Stroma Formation and Angiogenesis by Overexpression of Growth Factors, Cytokines, and Proteolytic Enzymes in Human Skin Grafted to SCID Mice

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Reorganization of skin during wound healing, inflammatory disorders, or cancer growth is the result of expression changes of multiple genes associated with tissue morphogenesis. We wanted to identify proteins involved in skin remodeling and select those that may be targeted for agonistic or antagonist therapeutic approaches in various disease processes. Full-thickness human skin was grafted to severe combined immunodeficient mice and injected intradermally with 38 different adenoviral vectors inserted with 37 different genes coding for growth factors, cytokines, proteolytic enzymes and their inhibitors, adhesion receptors, oncogenes, and tumor suppressor genes. Responses were characterized for infiltration of inflammatory cells, vascular density, matrix formation, fibroblast-like cell proliferation, and epidermal hyperplasia. Of the 17 growth factor vectors, 16 induced histological changes in human skin. Members of the VEGF and angiopoietin families induced neovascularization. PDGFs and TGF-βs stimulated connective tissue formation, and the chemokines IL-8 and MCP-1 attracted inflammatory neutrophils and monocytes, respectively. The serine protease uPA induced a vascular response similar to that of VEGF. Vectors with adhesion receptors, oncogenes and tumor suppressor genes had, with few exceptions, little effects on skin architecture. The overall results suggest that adenoviral vectors can effectively remodel the architecture of human skin for studies in morphogenesis, inflammatory skin disorders, wound healing, and cancer development. ***Keywords: adenovirus/human skin graft/growth factor/skin remodelling. J Invest Dermatol 120:683–692, 2003***

Human skin has a unique architecture and is very different from skin of laboratory animals, including mice. It has a multilayered epidermis with keratinocytes, melanocytes, and Langerhans cells, and a thick, well-vascularized dermis. By comparison, the mouse skin has only 3–4 keratinocyte layers and a thin dermis. Studies of tissue reorganization in wound healing, cancer and inflammation are difficult to compare between mice and humans. We therefore developed a human skin/SCID mouse chimera model in which full-thickness human skin is grafted to immunodeficient mice (Juhasz *et al*, 1993). The human epidermis remains unchanged, whereas the dermis becomes infiltrated by murine blood vessels and some murine inflammatory cells (Yan *et al*, 1993). The human skin graft maintains its unique architecture throughout the lifetime of the animal, and it has successfully been used for studies of wound healing (Juhasz *et al*, 1993; Sylvester *et al*, 2000), inflammation (Oka *et al*, 2000), carcinogenesis (Atillasoy *et al*, 1998), tumor growth and metastasis (Sauter *et al*, 1999a). Increasingly, this model is being used for pre-clinical studies to predict the outcome in human skin following therapeutic intervention.

Pathological changes in skin are associated with profound changes in expression of a variety of genes that are associated with skin morphogenesis. Correlative studies testing for protein or mRNA expression have provided information of potentially important agents in skin remodeling processes. Injection of recombinant proteins into skin or their topical application to wounds has provided preliminary information on their potential

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for wound repair and skin remodeling. However, recombinant proteins are rapidly degraded and need frequent reapplications to have lasting effects. Therefore, gene therapy approaches are attractive alternatives for studies of protein functions. Adenoviral vectors readily infect most human skin cells and are widely accepted for efficient gene transduction (Setoguchi *et al*, 1994). Adenovirus-mediated gene transfer results in protein expression within 24 h with a maximum at 72 h (Berking *et al*, 2001a, 2001b; Nesbit *et al*, 2001a; Nesbit *et al*, 2001b). Depending on the proliferation of transduced cells and a potential immune response, forced gene expression in human skin grafts may last up to 3 weeks.

Wound healing, cutaneous inflammatory disorders such as psoriasis and scleroderma, or skin cancer formation are complex processes that are difficult to dissect into gene expression changes that cause tissue remodeling. However, investigations of these conditions are often hampered by the lack of adequate animal models, where single parameters can be experimentally modified. In this study, we have transduced human skin grafted to SCID mice with adenoviral vectors without prior wounding. We chose human skin for injection of adenoviral vectors instead of mouse skin because the mouse dermis is generally too thin for even small quantities of fluid requiring subdermal or subcutaneous deposition of the vector(s). The proteins under study were selected for their potential involvement in skin morphogenesis, wound healing, inflammation, and cancer. The full-thickness well-healed skin grafts were injected with adenoviral vectors for growth factors, chemokines, proteolytic enzymes, enzyme inhibitors, adhesion receptors, proto-oncogenes, tumor suppressor genes, several antagonists to growth factors, growth factor receptors or a receptor for a protease. The resulting remodeling of human skin demonstrates the utility of the presented approach for further in-depth studies of a variety of dermatological conditions. Inflammation, neovascularization, and connective tissue formation could be induced. These pathobiological reaction patterns are hallmarks of skin diseases such as psoriasis and scleroderma. The endogenous overexpression of genes produced strong responses.

MATERIALS AND METHODS

Adenovirus preparation Serotype 5, replication defective adenovirus vectors were used in this study. The backbone of adenoviruses had either E1 or E1 and E3 deleted regions and all the transgenes were driven by the cytomegalyvirus promoter. The parental adenoviruses include pMJ17 (E1 deleted), d17001, Adeasy-1 and in340 (E1 and E3 deleted) viruses. The viruses included in the study are listed in **Table 1**. All viruses were prepared from 293 cells, purified by double CsCl density centrifugation (Graham and Prevec, 1995) and stored in 50% glycerol containing 10 mM Tris, pH 8.0, 0.1 mM EDTA, and 0.1% BSA at − 80°C. Virus titers were determined by measuring absorbance at 260 nm and by plaque assay as described before (Satyamoorthy, *et al*, 1997). Virus preparations were dialyzed extensively against phosphate buffered saline before injection into animals. Expressions of all transgenes were measured as previously described (see references in **Table 1**) by Western analysis, ELISA, RIA, or Northern analysis wherever applicable. All animal experiments were approved by the IACUC (institutional review board for all animal experiments).

Human skin grafting C.B.-17 SCID mice between 4 and 6 wk of age were used for grafting of human foreskins from newborns (Atillasoy *et al*, 1998; Berking *et al*, 2001a, b). The 1.5–2 cm^2 full-thickness skin specimens were placed into size-matched wound beds on the dorsal torso of the mice. Each mouse received a separate graft, which was allowed to heal for 4–6 wk prior to injection of adenoviral vectors.

Gene transfer Human skin grafts were injected with 100 μL of virus suspension containing 5×10^8 plaque forming units (pfu) in phosphate-buffered saline (PBS). The viruses were injected intradermally with Hamilton gas-light syringes with 30-gauge needles (Reno, NY). Four to 15 grafts were injected with the same vector. For each set of 5 viruses one control *lac*Z group was included. Intradermal injection was confirmed by presence of an epidermal elevation. The area of injection was marked with indelible ink. Starting three d following injection, mice were sacrificed and grafts were harvested for histological analysis.

