

Overexpression of Vascular Permeability Factor/Vascular Endothelial Growth Factor and its Receptors in Psoriasis

By Michael Detmar, Lawrence F. Brown, Kevin P. Claffey, Kiang-Teck Yeo, Olivier Kocher, Robert W. Jackman, Brygida Berse, and Harold F. Dvorak

From the Department of Pathology, Beth Israel Hospital, Harvard Medical School, Boston, Massachusetts 02215

Summary

Psoriatic skin is characterized by microvascular hyperpermeability and angioproliferation, but the mechanisms responsible are unknown. We report here that the hyperplastic epidermis of psoriatic skin expresses strikingly increased amounts of vascular permeability factor (VPF; vascular endothelial growth factor), a selective endothelial cell mitogen that enhances microvascular permeability. Moreover, two VPF receptors, *kdr* and *flt-1*, are overexpressed by papillary dermal microvascular endothelial cells. Transforming growth factor α (TGF- α), a cytokine that is also overexpressed in psoriatic epidermis, induced VPF gene expression by cultured epidermal keratinocytes. VPF secreted by TGF- α -stimulated keratinocytes was bioactive, as demonstrated by its mitogenic effect on dermal microvascular endothelial cells in vitro. Together, these findings suggest that TGF- α regulates VPF expression in psoriasis by an autocrine mechanism, leading to vascular hyperpermeability and angiogenesis. Similar mechanisms may operate in tumors and in healing skin wounds which also commonly express both VPF and TGF- α .

Psoriasis is a common, chronic skin disease characterized by recurrent erythematous skin plaques that exhibit epidermal hyperplasia, a variable inflammatory cell infiltrate, and abnormalities of the papillary dermal vasculature (1–4). Microvessels in the papillary dermis of psoriatic plaques are elongate, dilated, and hyperpermeable (5, 6) and more closely resemble postcapillary venules than the capillary loops of normal skin (7, 8). Whereas vascular changes may precede inflammatory cell infiltration in developing psoriatic plaques, and may reappear before clinical relapse (9–11), there is increasing evidence that epidermal alterations precede capillary leakiness and vascular anomalies in the development of psoriatic skin lesions (12). Moreover, a previous study demonstrated that the angiogenic properties of psoriatic skin were associated with the epidermis, not the dermis (13).

Vascular permeability factor (VPF) is a 32–42-kD glycosylated protein that is overexpressed by many human and animal tumors (14–17) and by the epidermis of healing wounds (18), conditions that, like psoriasis, are associated with enhanced microvascular permeability and angiogenesis. Two tyrosine kinase receptors for VPF, *kdr* and *flt-1*, are also overexpressed in the microvessels of tumors that overexpress VPF (19–21). In vivo, VPF enhances microvascular permeability with a potency some 50,000 times that of histamine and induces angiogenesis (22–25). In vitro, VPF is a selective mitogen for cultured endothelial cells, hence its alternate name, vas-

cular endothelial growth factor (26, 27). We hypothesized that a cytokine with these properties might play an important role in the pathogenesis of psoriasis.

In this report, we demonstrate increased expression of VPF mRNA by the hyperplastic epidermis of lesional psoriatic skin and increased expression of two VPF receptors in psoriatic dermal microvessels. In vitro investigations showed that VPF expression and secretion of bioactive VPF by epidermal keratinocytes were induced by TGF- α , a cytokine known to be overexpressed in psoriatic epidermis (28, 29). Therefore, besides its mitogenic effect on epidermal keratinocytes, TGF- α likely regulates VPF expression in psoriatic epidermis by an autocrine mechanism, leading to increased vascular permeability and angiogenesis.

