Expression of Vascular Permeability Factor (Vascular Endothelial Growth Factor) by Epidermal Keratinocytes during Wound Healing

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Summary

Persistent microvascular hyperpermeability to plasma proteins even after the cessation of injury is a characteristic but poorly understood feature of normal wound healing. It results in extravasation of fibrinogen that clots to form fibrin, which serves as a provisional matrix and promotes angiogenesis and scar formation. We present evidence indicating that vascular permeability factor (VPF; also known as vascular endothelial growth factor) may be responsible for the hyperpermeable state, as well as the angiogenesis, that are characteristic of healing wounds. Hyperpermeable blood vessels were identified in healing split-thickness guinea pig and rat punch biopsy skin wounds by their capacity to extravasate circulating macromolecular tracers (colloidal carbon, fluoresceinated dextran). Vascular permeability was maximal at 2–3 d, but persisted as late as 7 d after wounding. Leaky vessels were found initially at the wound edges and later in the subepidermal granulation tissue as keratinocytes migrated to cover the denuded wound surface. Angiogenesis was also prominent within this 7-d interval. In situ hybridization revealed that greatly increased amounts of VPF mRNA were expressed by keratinocytes, initially those at the wound edge, and, at later intervals, keratinocytes that migrated to cover the wound surface; occasional mononuclear cells also expressed VPF mRNA. Secreted VPF was detected by immunofluoroassay of medium from cultured human keratinocytes. These data identify keratinocytes as an important source of VPF gene transcript and protein, correlate VPF expression with persistent vascular hyperpermeability and angiogenesis, and suggest that VPF is an important cytokine in wound healing.

Wound healing is a well-ordered response to injury characterized by diverse cellular activities that include acute and chronic inflammation, cell migration, angiogenesis, and matrix deposition (1–3). The initial wounding event injures blood vessels and results in focal hemorrhage. Fibrinogen in extravasated blood clots to form a fibrin gel into which other plasma proteins, notably fibronectin, may be incorporated (4). Inflammatory cells, fibroblasts, and new blood vessels migrate into this gel, digesting it while depositing cellular fibronectins, interstitial collagens, proteoglycans, and other components of mature stroma (2). In this manner, the provisional fibrin matrix is replaced by granulation tissue, and later, by a scar. Coincident with stroma generation, keratinocytes migrate to provide an epidermal covering that prevents fluid loss and retards bacterial invasion. This ordered sequence of events is thought to be orchestrated by interactions among cells, growth factors (e.g., platelet-derived growth factor, TGF-β, basic fibroblast growth factor), and extracellular matrix proteins (5).

A poorly appreciated feature of wound healing is that vascular hyperpermeability continues at wound sites for a considerable time after the cessation of overt bleeding (6, 7). Thus, vessels continue to leak plasma proteins for several days after the initiating trauma has ceased and even for a short time after a complete epidermal covering has been restored. Continued vascular hyperpermeability results in persistent extravasation of fibrinogen and ongoing deposition of provisional fibrin gel matrix, events that favor the continued induction of granulation tissue. The mechanisms responsible for initiating and maintaining the persistent vascular hyperpermeability characteristic of healing wounds have not been investigated.

Previously, our laboratory and others have described a protein, vascular permeability factor (VPF),[1] that enhances the permeability of local venules and small veins with a potency

[1] *Abbreviations used in this paper:* VEGF, vascular endothelial growth factor; VPF, vascular permeability factor.

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some 50,000 times that of histamine (8, 9). VPF is also a selective mitogen for endothelial cel-ls, hence, its alternative name, vascular endothelial growth factor (VEGF) (10, 11), and has been reported to be chemotactic for monocytes (12). Since increased vascular permeability and angiogenesis are characteristic features of wound healing (3), we considered the possibility that this mediator might also be present in healing wounds.