Tissue processing and histological evaluation The human skin grafts were dissected free from surrounding murine tissue and bisected perpendicular to the long axis of the graft. The grafts were fixed overnight in 10% neutral buffered formalin at 4°C, and half were paraffin embedded for histological assessment. The other half of the grafts were cryopreserved after dehydration in 20% sucrose overnight at 4°C.

Serial sections (5 μm) from each of the formalin-fixed, paraffin-embedded grafts were cut onto slides and stained with hematoxylin and eosin (H&E). The degree of acute inflammatory response, vascular response, connective tissue and epidermal reaction to the adenovirus-mediated transgenes was assessed independently by 2 observers (C.J.G. and J.L.). Uniform staining of sections was evaluated at low magnification (X100). Individual counts for inflammatory cells such as granulocytes, lymphocytes and monocytes, vessels, and fibroblasts were then taken at high magnification (X400). Counts were quantified as the number of structures/mm^2 (1 high power field = 0.0496 mm^2). Each count was performed independently on five representative high power fields by the two investigators. For evaluation of collagen in the skin tissues, Masson's trichrome stain was performed on formalin-fixed paraffin-embedded sections according to standard protocols. Collagen deposition was quantified using Image-Pro^{Æ}Plus analyzing software (Media Cybernetics, L.p., Silver Spring, MD). With the help of this image analyzing software, the collagen-specific color densitometric measurements were converted to optical density. Epidermal hyperplasia was evaluated by determining the number of papillae/mm epidermal surface and measuring epidermal thickness. Changes in skin were scored negative if both observers agreed the effect was below a prospectively set threshold for each evaluation criteria using skin injected with the *lacZ* as GFP adenoviral vectors and PBS (phosphate-buffered saline) as controls. To distinguish between human and mouse cells, sections were stained with Hoechst dye that provides characteristic staining for human vs. mouse cells (Atillasoy *et al*, 1998; Berking *et al*, 2001a, 2001b). Statistical comparisons between test group and control groups, lacZ/Ad5 or GFP/Ad5-injected skin, were done by Student's *t* test.

For β-Galactosidase histochemistry, cryosections on slides were fixed in 0.5% glutaraldehyde for 10 min, followed by rinsing twice in 1 mM $MgCl_2$ for 10 min each. The slides were then incubated for 1 h in the dark at 37°C in 1 mM $MgCl_2$, 20XKCl in PBS solution at pH 7.4, and β-galactosidase (5-bromo-4-chloro-3-indolyl β-galactopyranoside) at a final concentration of 1 mg/ml. They were then washed 3 times in tap water and coverslipped with mounting medium for microscopic evaluation.

Immunohistochemistry Immunohistochemistry was performed on serial sections using an avidin-biotin-peroxidase system kit (Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine tetrahydrochloride (Sigma) or 3-amino-9-ethylcarbazole (Vector) as chromogens. Antigens in the formalin-fixed tissues were retrieved by trypsin digestion at 37°C or microwave heat treatment in citrate buffer. Cryostat sections cut between 6 and 8 μm on a slide were air-dried and fixed in ice-cold acetone for 10 min prior to incubation with the primary antibodies in a humidified chamber at 4°C overnight or at room temperature for 1–2 h. Non-specific binding was blocked with 10% normal horse or 10% normal goat serum. Primary monoclonal antibodies used in this study were: mouse anticollagen type IV, antimurine PECAM-1, and antismooth muscle actin antibodies. A mouse IgG1 isotype antibody (P3) was used as negative control for each staining. Between each incubation step, slides were rinsed twice in PBS for 3–5 min. A biotin-labeled antimouse secondary antibody was applied for 30 min at room temperature followed by incubation with a preformed avidin-biotinylated enzyme complex for 30 min. After color development by adding the chromogen and counterstaining with Mayer's hematoxylin (Sigma), sections were mounted and evaluated under a light microscope.

RESULTS

Thirty-eight adenoviral vectors of different gene groups were prepared for injection into human skin grafts. The largest group of human proteins was represented by growth factors and cytokines followed by adhesion receptors, proteolytic enzymes, their inhibitors, the antisense uPA receptor, and oncogenes and tumor suppressor genes (**Table 1**). Intradermal injection of the 100 μL virus suspension created an elevation (papule) that disappeared within 1–2 days. Staining for β-galactosidase 3 days after injection of the *lac*Z control vector (*lac*Z/Ad5) revealed strong positivity

Table 1. Adenoviral Vectors For Over-Expression of Proteases, Inhibitors, Adhesion Receptors, Oncogenes, Tumor Suppressor Genes, and Miscellaneous Human Genes[a]

