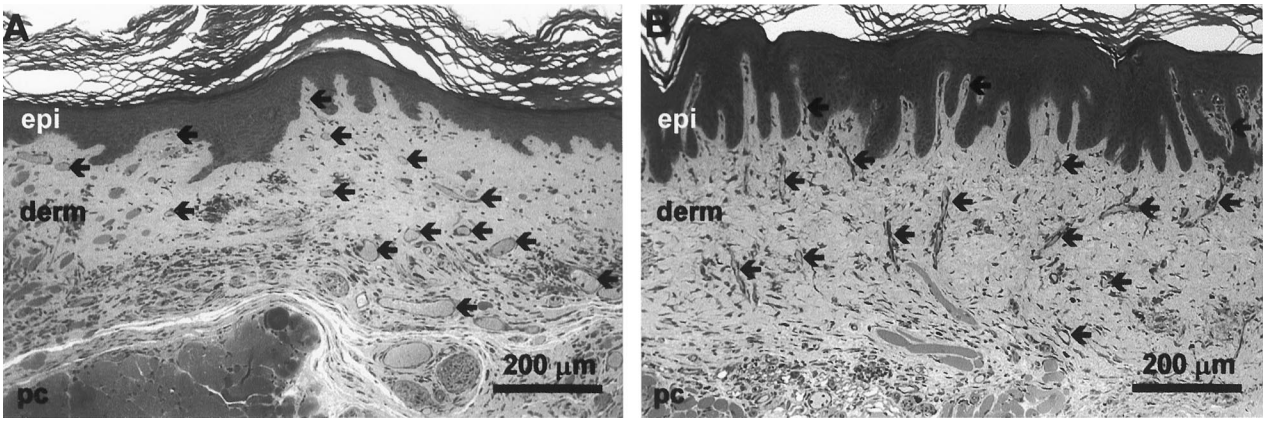

Figure 4. Histologies of cultured skin substitutes (CSS) at 14 weeks after grafting. Shown are light micrographs of histological sections stained with toluidine blue. A. Control CSS. B. vascular endothelial growth factor-modified CSS. The arrows point to some examples of dermal blood vessels. *epi*, epidermis; *derm*, dermis; *pc*, panniculus carnosus.

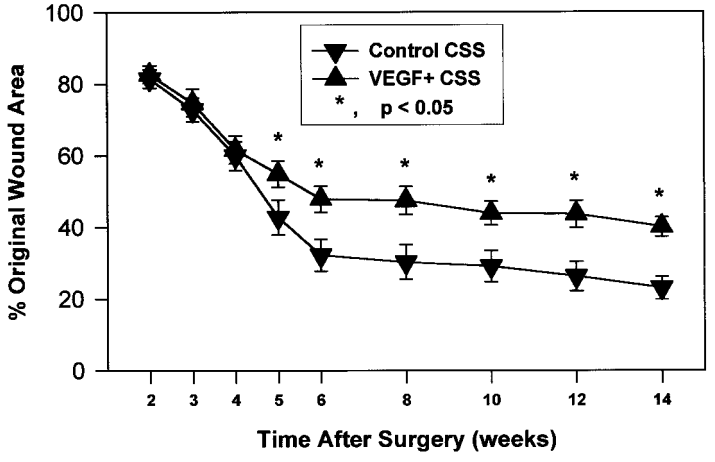

Figure 5. Wound area measurements in control and VEGF$^+$ CSS at multiple time points after grafting. Values were expressed as percent original wound area (100% at time of surgery). Mean values are plotted; error bars = ± SEM. Statistically significant differences between groups were found at weeks 5 through 14 after grafting ($P < 0.05$). *CSS*, cultured skin substitutes; *VEGF*$^+$, vascular endothelial growth factor-modified.

sion of the two retroviral transcripts is roughly equivalent in VEGF$^+$ CSS before grafting, only the 2.9 kb transcript from the CMV promoter/enhancer is detected at 14 weeks after transplantation to mice (Figure 6). Despite continued expression of the retroviral VEGF transcript in VEGF$^+$ CSS after grafting, human VEGF protein was not detectable in the serum of mice at 1, 2, or 4 weeks after surgery (data not shown).