Materials and Methods

Wounding and Preparation of Tissue for Histology and In Situ Hybridization. Partial thickness skin wounds were bored with a 4-mm biopsy punch on the shaved flanks of young adult female CD rats (200–250 g; Charles River Breeding Laboratories, Inc., Wilmington, MA) or strain 2 guinea pigs (500–600 g; National Cancer Institute, Bethesda, MD) anesthetized with 25 mg/kg ketamine HCl and 5 mg/kg xylazine. At various intervals after wounding, animals were killed by ether/carbon dioxide inhalation and tissues were processed for in situ hybridization (13, 14). All animal procedures had the approval of the Beth Israel Hospital Institutional Animal Care and Use Committee.

To assess vascular permeability, wound-bearing rats were injected intravenously with either colloidal carbon (0.5 cc/250 g; Gunther Wagner, Inc.) or 70-kD fluoresceinated FITC-dextran (2 μmol or 144 mg/250 g; Sigma Chemical Co., St. Louis, MO), and killed 70 or 30 min, respectively, after injection. Tissues from carbon-injected animals were fixed in paraformaldehyde-glutaraldehyde for preparation of 1 μm Epon sections; tissues from FITC-dextran-injected animals were fixed in 70% ethanol-formaldehyde for paraffin sections (15).

In Situ Hybridization. Rat cDNA for VPF was prepared using PCR as described previously (13), except that a different reverse primer (CCGGAATTCAGCGCCTCGGCTTGTC) was used. The resulting cDNA (393 bp) was subcloned into pGEM-3Zf(+) (Promega Biotec, Madison, WI), from which single-stranded antisense RNA probes were synthesized (~10^8 cpm/μg) with ^{35}S-UTP, purified on polyacrylamide gels, and used without reduction in length. This probe contains sequences common to all four of the VPF isoforms thus far described (16, 17). A probe of the same length but in the sense orientation served as a control. In situ hybridization on guinea pig wounds and human keratinocytes was carried out with probes to guinea pig and human VPF as previously described (13). These probes reacted both with a 4.5-kb band on Northern blots and with mRNA in tissue sections from the respective species (13).