Gene group	Abbreviation	Adenovirus	Description	Reference (source)[c]
Growth factors and cytokines[b]				
Basic fibroblast growth factor (18 kDa)	bFGF	dl7001	E1$^-$, E3$^-$, Ad5	Nesbit *et al*, 1999, Berking *et al*, 2001a
Basic fibroblast growth factor (18, 22, 23, 24 kDa)	bFGF(l)	dl7001	E1$^-$, E3$^-$, Ad5	Nesbit *et al*, 1999, Berking *et al*, 2001a
Platelet-derived growth factor A	PDGF-A	dl7001	E1$^-$, E3$^-$, Ad5	(Herlyn Lab)
Platelet-derived growth factor B	PDGF-B	dl7001	E1$^-$, E3$^-$, Ad5	Liechty *et al*, 1999; Nesbit *et al*, 2001a
Insulin-like growth factor-1	IGF-1	AdEasy-1	E1$^-$, E3$^-$, Ad5	Satyamoorthy *et al*, 2001a, b
Monocyte chemoattractant protein-1	MCP-1	dl7001	E1$^-$, E3$^-$, Ad5	Nesbit *et al*, 2001b
Interleukin-8	IL-8	dl7001	E1$^-$, E3$^-$, Ad5	Oka *et al*, 1999
Vascular endothelial cell growth factor-121	VEGF	dl7001	E1$^-$, E3$^-$, Ad5	Herlyn Lab
Vascular endothelial cell growth factor C	VEGF-C	AdEasy-1	E1$^-$, E3$^-$, Ad5	(Herlyn/Detmar Labs.)
Vascular endothelial cell growth factor D	VEGF-D	AdEasy-1	E1$^-$, E3$^-$, Ad5	(Herlyn/Detmar Labs.)
Pleiotrophin[d]	Pleiotrophin	dl7001	E1$^-$, E3$^-$, Ad5	Satyamoorthy *et al*, 2000
Transforming growth factor beta-1, active porcine	TGF-β1		E1$^-$, E3$^-$, Ad5	Lee *et al.*, 1998, Berking *et al*, 2001a
Transforming growth factor beta-3	TGF-β3	AdEasy-1	E1$^-$, E3$^-$, Ad5	(Cromblehome Lab.)
Hepatocyte growth factor/Scatter factor	HGF/SF	dl7001	E1$^-$, E3$^-$, Ad5	Phaneuf *et al*, 2000
Angiopoietin-1	Ang-1	in340	E1$^-$, E3$^-$, Ad5	Regeneron
Endothelin-3	ET-3	AdEasy-1	E1$^-$, E3$^-$, Ad5	(Herlyn Lab)
Stem cell factor	SCF	AdEasy-1	E1$^-$, E3$^-$, Ad5	(Herlyn Lab)
Proteolytic enzymes, inhibitors, and antagonists				
Matrix metalloproteinase-9	MMP-9	dl7001	E1$^-$, E3-Ad5	(Herlyn Lab.)
Urokinase type plasminogen activator	uPA	pJM17	E1$^-$, Ad5	Nagai *et al*, 1999
Plasminogen activator inhibitor-1	PAI-1	pJM17	E1$^-$, Ad5	Carmeliet *et al*, 1997
Antisense urokinase type plasminogen activator receptor	ASuPAR	pJM17	E1$^-$, Ad5	Mohan *et al*, 1999
Tissue inhibitor of matrix metalloproteinase-1	TIMP-1	pJM17	E1$^-$, Ad5	Baker *et al*, 1996
Adhesion receptors				
N-Cadherin[d]	N-Cadherin	dl7001	E1$^-$, E3$^-$, Ad5	Li *et al*, 2001
E-Cadherin[d]	E-Cadherin	dl7001	E1$^-$, E3$^-$, Ad5	Hsu *et al*, 2000
β3-Integrin[d]	β3	dl7001	E1$^-$, E3$^-$, Ad5	Hsu *et al*, 1998
α2 Integrin[d]	α2	dl7001	E1$^-$, E3$^-$, Ad5	Nesbit *et al*, 2001a
α5 Integrin[d]	α5	dl7001	E1$^-$, E3$^-$, Ad5	Nesbit *et al*, 2001a
Melanoma cell adhesion molecule (MUC18, CD146, S-Endo-1)[e]	Mel-CAM	AdEasy-1	E1$^-$, E3$^-$, Ad5	Satyamoorthy *et al*, 2001b
Oncogenes, tumor suppressor genes and others				
Human papillomavirus type 16-E6 oncogene[d]	HPV16-E6	dl7001	E1$^-$, E3$^-$, Ad5	Prabhu *et al*, 1998
Human papillomavirus type 16-E7 oncogene[d]	HPV16-E7	dl7001	E1$^-$, E3$^-$, Ad5	(Eck Lab.)
Tumor suppressor protein p16^{INK4A}[d]	p16^{INK4A}	dl7001	E1$^-$, E3$^-$, Ad5	Mitra *et al*, 1999
Tumor suppressor protein p21$^{Wafl/cip1}$[d]	p21$^{Wafl/cip1}$	pJM17	E1$^-$, Ad5	Prabhu *et al*, 1996
Tumor suppressor protein p53[f]	p53	pJM17	E1$^-$, Ad5	Blagosklonny *et al*, 1996
Antisense cyclin D1 (AS)[d]	Cyclin D1 (AS)	dl7001	E1$^-$, E3$^-$, Ad5	Sauter *et al*, 1999b
Cellular homologue of avian myelocytomatosis virus oncogene[f]	*c-myc*	pMJ17	E1$^-$, Ad5	Mitchell *et al*, 1999
Fibroblast growth factor binding protein	FGF-BP	dl7001	E1$^-$, E3$^-$, Ad5	Sauter *et al*, 2001
Control vectors				
LacZ	*LacZ*	dl7001	E1$^-$, E3$^-$, Ad5	Satyamoorthy *et al*, 1997
Green fluorescence protein[d]	GFP	dl7001	E1$^-$, E3$^-$, Ad5	De Martin *et al*, 1997

[a] CMV promoter was used for all adenoviral constructs.
[b] All genes were of human origin unless otherwise indicated.
[c] References refer to characterization of the adenoviral vector.
[d] Reactivity in human skin was similar to the *LacZ*/Ad5 vector. Therefore, results in figures are not listed.
[e] Induction of angiogenesis at 195 ± 12 vessels/mm^2.
[f] Induction of hyperplasia at 0.17 mm/mm^2. *c-myc*/Ad5 also induced thrombosin at 75 vessels/mm^2.

in the dermis, whereas the epidermal cells were not stained (not shown). A mild inflammatory infiltrate containing monocytes/macrophages and neutrophils was invariably seen 3 d after adenovirus injection regardless of the type of the vector, which was not seen after the injection of PBS. Nine of the 17 growth factors and cytokines induced macroscopic changes in the human skin that were first visible 48–72 h after injection of the adenoviral vectors. The clinical responses were characterized as swelling (PDGF-A, PDGF-B, VEGF-D, TGF-β_1, TGF-β_3), erythema (VEGF, VEGF-C) and/or edema (VEGF, MCP-1, IL-8) and lasted for one wk and up to 8 wk (**Table 2**). Histological changes peaked between d 3 and 7 after injection and lasted for a maximum of 6 wk. Due to the episomal nature of adenovirus-mediated gene expression, human proteins could be detected up to 2 wk (Nesbit *et al*, 2001b). After 10–14 d, histological responses began to decrease. The longest lasting responses were seen for PDGF-B and VEGF. Most likely, TGF-β_1 also induced long-lasting changes. However, injection of 5×10^8 pfu TGF-β_1/Ad5, lead to the death of animals on d 10 (Berking *et al*, 2001b). The adenoviral vector for the growth factor pleiotrophin did not induce any changes (Satyamoorthy *et al*, 2000). In addition, all adhesion receptors, N-cadherin, E-cadherin, β3 integrin, α2 integrin, α5 integrin, MUC18 (**Table 1**) did not induce any changes in skin with the exception of a moderate angiogenic response by Mel-CAM. Of the oncogenes and tumor suppressor genes listed in **Table 1**, only p53 and c-myc induced mild hyperplasia and thrombosis.

Table 2. Response in human skin grafts to adenoviral vector-mediated over-expression of growth factors, chemokines and proteolytic enzymes and their inhibitors.

Adenoviral vector[a]	No. of human skin grafts[b]	Clinical response[c]	Histological response[d]	
			Peak	Duration
bFGF	12	NR[e]	14	42
PDGF-A	4	7	3–5	28
PDGF-B	15	21	3–5	42
IGF-1	6	NR	3–7	21
SCF	8	NR	3–7	21
ET-3	8	NR	3–7	21
HGF/SF	4	NR	3	3
VEGF	10	42	3–5	60
VEGF-C	4	7	3–7	7[f]
VEGF-D	4	14	3–7	7[f]
Ang-1	4	NR	3–7	7[f]
TGF-β_1	12	>10[g]	3–7	10[g]
TGF-β_3	6	7	3–7	14
IL-8	8	14	3	8-14
MCP-1	10	10	3	8-14
uPA	8	NR	3–7	7[f]
PA1-1	4	NR	3–7	7[f]
ASuPAR	4	NR	3–7	7[f]
MMP-9	4	NR	3–7	7[f]
TIMP-1	4	NR	3[h]	3
lacZ[i]	15	NR	3[h]	3
GFP[i]	15	NR	3[h]	3

[a]5 X 10^8 pfu in 100 µl PBS were injected intradermally.
[b]Each human skin graft was cut between 1 and 2 cm^2 and was injected with a single vector. At the end of each experiment, mice were sacrificed and the grafts were examined histologically and immunohistologically.
[c]Days when changes in color, swelling, consistency were noted.
[d]Days of histological changes described in Figure
[e]NR, no visible response.
[f]Maximal observation time was day 7.
[g]Maximal observation time because animals died between 10 and 14 days after injection even if 10-fold lower concentration of virus was injected.
[h]Response was restricted to mild and transient inflammatory cell infiltrate.
[i]Negative controls.