Materials and Methods

In Situ Hybridization and Immunohistochemistry. 6-mm punch biopsies were taken, after obtaining informed consent, from the involved and uninvolved skin of six patients with chronic plaque-type psoriasis that had not been treated for a period of at least 6 wk and from the normal skin of six healthy volunteers. In situ hybridization was performed on 6 μ m fresh-frozen tissue sections using single-stranded antisense or control sense riboprobes as described (20). The VPF probe was prepared from a 204-bp VPF cDNA fragment isolated from human HT 1080 cells (30). Riboprobes for the VPF receptors, *kdr* and *flt-1*, were prepared from cDNA fragments

as described (21). Immunohistochemistry was performed on fresh-frozen tissue sections reacted with an affinity-purified rabbit antibody against a 26-amino acid peptide corresponding to the NH₂-terminus of human VPF as described (31). The specificity of this antibody has been documented by Western blotting (32). As a negative control, primary anti-VPF antibody was replaced with an equivalent concentration of normal rabbit IgG.

Cell Culture. Human epidermal keratinocyte cultures were established from normal adult body skin of healthy volunteers and propagated in serum-free keratinocyte growth medium (KGM) (Clonetics Corp., San Diego, CA) as described (33). Second passage keratinocytes were used in all experiments. Human dermal microvascular endothelial cells (HDMEC) were isolated from neonatal foreskins after routine circumcisions as described (34) and cultured on fibronectin-coated culture dishes in endothelial basal (EBM) (Clonetics Corp.), supplemented with 20% FCS, 1 μ g/ml hydrocortisone acetate, and 10 μ M dibutyryl-cAMP (all from Sigma Chemical Co., St. Louis, MO).

Northern Blot Analysis. Total cellular RNA was extracted from confluent, second-passage keratinocytes cultured in 100-mm dishes for 24 h in KBM without added growth factors, with 0.1–100 ng/ml recombinant human TGF- α (British Biotechnology, Abington, UK) or with 10 ng/ml recombinant human epidermal growth factor (EGF; Clonetics Corp.) as described (35). Northern blots were hybridized with a human VPF cDNA probe (546 bp) obtained by reverse transcription-PCR of RNA from human glioblastoma U-373 cells, using the oligonucleotide primers 5'-TCCGAATTCGACCCATGGCAAGAA-3' and 5'-TTCGAATTCCTGAGGGA-GCTCC-3'. Control hybridization was performed with ribosome-associated protein cDNA, 36B4 (36) to demonstrate equivalent RNA loading.

VPF Immunofluorometric Assay. Confluent second passage keratinocytes were cultured in 24-well plates for 48 h in KBM without added growth factors, or with added TGF- α (0.1–100 ng/ml), or with TGF- α (100 ng/ml) plus 10 μ g/ml rabbit anti-EGF receptor antibody (UBI, Lake Placid, NY). VPF was quantitated in conditioned media (CM) by a modified two-site, time-resolved immunofluorometric assay (37) using a polyclonal antibody against recombinant human VPF for both capture and detection. Values were normalized for total cell numbers after culture. Data are expressed as pM VPF/10⁵ cells. In these and other experiments, statistical significance was calculated with the Dunnett multiple comparison test.

Bioassay for Mitogenic Activity of Keratinocyte CM. CM was collected from confluent keratinocyte cultures in 100-mm dishes incubated for 48 h in 5 ml KBM medium supplemented with 100 ng/ml TGF- α and stored frozen at –80°C for up to 2 wk. As control, medium was added to culture dishes without cells and incubated and processed identically. Second passage HDMEC were seeded at 5 \times 10³ cells/cm² in 24-well plates in EBM medium supplemented with 2% FCS. After 16 h, varying amounts (1–30%) of keratinocyte conditioned or control medium were added to quadruplicate wells for 48 h. 1 μ Ci/ml methyl-[³H]thymidine (sp act 82.4 Ci/mmol; DuPont NEN, Boston, MA) was added during the final 6 h of culture, and the thymidine incorporation into DNA was determined as described (38). Data are expressed as a stimulatory index: ratio of net [³H]thymidine incorporation by HDMEC cultured with conditioned or control medium.

VPF Depletion by Solid Phase Immunoabsorption. CM from keratinocyte cultures treated with 100 ng/ml TGF- α (see above) were depleted of VPF by solid phase immunoabsorption as described (32), using the same affinity-purified anti-VPF antibody as for immunohistochemistry, coupled to protein A-Sepharose beads (Pharmacia, Uppsala, Sweden). As a control, CM was depleted of TGF- α with

a specific polyclonal antibody (PeproTech, Rocky Hill, NJ) or incubated with Sepharose beads alone. Control medium was treated identically. The capacity of these CM to stimulate HDMEC thymidine incorporation was measured as described above.