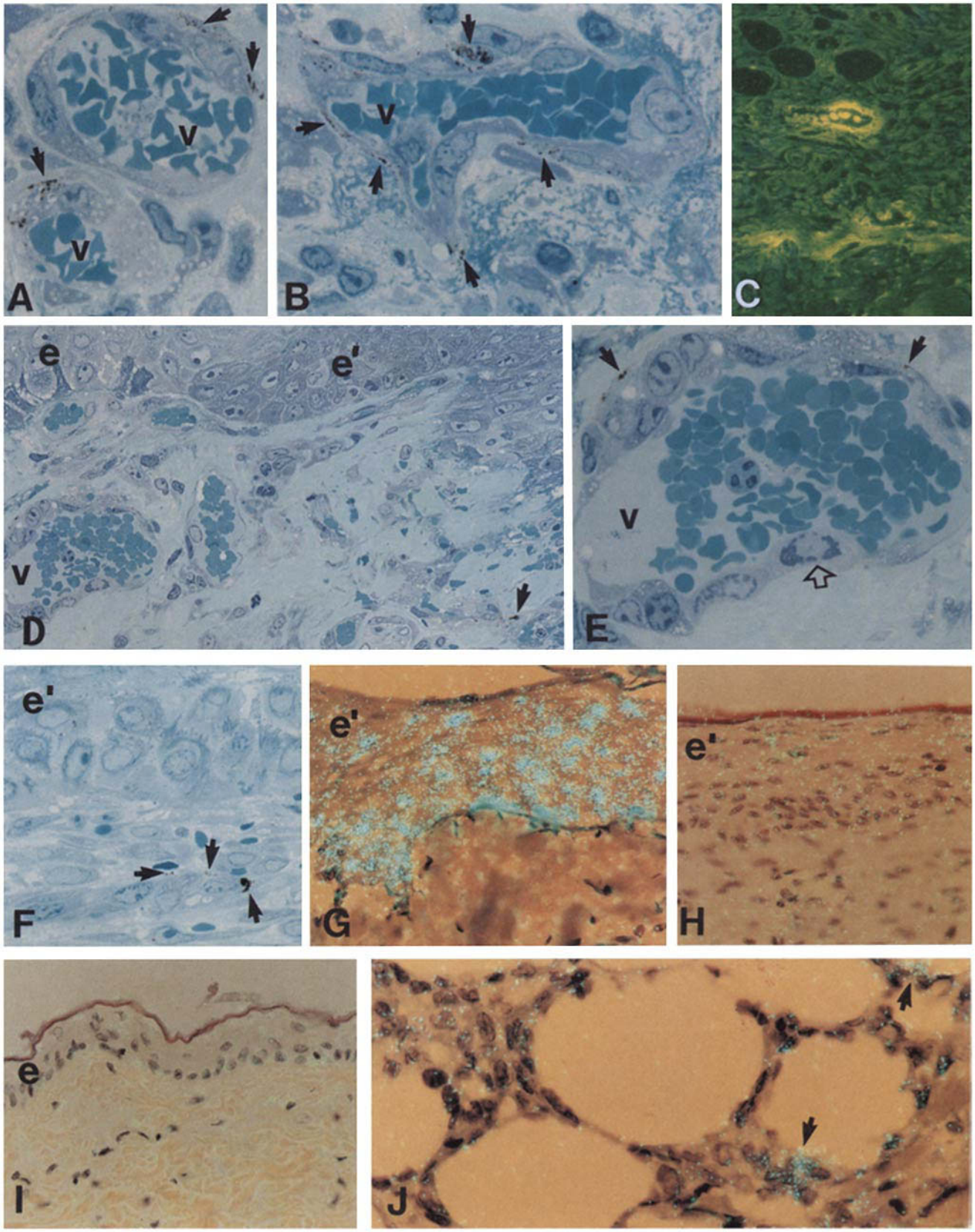
Keratinocyte Culture and Immunoassay of VPF. Human epidermal keratinocytes (Clonetics, Inc., San Diego, CA) were maintained for two to four passages in serum-free keratinocyte growth medium (KGM; Clonetics, Inc.) at 37°C, 5% CO_2. For VPF quantitation, cells were subcultured in 24-well plates (Costar, Cambridge, MA) at 42,000 cells per well, and, after overnight culture, medium (1 ml) was replaced with fresh KGM. At varying intervals thereafter, the conditioned medium was harvested and the cell layer extracted with 0.5 ml lysis buffer (0.5% Triton X-100 in PBS, pH 7.2, containing 0.24 TIU/ml aprotinin). Samples were centrifuged (10,000 *g*, 4°C) and stored (−70°C) for immunoassay. Replicate 50-μl samples of human keratinocyte culture media were assayed for VPF in a time-resolved immunofluorometric assay as previously described (18). Affinity-purified polyclonal rabbit antibodies directed against the COOH-terminal region of guinea pig VPF served as the "capture" antibodies. Affinity-purified polyclonal rabbit antibodies prepared against a peptide corresponding to the NH_2 terminus of human VPF (8) were covalently linked to Eu^{3+} chelate to permit fluorometric detection as second antibodies (18). Results of the immunoassay of conditioned medium were normalized for total cell protein as determined by the BCA assay (Pierce Chemical Co., Rockland, IL). Recombinant human VPF served as a standard (gift of Dr. Steven Ledbetter, Upjohn Laboratories, Kalamazoo, MI) to quantitate the VPF secreted by human keratinocytes.

Results and Discussion

Rat and guinea pig skin wounds healed with similar kinetics in accord with prior descriptions (14, 19). Within minutes of punch biopsy the wound defect was filled by clotted blood and bleeding stopped. Tracer studies with either colloidal carbon or 70-kD FITC-D confirmed earlier reports that significant vascular hyperpermeability persisted for some days after wounding in both rat and guinea pig skin (6) and localized leakage to specific blood vessels that most closely resembled venules and small veins (Fig. 1, *A–F*). With both tracers, extensive leakage was evident in 2–3-d wounds (Fig. 1, *A–C*), and vascular hyperpermeability persisted at decreasing levels through day 7 (Fig. 1, *D–F*). Leaky vessels were located in the superficial dermis at the wound edges (Fig. 1, *D* and *E*) and more centrally in the granulation tissue of the wound bed (Fig. 1, *A–C* and *F*). The endothelial cells lining hyperpermeable vessels were enlarged and occasionally exhibited mitotic figures, indicating that they were participating in the angiogenic response (Fig. 1, *A*, *B*, and *D–F*); thus, an extensive temporal and spatial overlapping of vascular hyperpermeability and angiogenesis occurred.