Growth factors induced new blood vessels, but extent and dynamics of induction varied. bFGF did not induce blood vessels within 3 d (**Fig 1*A***), but it showed strong angiogenic activities after three injections at weekly intervals (Berking *et al*, 2001a). Both PDGF-A and PDGF-B were angiogenic, as were SCF and ET-3, whereas neither IGF-1 nor HGF/SF induced new blood vessels. Whereas the peak induction of stroma formation by PDGF-B was within 3 to d after injection of the adenoviral vector (**Table 2**), the angiogenic response was delayed by several days apparently due to the PDGF-B-mediated stimulation of VEGF (Nesbit *et al*, 2001b). VEGF is the strongest angiogenic growth factor inducing an intense erythema within 3 d (**Fig 2*B***) when compared to *LacZ*/Ad injected skin (**Fig 2*A***). The skin was severely swollen for two wk (**Fig 2*C*,*D***), and the edema subsided by day 28 (**Fig 2*E***). Its appearance changed by 60 d to a pink glazed color (**Fig 2*F***). Histologically, VEGF-induced vessels were large and dilate with the endothelial cells on day 3 being swollen and bulging into the lumen (**Fig 2*G***). The dermis developed edema, and minimal hypoplasia of the epidermis was seen. Endothelial cell proliferation continued for at least 4 wk. At that time, the vascular spaces decreased in size. Injection of *LacZ*/Ad5 or GFP/Ad5 induced no increase in blood vessels and resulted in only mild dermal edema with mild mixed inflammatory infiltrate (**Fig 2*H***). At 60 d the VEGF-injected grafts showed a reduction in the number of vessels with a thinning of the epidermis and loss of ridge structures indicative of scarring. The other members of the VEGF family tested, VEGF-C and VEGF-D had similar but milder effects on vessel formation. Ang-1 and TGF-β_1 also showed moderate induction (Berking *et al*, 2001b), whereas TGF-β_3 and the chemokines IL-8 and MCP-1 showed little effects on vessel numbers when compared to control vectors. Indirect effects may lead to vessel formation by factors that are not angiogenic on their own. For example, MCP-1 expressed by melanoma cells attracts monocytes/macrophages that can secrete angiogenic cytokines such as TNF-α (Nesbit *et al*, 2001a).

The serine protease uPA induced strong vessel formation (**Fig 3*B*,*C***), which was similar to VEGF when expressed as number of vessels/mm^2. However, the vessels were smaller and there was no erythema visible in the skin. Few vessels are seen when LacZ/Ad5 was injected into the dermis (**Fig 3*D***). Neither PAI-1, an antisense constructs for the uPA receptor, MMP-9, or TIMP showed angiogenic activities.

Dermal stroma reactions were characterized by proliferation of noninflammatory cells such as fibroblasts, smooth muscle cells and pericytes (**Fig 1*B***). bFGF, PDGF-A, PDGF-B, and IGF-1 all stimulated stromal cells, whereas SCF, ET-3, HGF/SF, VEGF, VEGF-C, VEGF-D, and Ang-1 showed little or no induction. Strong inducers of fibroblastic cells were both TGF-β_1 and TGF-β_3, whereas the chemokines IL-8 and MCP-1 showed no activities. None of the two proteolytic enzymes, their inhibitors or the antisense uPAR vector induced stromal cells.

Induction of matrix formation coincided with stimulation of noninflammatory cells by growth factors and cytokines (**Fig 1*C***). Matrix deposition, measured after trichrome staining for all collagens, was strongest for those vectors that stimulated noninflammatory cells. Stimulatory activities had bFGF, PDGF-A, PDGF-B, IGF-I, VEGF-D, and TGF-β_1 and TGF-β_3. Several growth factors that were angiogenic did not induce a significant connective tissue reaction, including VEGF, VEGF-C, SCF or ET-3. Similarly, uPA that was highly angiogenic did not induce a significant proliferation of fibroblasts nor did it induce matrix formation. An example for induction of matrix is given for IGF-1