Vascularization of CSS After Grafting

For evaluation of vascularization, murine endothelial cells in sections of grafts excised from mice were stained with an antibody against murine PECAM-1. Examples of PECAM-1 immunostaining are shown in Figure 7A. Quantitative image analysis was used to measure the percent area staining positive for PECAM-1. If this analysis was performed on the entire dermal regions of grafted CSS (Figure 7A, solid white outlines), there were significantly higher densities of endothelial cells in the VEGF$^+$ grafts compared with controls at 1 week after grafting (Figure 7B). The differences in the values for entire dermal regions were not statistically significant at later time points. However, if the analysis was focused on just the upper part of the dermis (Figure 7A, dashed white lines), differences in endothelial cell density were seen which persisted up to 4 weeks after grafting and were even seen at 14 weeks after grafting (Figure 7C). This indicates a difference in distribution of blood vessels

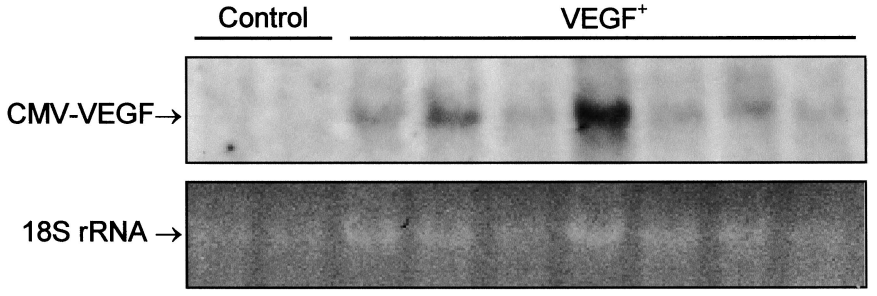

Figure 6. Expression of retroviral VEGF RNA 14 weeks after grafting to mice. RNA samples were prepared from cultured skin substitutes that showed positive HLA-ABC staining, indicating engraftment of human keratinocytes. A Northern blot containing total RNA (5 μg/lane) from control (left 2 lanes) and VEGF$^+$ (right 7 lanes) hybridized to a VEGF cDNA probe is shown (top). The position of the 2.9 kb VEGF transcript originating from the retroviral CMV promoter/enhancer is shown (arrow, top). A photograph of the ethidium bromide-stained gel showing the 18S ribosomal RNA band is shown (bottom). *VEGF*, vascular endothelial growth factor

between VEGF$^+$ and control grafts at later time points after surgery, with greater densities of endothelial cells near the dermal-epidermal junction in VEGF$^+$ CSS, although differences in overall vascularization were not present.

DISCUSSION

Genetic modification resulted in greatly increased expression of VEGF mRNA in cultured keratinocytes and in CSS prepared with modified cells. High levels of VEGF mRNA expression were maintained in the VEGF$^+$ CSS through the in vitro culture period, and VEGF protein secretion was significantly higher than in control CSS at the time of grafting to mice. The VEGF protein secreted by keratinocytes modified with the L-CVEGF-SN vector was previously shown to be bioactive in a microvascular endothelial cell growth assay.[26] The level of VEGF protein measured in media conditioned by control keratinocytes in this study, 0.9 ng/ml, is similar to levels observed in human keratinocyte cultures by other investigators.[28] This concentration roughly corresponds to the level reported to be required in vitro for half-maximal stimulation of DNA synthesis in endothelial cells (~1.0 ng/ml).[27] Maximal response to VEGF in vitro has been reported at concentrations of approximately 10 ng/ml, the level at which saturation of binding to human dermal microvascular endothelial cells occurs.[28] Thus, a five-fold increase in VEGF secretion in genetically modified keratinocytes, as was observed in the present study, would be expected to have a significant effect on the growth of microvascular endothelial cells. The level of VEGF secreted by control CSS at the time of grafting to mice, 0.19 ng/ml, is below the minimal effective concentration reported for stimulation of endothelial cells (~0.3 ng/ml).[28] Preparation of CSS with VEGF-modified keratinocytes resulted in a roughly 38-fold increase in VEGF secretion in vitro, with an average concentration of 7.2 ng/ml. Thus, genetic modification resulted in a level of VEGF secretion sufficient to elicit a mitogenic response in microvascular endothelial cells in vitro. Maintenance of this level of VEGF production in VEGF$^+$ CSS after grafting could hypothetically stimulate enhanced growth of endothelial cells in vivo.