Preliminary experiments, using a time-resolved immunofluorometric assay (18), indicated that VPF protein was detectable in wound extracts. In situ hybridization was then performed to define the cellular source(s) of the VPF expressed in rat and guinea pig healing wounds, and identical results were obtained with both species using the appropriate probes. Antisense probes revealed strikingly elevated VPF mRNA in keratinocytes at the wound edges as early as 1 d after wounding, and abundant VPF expression was a prominent

Figure 1. Vascular hyperpermeability and in situ expression of VPF mRNA in healing rat (*A*, *B*, and *D-J*) and guinea pig (*C*) skin wounds. (*A–C*) Leaky vessels (*v*) present in early granulation tissue underlying 3-d rat (*A* and *B*) or guinea pig (*C*) skin wounds as detected by colloidal carbon (*A* and *B*) or 70-kD FITC-D (*C*) extravasation. Arrows (*A* and *B*) indicate carbon deposited in vessel walls. (*D* and *E*) Filled arrows identify colloidal carbon deposits in vessels of a 5-d wound; one of these (*v*) contains a mitotic endothelial cell (*open arrow*) and is illustrated at higher magnification in *E*. Note proximity of these leaky vessels to the junction between wound epidermis (*e', right*) and normal epidermis (*e, left*), a distinction made obvious by different staining properties. (*F*) A persistent carbon-labeled vessel (*arrows*) underlying newly migrated epidermis covering a 7-d wound. (*G–J*) In situ hybridization of 3-d (*G* and *J*) and 7-d (*H*) rat skin wounds and normal skin adjacent to a 3-d wound (*I*) hybridized with a ^{35}S-labeled antisense probe to VPF. (*G*) Strong keratinocyte labeling is observed throughout the thickened epidermis (*e'*) that is proliferating and migrating to cover the wound defect; by contrast, the epidermis (*e*) of nearby normal skin is unlabeled (*I*). Rare mononuclear cells, probably macrophages (*arrows*),



in the 3-d wound bed (*J*) are also strongly labeled. In 7-d wounds (*H*), epidermal (*e'*) thickening persists but keratinocyte labeling for VPF mRNA has decreased markedly, though still remaining above background levels. (*A*, *B*, and *D–F*) 1-μm thick, Giemsa stained, Epon sections; (*C*) fluorescence microscopy of unstained, 20-μm paraffin section. (*G–H*) Hematoxylin and eosin sections of radioautographs viewed by combined epipolarization and low light level bright field microscopy. (*A* and *B*) ×1,100; (*C* and *D*) ×360; (*E*) ×850; (*F*) ×680; (*G–I*) ×260; (*J*) ×430.

and consistent feature of epidermal cells migrating to cover the wound defect. Epidermal labeling was maximal on days 2–3, and keratinocytes of the basal and the more superficial, differentiated layers expressed VPF mRNA with equal intensity (Figs. 1 *G* and 2 *A*). By day 7, when epidermal covering was complete, keratinocyte expression of VPF mRNA persisted at above normal levels (Fig. 1, *H* vs. *I*), though in much lower amounts than at earlier stages of wound healing. In addition to keratinocytes, occasional mononuclear cells with morphological features of macrophages in the wound bed also expressed VPF mRNA (Fig. 1 *J*). Neither keratinocytes, macrophages, nor any other cell type in rat or guinea pig wounds or in normal skin labeled with sense riboprobes (Fig. 2 *B*). To obtain independent evidence that keratinocytes synthesize and secrete VPF, immunoassays were performed on isolated human keratinocytes. The immunofluorometric assay used for these experiments employs an antipeptide antibody directed to the COOH terminus of VPF as the "capture" antibody and a second antipeptide antibody to the NH_2 terminus of VPF as the "reporter" antibody (18). Using this assay, we found that human keratinocytes (42,000 per well) cultured in serum-free medium for 120 h secreted ~6 ng VPF. Using a human VPF probe, we also carried out in situ hybridization on cells grown under these conditions and demonstrated the presence of VPF mRNA. These results demonstrate that the VPF gene is transcribed and that VPF is synthesized and secreted by cultured keratinocytes.