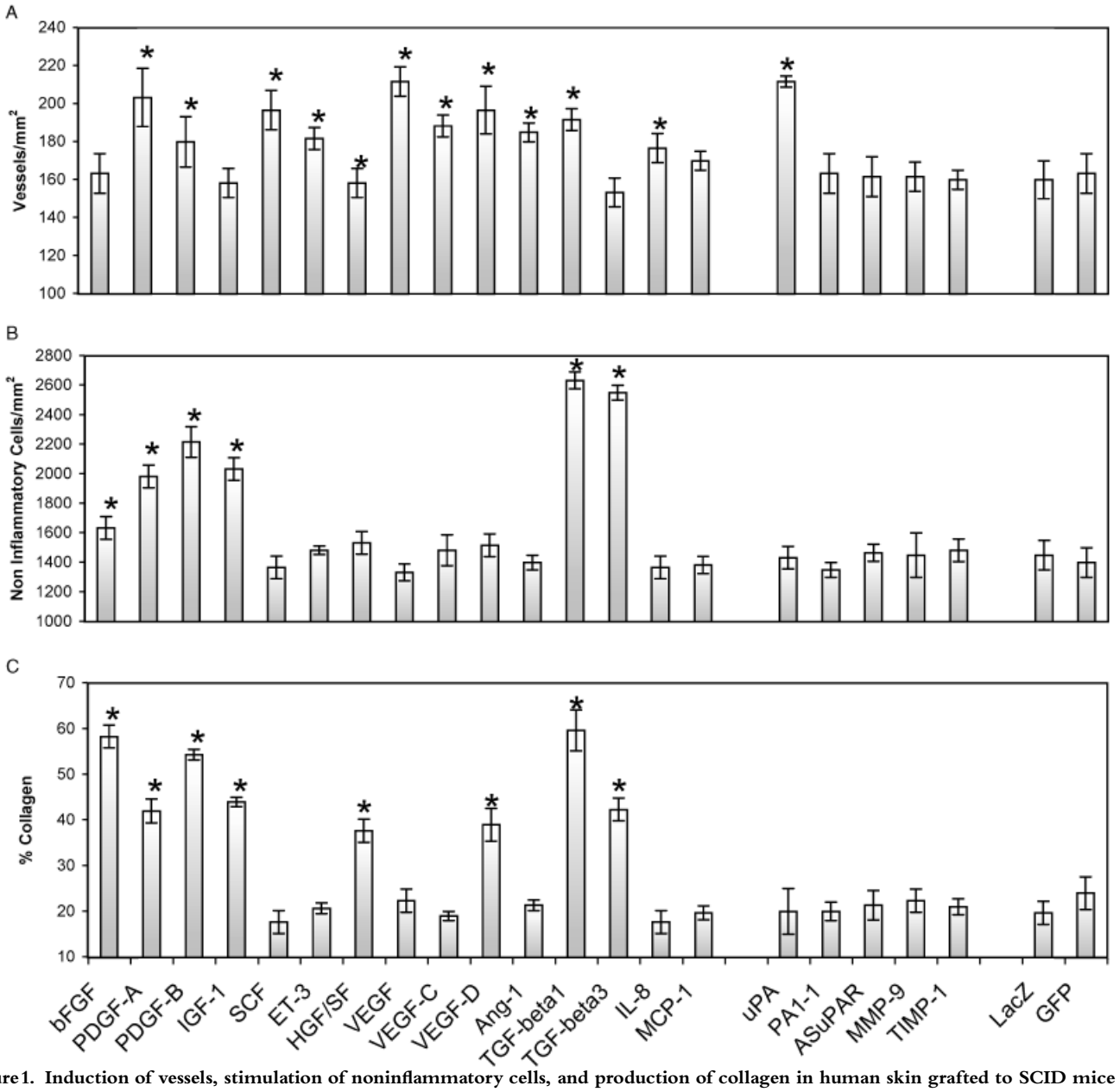

Figure 1. Induction of vessels, stimulation of noninflammatory cells, and production of collagen in human skin grafted to SCID mice after injection of replication-deficient adenoviral vectors coding for growth factors, cytokines, proteolytic enzymes and their inhibitors. The adenoviral vectors were injected intradermally at 5×10^8 pfu/100 μL, and the grafts were harvested 3 days later for histological and immunohistological analyzes. All vectors from Table 1 not listed here were nonreactive. (*A*) Vessel formation was initially determined on H&E-stained section and was confirmed by staining with anticollagen IV antibodies (not shown), and, in part, with antihuman and antimouse PECAM-1 (CD31). Adenoviral vectors for lacZ and GFP served as negative controls. Bars indicate number of vessels ± SD counted on five high power fields of histological sections through the center of injected grafts. All values >175 vessels/mm^2 are significant ($p < 0.05$). (*B*). Presence of cells other than inflammatory cells in human skin grafts 3 days after adenoviral vector injection. Cells include fibroblast-like cells, smooth muscle cells, pericytes and single endothelial cells and their precursors. Results are expressed as number of noninflammatory cells/mm^2 ± SD. Values >1650/mm^2 are significantly different from controls ($p < 0.05$). (C). Collagen in skin grafts was stained with Trichrome. Results are expressed as percentage collagen ± SD as quantified by image analyzes. Values >25% are significant ($p < 0.05$) from those found with control vectors.

(**Fig 3*E***) which shows strong collagen induction when compared to the lacZ control group (**Fig 3*A***).

The inflammatory response was correlated with the degree of infiltration of inflammatory cells such as neutrophils and monocytic cells (**Fig 4*A***). Growth factors that induced angiogenesis and stimulated fibroblastic cells to produce increased collagen were generally not inducing inflammatory cells. Growth factors that did not activate inflammation included bFGF, PDGF-A, PDGF-B, IGF-1, ET-3, HGF/SF, VEGF, VEGF-C, VEGF-D and TGF-β_3, whereas SCF, Ang-1 and TGF-B_1, not only induced increased infiltration of inflammatory cells but also vessel thrombosis with concomitant necrosis of the surrounding area (**Fig 4*B***).

The cytokines with chemoattractive activity for either monocytes/macrophages (MCP-1) or neutrophils (IL-8) were the strongest inducers of inflammatory cells (Oka *et al*, 2000; Nesbit *et al*, 2001a). Examples of neutrophil infiltration after IL-8 overexpression are given in **Fig 3*F***, and for monocyte infiltration after MCP-1 overexpression in **Fig 3*G***. A similarly strong reaction was induced by the serine protease uPA, its inhibitor, PAI-1. Thrombosis with a mild inflammatory cell infiltrate was observed with the antisense vector for the uPA receptor,