The present study confirmed previous results[26] showing enhanced vascularization of VEGF$^+$ CSS after grafting. This study not only examined vascularization of the upper dermis, as was done previously for early time points after grafting,[26] but also quantified vascularization of the entire dermal region. Although the difference between control and VEGF-modified grafts in vascularization of the entire dermal regions was significant 1 week after surgery, similar levels were seen after stable engraftment was completed. This suggests that that VEGF overexpression acts by accelerating early graft vascularization. Although the overall level of vascularization was the same for control and VEGF-modified grafts at later time points after surgery, the distribution of vessels was different. There were more vessels close to the dermal-epidermal junction in VEGF-modified grafts. This observation is most likely because of the chemotactic effects[32,33] on the murine endothelial cells of a gradient of VEGF protein generated by VEGF-modified epidermal keratinocytes.

Expression of the retroviral VEGF gene was detected in grafted VEGF$^+$ CSS up to 14 weeks after grafting. Interestingly, the predominant retroviral VEGF transcript expressed at 14 weeks after surgery corresponds to the size predicted for expression from

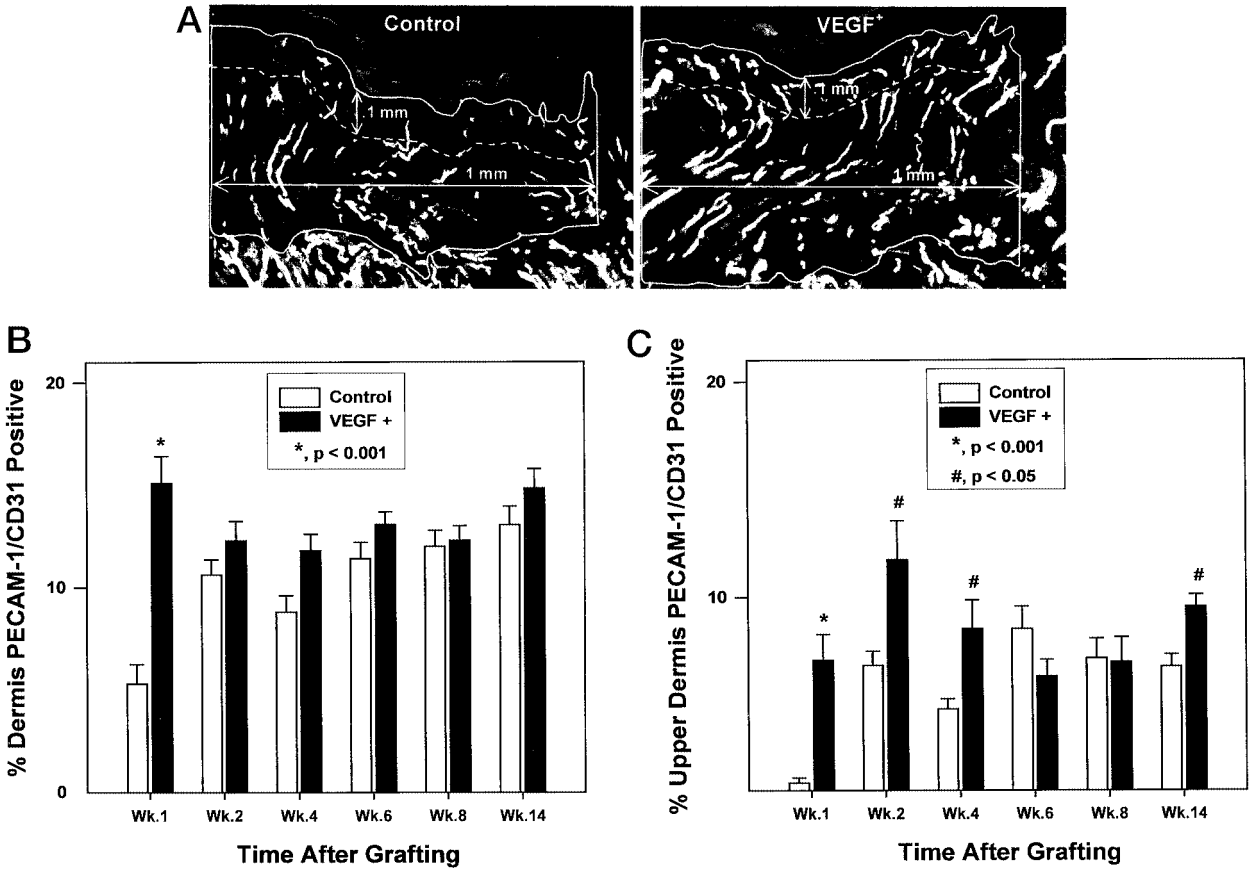

Figure 7. Evaluation of vascularization in control and VEGF-modified CSS after grafting. A. Examples of PECAM-1/CD31 immunostaining in sections of control (left) and VEGF-modified (right) CSS excised 14 weeks after grafting. Dermal regions corresponding to 1 mm graft lengths are outlined with solid white lines. The upper dermis, corresponding to a depth of 0.1 mm below the dermal/epidermal junction, is delimited here in each section by a dashed white line. Vascularization was analyzed by measuring the percent area of either whole dermal regions or upper dermal regions staining positive for PECAM-1/CD31. B. Quantitation of endothelial cell densities of entire dermal regions. Shown are mean values for each group at each time point after grafting; error bars = ± SEM. A statistically significant difference was found at 1 week after grafting ($P < 0.001$). C. Quantitation of endothelial cell densities of upper dermal regions (top 0.1 mm of dermis). Shown are mean values for each group at each time point after grafting; error bars = ± SEM. Statistically significant differences were found at 1 week ($P < 0.001$) and 2, 4, and 14 weeks ($P < 0.05$) after grafting. *CSS*, cultured skin substitutes; *VEGF*$^+$, vascular endothelial growth factor-modified; *Wk*, week.