Results and Discussion

Initial experiments were performed to determine whether VPF was overexpressed in psoriatic skin. In situ hybridization (ISH) demonstrated enhanced expression of VPF mRNA in the hyperplastic epidermis of all psoriatic patients biopsied (Fig. 1 A). As in healing skin wounds, differentiated keratinocytes of the suprabasal layers labeled most intensely, but VPF mRNA was also expressed focally by basal keratinocytes and by rare mononuclear inflammatory cells infiltrating the upper dermis. In contrast, little or no VPF mRNA was expressed in the epidermis of all normal volunteers (Fig. 1 C). ISH performed on areas of skin from psoriatic patients that appeared clinically normal also revealed focally increased expression of VPF mRNA. However, these biopsies also showed microscopic abnormalities suggestive of early psoriatic activity. Consistent with these ISH findings, immunohistochemistry of clinically involved psoriatic skin revealed strong cytoplasmic staining for VPF, predominantly in suprabasal epidermal keratinocytes (Fig. 1 E). ISH was also employed to identify sites of VPF receptor mRNA expression. Strong labeling for the mRNAs of both kdr and flt-1 was observed in the papillary dermal microvessels of active psoriatic lesions (Fig. 1 D). In contrast, little or no receptor expression was detected in deeper vessels of psoriatic skin or in vessels of normal skin.

Because increased VPF expression likely contributes to the microvascular hyperpermeability and angiogenesis of tumors and healing skin wounds (14–17), we reasoned that a common mediator might be responsible for upregulating VPF expression in these disorders as well as in psoriasis. A likely candidate was TGF- α , a cytokine with angiogenic properties in vivo (39) and one known to be overexpressed in the epidermis of psoriatic skin lesions and of healing skin wounds and in many epithelial tumors (28, 29, 40).

To test the possibility that TGF- α might regulate VPF expression in psoriasis, we performed Northern analyses on RNA extracted from confluent human epidermal keratinocytes, cultured with or without TGF- α . VPF mRNA expression was upregulated by TGF- α in a dose-dependent fashion at concentrations \geq 1 ng/ml (Fig. 2 A). EGF also upregulated VPF mRNA expression by cultured keratinocytes (Fig. 2 A); TGF- α is structurally related to EGF and reacts with the EGF receptor (41, 42).

To determine whether TGF- α also increased synthesis and secretion of VPF protein, we tested keratinocyte CM for VPF by immunoassay. After 48 h of culture in the presence of TGF- α , keratinocyte CM contained significantly increased amounts of immunoreactive VPF protein. At 100 ng/ml, TGF- α increased VPF levels fivefold, and a statistically significant increase was noted with concentrations as low as 3 ng/ml (Fig. 2 B). This effect was mediated by TGF- α binding to the EGF receptor, since addition of an anti-EGF receptor