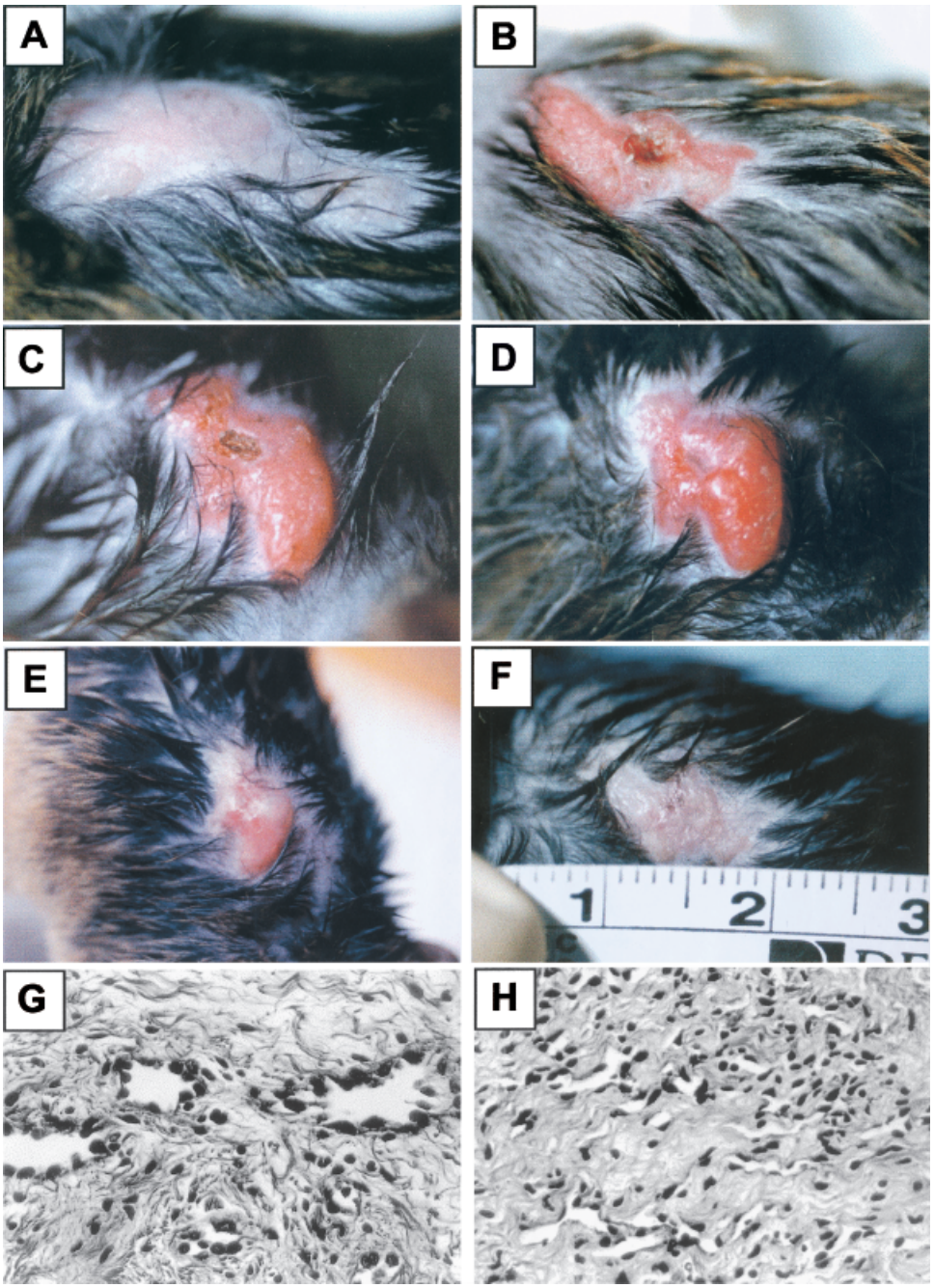

Figure 2. Dynamics of changes in human skin after adenovirus vector-mediated overexpression of VEGF. Human foreskin xenograft injected with recombinant adenovirus. (*a*) Xenograft before injection. (*b–f*) Same xenograft after intradermal injection of VEGF/Ad5 at 3 days (*b*), 7 days (*c*), 14 days (*d*), 28 days (*e*), and 60 days (*f*). (*g*)Histological changes in human skin 3 days after VEGF/Ad5 injection. Edema and dilated vascular spaces are observed. Endothelial cells are plump and bulge into the lumina (H&E staining, × 400). (*h*) Three days after *Lac*Z/Ad5 injection, cellular infiltrate and edema are limited (H&E, × 300).

ASuPAR. Strong thrombosis but little cellular infiltration was induced by MMP-9, whereas the inhibitor TIMP-1 was negative.

Several proteins induced thickening of the epidermis (acanthosis) (**Fig 4*C***). The stimulation of epidermal cells was likely mediated through indirect mechanisms, i.e., paracrine stimulation of the epidermal cells by soluble factors released by the transduced dermal cells (Liechty *et al*, 1999; Sylvester *et al*, 2000; Berking *et al*, 2001a). There is little evidence of transduction of epidermal cells (Berking *et al*, 2001a). bFGF, PDGF-B, SCF and ET-3 most strongly induced thickening of the epidermis. The mechanisms for epidermal stimulation are not clear. Dermal fibroblasts can produce a variety of growth factors that can be mitogenic for keratinocytes and/or that inhibited differentiation. Factors include TGF-α, KGF or ET (Ruiter *et al*, 2002). Epidermal hyperplasia was also noted after injection of the chemokines IL-8 and MCP-1, apparently due to the release of keratinocyte mitogen by neutrophils and monocytic cells, respectively. Strong responses were seen for the ASuPAR adenoviral vector (**Fig 4*C***). It is plausible that disruption of the expression of the uPA receptor (Mohan *et al*, 1999) activated keratinocyte mitogens in the fibroblasts. The vector stimulated both epidermal thickness and papillomatosis. On the other hand, neither uPA nor PAI-1 had any effects. Similar to ASuPAR, the growth factors SCF and ET-3 stimulated the epidermal cells whereas the activities of PDGF B, VEGF-D, and bFGF were weaker. The proto-oncogene c-myc induced both epidermal hyperplasia and papillomatosis

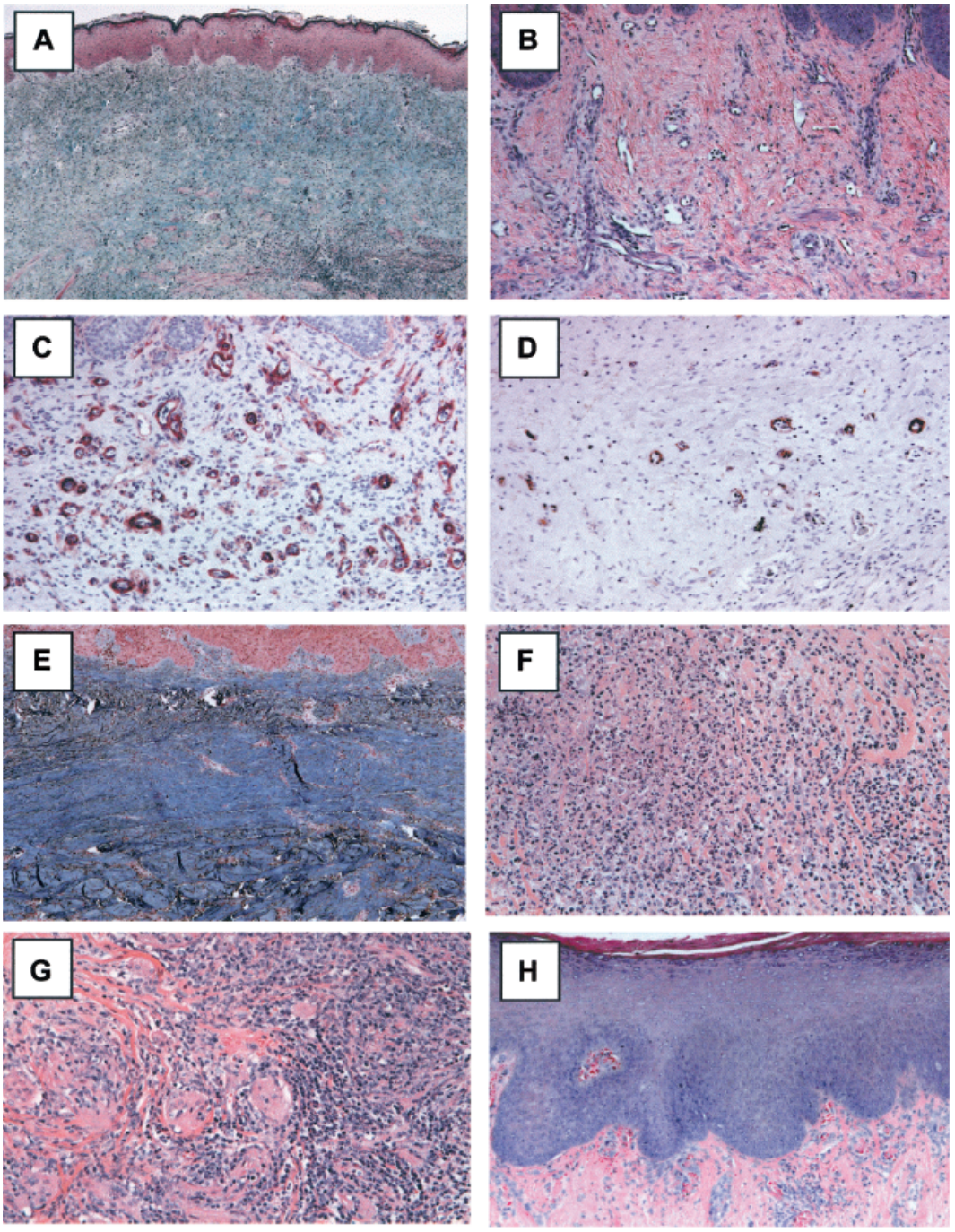

Figure 3. Staining of fixed sections of human skin grafts injected 3 days earlier with adenoviral vectors. (*a*) Human skin expressing *lacZ* stained with trichrome for collagens, X50. (*b*) Human skin graft expressing uPA, H&E staining, X100. (*c*) Graft expressing uPA and stained with anticollagen type IV antibodies to delineate vessel formation, X100. Vessel formation was seen similarly when sections were stained with anti-PECAM-1 (CD31) antibodies (not shown). (*d*) Staining of grafts expressing *lacZ* using anticollagen type IV antibodies indicates few vessels. (*e*) Trichrome staining of human skin grafts for all collagens after overexpression of IGF-1, X50. (*f*) IL-8 overexpression induced a strong neutrophilic infiltrate, H&E staining, X200. (*g*) MCP-1 - induced macrophage infiltration, H&E staining, X200. (*h*) ASuPAR induced epidermal hyperplasia and papillomatosis, H&E staining, X100.