the CMV promoter/enhancer. This suggests that the CMV promoter/enhancer is more effective for driving long-term expression of exogenous genes in grafted keratinocytes than the retroviral LTR promoter. Despite prolonged expression of the retroviral VEGF mRNA, human VEGF protein was below the level of detection by ELISA (~9 pg/ml) in the serum of grafted mice analyzed up to 4 weeks after surgery. This indicates that overexpression of VEGF in grafted CSS resulted in localized protein expression that did not reach significant levels in the circulation. However, the increased vascularization seen in VEGF$^+$ CSS suggests that the VEGF protein secreted by modified epidermal keratinocytes was able to penetrate the dermal/epidermal basement membrane and elicit a response from murine endothelial cells present in the wound bed.

CONCLUSIONS

Overexpression of VEGF in modified cultured skin resulted in reduced contraction after grafting. In addition, positive HLA-ABC immunostaining was seen in a greater percentage of VEGF-modified grafts compared with controls. Taken together, these results suggest that enhanced early vascularization can lead to greater graft stability and improved engraftment of cultured skin. Therefore, overexpression of

VEGF in genetically modified cultured skin substitutes can contribute to improved healing of full-thickness skin wounds in athymic mice. Because the impact on healing may be caused by accelerated early vascularization, future studies should examine ways to achieve short-term VEGF overexpression in modified CSS. Negative effects of VEGF overexpression in mice up to 14 weeks after grafting were not observed in this study; however, because VEGF has been postulated to play a role in tumor angiogenesis,[34,35,36] potential hazards of chronic VEGF overexpression cannot be ignored. Benefits to vascularization and wound healing may be obtained by increased VEGF expression in only the first few weeks after grafting. The use of weaker promoters or inducible vector systems should be explored to allow regulation of VEGF overexpression after grafting of genetically modified CSS.