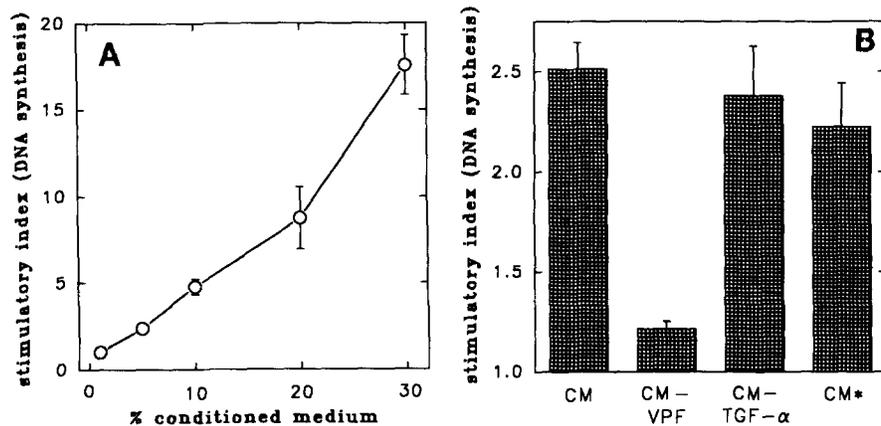


Figure 3. (A) Stimulation of DNA synthesis in cultured human dermal microvascular endothelial cells (HDMEC) by conditioned medium (CM) from keratinocytes cultured 48 h in serum-free medium supplemented with 100 ng/ml TGF- α . DNA synthesis is expressed as a stimulatory index (mean \pm SD): ratio of net thymidine incorporation by HDMEC cultured with added conditioned/control medium ($n = 4$). (B) Depletion of VPF by immunoadsorption significantly suppressed the stimulatory effect of 10% CM from TGF- α -treated keratinocytes on HDMEC [3 H]thymidine incorporation ($p < 0.01$). Analogous depletion of TGF- α or incubating CM with Sepharose beads alone (*) did not significantly alter the stimulatory effect of keratinocyte CM. Data are expressed as a stimulatory index as described above (mean \pm SD): ratio of net thymidine incorporation by HDMEC cultured with added 10% conditioned/control medium ($n = 4$).

antibody inhibited VPF induction by TGF- α (Fig. 2 B). Though somewhat less effective, EGF also increased the amount of VPF released by cultured keratinocyte (data not shown). Inasmuch as TGF- α and EGF receptors (but not EGF itself) are overexpressed in psoriatic skin (28, 29, 43), our findings suggest that TGF- α may stimulate VPF synthesis and secretion by epidermal keratinocytes in psoriasis. This is further supported by the almost identical localization of keratinocytes expressing VPF mRNA and TGF- α mRNA (44) within the epidermis. In contrast, neither IL-6 nor -8, other cytokines increased in lesional psoriatic epidermis, modulated VPF levels in keratinocyte CM.

The VPF identified in keratinocyte CM by immunoassay was biologically active, as measured by its capacity to stimulate thymidine incorporation in cultured HDMEC. HDMEC strongly express VPF receptors and proliferate in response to graded doses of VPF (Detmar, M., unpublished data). CM obtained from TGF- α -treated keratinocyte cultures was potently mitogenic for HDMEC in a concentration-dependent fashion (Fig. 3 A). Because the mitogenic effect of keratinocyte CM might have resulted from secreted products other than VPF, possibly including TGF- α itself, additional experiments were performed to demonstrate specificity. Depletion of VPF by specific antibodies removed more than 80% of the endothelial cell mitogenic activity present in CM derived from TGF- α -treated keratinocyte cultures. In contrast, depleting CM of TGF- α was without effect (Fig. 3 B).

Our findings indicate that both VPF and two of its receptors are strikingly overexpressed in psoriatic skin and, by this mechanism, likely induce the increased numbers of hyperpermeable blood vessels required to meet the increased nutritional needs of the hyperplastic psoriatic epidermis. It remains to be established whether additional angiogenic factors also contribute to the microvascular alterations in psoriasis. Whereas production of platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) by epidermal keratinocytes in vitro, as well as increased expression of PDGF receptors by dermal psoriatic vessels, have been previously demonstrated (45, 46), elevated levels of these factors in psoriatic skin have not been reported. Moreover, neither PDGF nor bFGF is known to increase microvascular permeability.

Our in vitro findings indicate that TGF- α potently upregulates VPF expression in epidermal keratinocytes. Because TGF- α and the EGF receptor with which it interacts are also upregulated in psoriatic epidermis (28, 29, 43), it is likely that TGF- α , in addition to its mitogenic effect on epidermal keratinocytes, upregulates VPF expression in psoriasis by an autocrine mechanism. These findings have potential significance for, in addition to psoriasis, the pathogenesis of the vascular hyperpermeability and angiogenesis that characterize many tumors and healing wounds, conditions in which VPF, TGF- α , and their receptors are also overexpressed (14–20, 40, 43).

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