(not shown). The mechanisms for this stimulation are not clear. The tumor suppressor gene p53 induced a response in the epidermal keratinocytes (not shown), but the mechanisms for this stimulation are also not clear.

DISCUSSION

The human skin/SCID mouse chimera model is well suited for studies of skin reorganization by growth factors, cytokines, enzymes, inhibitors and a variety of other proteins or antagonists associated with growth, differentiation and transformation. The advantage of the human skin/SCID mouse model is the maintenance of normal skin architecture and the rapid response to gene modulation. The disadvantages are the lack of an immune response and potentially incomplete responses to murine cytokines that do not bind or bind only weakly to the human receptors of the skin grafts. All adenoviral vectors for growth factors and cytokines used in this study expressed proteins that are cross-reactive between mice and humans. On the other hand,

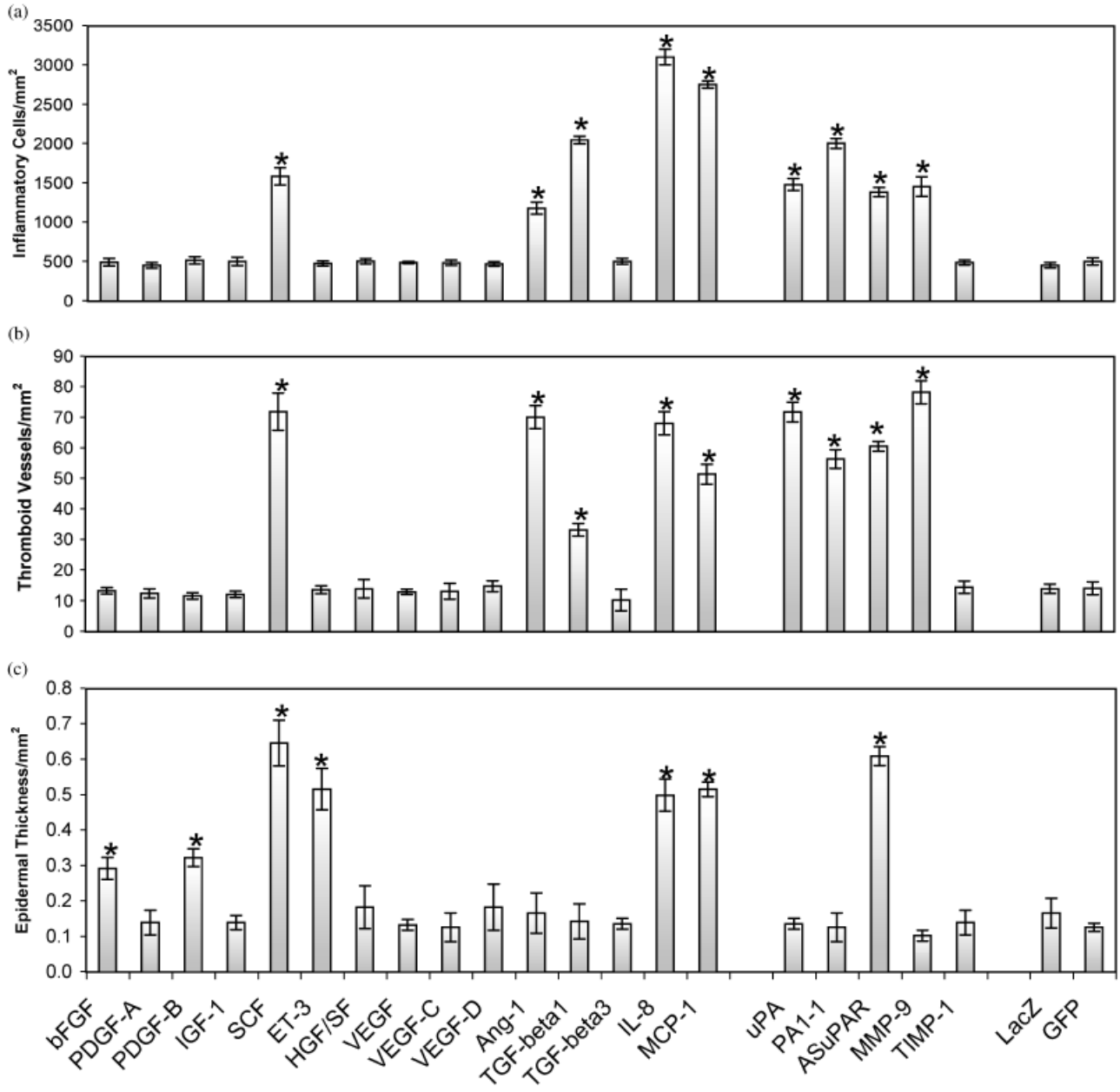

Figure 4. Inflammation and acanthosis 3 days after intradermal injection of adenoviral vectors for growth factors and proteolytic enzymes into human skin grafts. (*a*) Infiltration of inflammatory cells into the dermis of adenoviral vector injection. Results are expressed as number of inflammatory cells including neutrophils and monocytic cells/mm^2 ± SD values > 780 cells/mm^2 are significant ($p < 0.05$). (*b*) Thrombosis of vessels in human skin grafts, which was frequently associated with local necrosis. Results are expressed as number of thrombosed vessels/mm^2 ± SD. All values > 25 are significant ($p < 0.05$). (*c*) Epidermal thickness after intradermal overexpression of growth factors and proteolytic enzymes. Results are expressed in mm thickness ± SD with all values > 0.16 mm being significant ($p < 0.05$).

inflammatory molecules such as IL-1 or TNF-α are potentially released by activated cells in the dermis and have no biological effects on the target cells because the human ligands may not bind to the mouse receptors and vice versa.