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REFERENCES

1. Barret JP, Wolf SE, Desai MH, Herndon DN. Cost-efficacy of cultured epidermal autografts in massive pediatric burns. Ann Surg 2000;23:869–76.
2. Carsin H, Ainaud P, Le Bever H, et al. Cultured epithelial autografts in extensive burn coverage of severely traumatized patients: a five year single-center experience with 30 patients. Burns 2000;26:379–87.
3. Braye F, Pascal P, Bertin-Maghit M, Colpart JJ, Tissot E, Damour O. Advantages of using a bank of allogeneic keratinocytes for the rapid coverage of extensive and deep second-degree burns. Med Biol Eng Comput 2000;38:248–52.
4. Phillips TJ, Bhawan J, Leigh IM, Baum HJ, Gilchrest BA. Cultured epidermal autografts and allografts: a study of differentiation and allograft survival. J Am Acad Dermatol 1990;23:189–98.
5. Herndon DN. Perspectives in the use of allograft. J Burn Care Rehabil 1997;18:S6.
6. Wainwright D, Madden M, Luterman A, et al. Clinical evaluation of an acellular allograft dermal matrix in full-thickness burns. J Burn Care Rehabil 1996;17:124–36.
7. Stern R, McPherson M, Longaker MT. Histologic study of artificial skin used in the treatment of full-thickness thermal injury. J Burn Care Rehabil 1990;11:7–13.
8. Soejima K, Nozaki M, Sasaki K, Takeuchi M, Negishi N. Reconstruction of burn deformity using artificial dermis combined with thin split-skin grafting. Burns 1997;23: 501–4.
9. Horch RE, Debus M, Wagner G, Stark GB. Cultured human keratinocytes on type I collagen membranes to reconstitute the epidermis. Tissue Eng 2000;6:53–67.
10. Horch RE, Bannasch H, Kopp J, Andree C, Stark GB. Single-cell suspensions of cultured human keratinocytes in fibrin-glue reconstitute the epidermis. Cell Transplant 1998;7:309–17.
11. Hansbrough JF, Boyce ST, Cooper ML, Foreman TJ. Burn wound closure with cultured autologous keratinocytes and fibroblasts attached to a collagen-glycosaminoglycan substrate. JAMA 1989;262:2125–30.
12. Boyce ST, Greenhalgh DG, Kagan RJ, et al. Skin anatomy and antigen expression after burn wound closure with composite grafts of cultured skin cells and biopolymers. Plast Reconstr Surg 1993;91:632–41.
13. Kuroyanagi Y, Kenmochi M, Ishihara S, et al. A cultured skin substitute composed of fibroblasts and keratinocytes with a collagen matrix: preliminary results of clinical trials. Ann Plast Surg 1993;31:340–51.
14. Boyce ST, Goretsky MJ, Greenhalgh DG, Kagan RJ, Rieman MT, Warden GD. Comparative assessment of cultured skin substitutes and native skin autograft for treatment of full-thickness burns. Ann Surg 1995b;222:743–52.
15. Zacchi V, Soranzo C, Cortivo R, Radice M, Brun P, Abatangelo G. *In vitro* engineering of human skin-like tissue. J Biomed Mater Res 1998;40:187–94.
16. Caruso DM, Schuh WH, Al-Kasspooles MF, Chen MC, Schiller WR. Cultured composite autografts as coverage for an extensive body surface area burn: case report and review of the technology. Burns 1999;25:771–79.
17. Boyce ST, Kagan RJ, Meyer NA, Yakuboff KP, Warden GD. The 1999 Clinical Research Award: Cultured skin substitutes combined with Integra Artificial Skin™ to replace native skin autograft and allograft for the closure of excised full-thickness burns. J Burn Care Rehabil 1999;20:453–61.
18. Young DM, Greulich KM, Weier HG. Species-specific in situ hybridization with fluorochrome-labeled DNA probes to study vascularization of human skin grafts on athymic mice. J Burn Care Rehabil 1996;17:305–10.
19. Boyce ST. Cultured skin substitutes: a review. Tissue Eng 1996;2:255–66.
20. Boyce ST, Supp AP, Harriger MD, Greenhalgh DG, Warden GD. Topical nutrients promote engraftment and inhibit wound contraction of cultured skin substitutes in athymic mice. J Invest Dermatol 1995;104:345–9.
21. Morgan JR, Barrandon Y, Green H, Mulligan RC. Expression of an exogenous growth hormone gene by transplantable human epidermal cells. Science 1987;237:1476–9.
22. Vogt PM, Thompson S, Andree C, et al. Genetically modified keratinocytes transplanted to wounds reconstitute the epidermis. Proc Natl Acad Sci U S A 1994;91:9307–11.
23. Eming SA, Lee J, Snow RG, Tompkins RG, Yarmush ML, Morgan JR. Genetically modified human epidermis overexpressing PDGF-A directs the development of a cellular and vascular connective tissue stroma when transplanted to athymic mice—implications for the use of genetically modified keratinocytes to modulate dermal regeneration. J Invest Dermatol 1995;105:756–63.
24. Choate KA, Medalie DA, Morgan JR, Khavari PA. Corrective gene transfer in the human skin disorder lamellar ichthyosis. Nature Med 1996;2:1263–7.
25. Eming SA, Medalie DA, Tompkins RG, Yarmush ML, Morgan JR. Genetically modified human keratinocytes overexpressing PDGF-A enhance the performance of a composite skin graft. Hum Gene Ther 1998;9:529–39.
26. Supp DM, Supp AP, Bell SM, Boyce ST. Enhanced vascularization of cultured skin substitutes genetically modified to overexpress vascular endothelial growth factor. J Invest Dermatol 2000;114:5–13.
27. Conn G, Soderman DD, Schaeffer M-T, Wile M, Hatcher VB, Thomas KA. Purification of a glycoprotein vascular endothelial cell mitogen from a rat glioma-derived cell line. Proc Natl Acad Sci U S A 1990;87:1323–7.
28. Detmar M, Yeo K-T, Nagy JA, et al. Keratinocyte-derived vascular permeability factor (vascular endothelial growth factor) is a potent mitogen for dermal microvascular endothelial cells. J Invest Dermatol 1995;105:44–50.
29. Boyce ST, Ham R. Cultivation, frozen storage and clonal growth of normal human epidermal keratinocytes in serum-free media. J Tissue Cult Methods 1985;9:83–93.