Taken together, these data indicate that VPF and its mRNA are expressed in normal keratinocytes, both in situ and in tissue culture, and that VPF expression is markedly increased in the activated, migrating keratinocytes involved in wound healing. Occasional mononuclear cells, probably macrophages, in the wound bed also expressed VPF mRNA. This VPF expression is likely to have important biological significance because vessels exhibiting increased permeability were in close proximity (<0.5 mm) to VPF-expressing keratinocytes and/or macrophages, and the time course of VPF mRNA expression, microvascular hyperpermeability, and angiogenesis overlapped. VPF may be expected to induce angiogenesis in two ways: directly by serving as an endothelial cell mitogen, and indirectly by increasing vascular hyperpermeability and thereby promoting the deposition of an extravascular fibrin matrix, which is itself a potent angiogenic stimulus (20). Keratinocytes have increasingly come to be recognized as important regulators of inflammation as they have been found to contain and to express cytokines that promote chemotaxis, activate macrophages and bone marrow elements, and induce lymphocyte homing (21–23). The interplay between VPF and these other cytokines in wound healing now requires investigation.

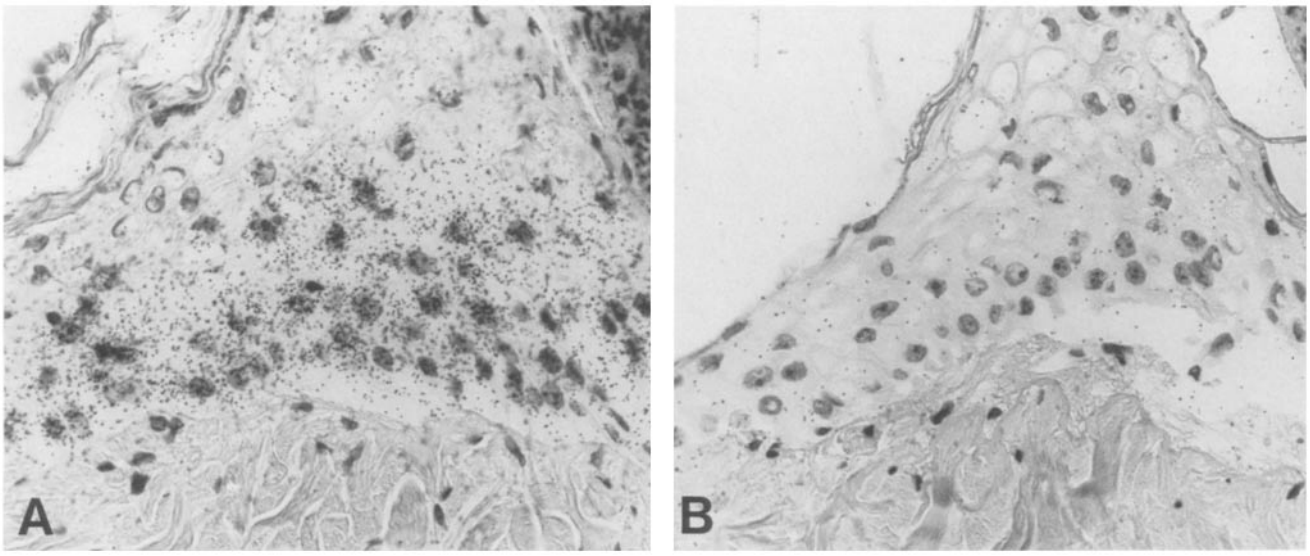


Figure 2. In situ hybridization of keratinocyte layer at edge of a 3-d rat skin wound hybridized with ^{35}S-RNA antisense (*A*, strongly positive) and sense (*B*, negative) VPF probes.

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