The approach of intradermal injection was restricted to overexpression in cells of the dermis because the human epidermis is too thin for injection. Injection of the viral vectors in 100 μL of buffer resulted in dissemination of the virus throughout the 1.5–2 cm^2 grafts (Sylvester *et al*, 2000). The local elevation that was created may have slightly separated the dermal matrix leading to blister formation by the injected fluid, but healing occured very quickly and did not cause any significant inflammatory reaction. Prior to infection with adenoviral vectors, infiltration of the human skin by mouse cells was limited to endothelia and few inflammatory cells. Although the dermis contains both a human and mouse vasculature (Yan *et al*, 1993), the mouse vasculature is more activated by angiogenic growth factors, apparently due to the continuous recruitment of endothelial cells from the circulation (Asahara *et al*, 1999). Similarly, stimulation of fibroblasts with mitogens delivered by adenoviral gene transfer also activated both human and mouse cells in the human skin grafts (data not shown). Thus, despite a maintenance of the human architecture after activation of skin for remodeling, the new cellular components are of both mouse and human origins. This is apparently due to the innate high activation state of mouse cells, rapid migration of murine skin cells into the graft, and the homing of precursors of endothelial cells and fibroblasts from the peripheral blood and bone marrow.

The observation period of 3 d for histological evaluation was relatively short and many processes of skin remodeling may

occur over longer time periods. However, adenovirus-mediated gene transfer results in maximum protein expression after 72 h and longer observation periods often resulted in the subsequent activation of a battery of downstream targets, e.g., growth factors and cytokines that obscure activity of the primary factor (Berking *et al*, 2001a, 2001b; Nesbit *et al*, 2001a, 2001b). The 3-d time point chosen reflects best the activity of the transduced growth factor gene. With the exception of bFGF and PDGF-B, we have not seen qualitative changes at time points later than day 3. bFGF does not have a signal sequence and induces in fibroblasts the expression of other growth factors that can have secondary effects. Similarly, secondary growth factors are induced by PDGF-B. Total expression of the transgenes was limited to approximately 2 wk due to the episomal nature of adenovirus-mediated expression. On the other hand, cellular changes continued to be visible for up to 8 wk as was demonstrated with the adenoviral vector for VEGF. Milder changes may be reversed shortly after cessation of adenovirus vector-mediated gene expression.

The 38 vectors representing different gene factors showed three groups of reactivities: (a) no response in any of the four main criteria for characterization, which include increase in blood vessel formation, fibroblast proliferation with matrix formation, inflammatory cell infiltrate with thrombosis or necrosis, and epidermal hyperplasia with papillomatosis; (b) strong response for two or more criteria; and (c) one response (for a single criterion). No changes were seen after overexpression of integrin subunits β_3, α_2, and α_5, and of E- and N-cadherins. This is apparently due to the confined nature of gene expression in which the genes cannot utilize their functions outside of the expressing cells and the natural tissue context.

Overexpression of the oncogenes from human papilloma virus type 16, E6 and E7 had no morphogenetic consequences because dermal fibroblasts in contrast to keratinocytes and melanocytes are nonpermissive for the tumorigenic activity of these oncogenes (Brandsma *et al*, 1995; Sexton *et al*, 1995). This suggested that induced expression of the gene has to occur in the appropriate tissue compartment. Single injections for gene overexpression were insufficient to cause significant remodeling in the three-d period selected here for evaluation. However, even weekly injections for 12 wk of the E6 and E7 adenoviral vectors into the human skin grafts together with thrice weekly irradiation of the skin with ultraviolet light did not induce any significant morphological changes (Berking *et al*, 2001a). While single injections of the adenoviral vector for bFGF as shown here induced little changes, three injections induced strong angiogenesis and stroma formation (Berking *et al*, 2001a). Thus, gene products that are slowly released from cells for activation of the cellular environment may require repeated injections.

This study was not intended to investigate the mechanisms of action for selected genes in skin disorders. An advantage of the presented approach, however, is the discovery of genes with unexpected reactivities. The most unpredicted result was the stimulatory activity of uPA for vessel formation. The enzyme can be produced by all skin cells, including keratinocytes, melanocytes, fibroblasts and endothelial cells. The mechanisms through which it induced angiogenic responses are not clear. It appears to activate cell- or matrix-bound angiogenic factors. A chief example is bFGF, which is bound to the extracellular matrix protein heparan sulfate proteoglycan (Vlodavsky *et al*, 1996; Tumova *et al*, 2000) and released through proteolytic cleavage. Whether the release of bFGF is critical or whether activation of additional factors is initiated awaits further studies. Another surprising result occured when the receptor for uPA was suppressed with an adenoviral antisense construct vector (Mohan *et al*, 1999). In this case, a strong stimulation of the epidermis resulted through the inactivation or activation of yet undefined factors that stimulate growth and prevent differentiation of keratinocytes. Regarding the apparent paradox as to the proinflammatory effects of both uPA and PAI-1, the various effects of the uPAR-uPA-PAI-1 system depend on a complex interplay of intermolecular associations and are subjected to fine regulation. PAI-1 is a multifaceted proteolytic factor (Blasi, 1997). It not only functions as an inhibitor of uPA (with PAI-2), but also plays an important role in signal transduction, cell adherence and cell migration. In addition to its serpin activity as an inhibitor of receptor-bound uPA, PAI-1 prevents integrin $\alpha v\beta 3$ association to vitronectin and appears to regulate VEGF expression. Using adenovirus technology and PAI-1-deficient mice, Bajou and colleagues showed that PAI-1 mutants (1) either bound vitronectin normally but failed to inhibit uPA or (2) inhibited uPA normally but had negligible binding to vitronectin (Bajou *et al*, 2001). In tumors, the concerted action of uPA, uPAR and PAI-1 facilities invasion and metastasis (Blasi, 1997). While the cellular events associated with the wound healing process are similar between wild type and PAI-1 −/− mice, the rate of wound closure is significantly accelerated in PAI-1 −/− mice (Chan *et al*, 2001). More recently, Devy and colleagues showed that the pro- or antiangiogenic effect of PAI-1 is dose dependent. These observations underscore the pleiotropic effects of PAI-1. Thus, PAI-1 may have various cellular and molecular effects that depend on a finely tuned spatial and temporal expression, the net being an inflammatory infiltrate in our model.

A variety of molecules for which a role in scleroderma has been described (Kahaleh and Leroy, 1999), e.g., TGF-β and PDGF, cause a connective tissue reaction in our model, characterized by a proliferation of fibroblasts and matrix formation. This observation thus underscores the usefulness of the presented experimental approach for the study of this skin disorder. Another unexpected finding was the involvement of ET-3 in the vascular response and the thickening of the epidermis (acanthosis). Both are histopathologic hallmarks of psoriasis (Nickoloff, 2000). A third characteristic is leukocyte infiltration (Ortonne, 1999) which was not influenced by ET-3 in our model. Nevertheless, these observations make ET-3 an attractive, yet still speculative, novel candidate for a pathogenetic role in psoriasis and psoriasiform tissue reactions.

In summary, our approach has shown the feasibility of the human skin/SCID mouse chimera model to investigate the activity of individual genes for skin remodeling and dermatoses such as psoriasis and scleroderma. This notion is in line with other observations (Nickoloff, 2000). The confirmation of expected activities for selected genes demonstrates that functions of unknown genes, which may have biological significance for skin cells can be discovered. Our studies then point to several genes that show unexpected functions in skin reorganization and dermatological disorders.

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