30. Boyce ST, Foreman TJ, English KB, et al. Skin wound closure in athymic mice with cultured human cells, biopolymers and growth factors. Surgery 1991;110:866–76.
31. Swope VB, Supp AP, Greenhalgh DG, Warden GD, Boyce ST. Expression of insulin-like growth factor-1 by cultured skin substitutes does not replace the physiologic requirement for insulin in vitro. J Invest Dermatol 2001;116:650–7.
32. Yoshida A, Anand-Apte B, Zetter BR. Differential endothelial migration and proliferation to basic fibroblast growth factor and vascular endothelial growth factor. Growth Factors 1996;13:57–64.
33. Senger DR, Ledbetter SR, Claffey KP, Papadopoulos-Sergiou A, Peruzzi CA, Detmar M. Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the alpha v/beta 3 integrin, osteopontin, and thrombin. Am J Pathol 1996;149:293–305.
34. Claffey KP, Brown LF, del Aguila LF, et al. Expression of vascular permeability factor/vascular endothelial growth factor by melanoma cells increases tumor growth, angiogenesis, and experimental metastasis. Cancer Res 1996;56: 172–81.
35. Larcher F, Robles AI, Duran H, et al. Up-regulation of vascular endothelial growth factor/vascular permeability factor in mouse skin carcinogenesis correlates with malignant progression state and activated H-ras expression levels. Cancer Res 1996;56: 5391–6.
36. Larcher F, Murillas R, Bolontrade M, Conti CJ, Jorcano JL. VEGF/VPF overexpression in skin of transgenic mice induces angiogenesis, vascular hyperpermeability and accelerated tumor development. Oncogene 1998;17: 303–11.