Transforming growth factor- α -induced transcriptional activation of the vascular permeability factor (VPF/VEGF) gene requires AP-2-dependent DNA binding and transactivation

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The endothelial cell-specific mitogen vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) represents a central regulator of cutaneous angiogenesis. Increased VPF/VEGF expression has recently been reported in psoriatic skin and healing wounds, both conditions in which transforming growth factor- α (TGF α) and its ligand, the epidermal growth factor receptor, are markedly up-regulated. Since TGF α strongly induces VPF/VEGF synthesis in keratinocytes, TGF α -mediated VPF/VEGF expression is likely to play a significant role in the initiation and maintenance of increased vascular hyperpermeability and hyperproliferation in skin biology. The objectives of the present studies were to determine the molecular mechanisms responsible for TGF α -induced transcriptional activation of the VPF/VEGF gene. We have identified a GC-rich TGF α -responsive region between –88 bp and –65 bp of the VPF/VEGF promoter that is necessary for constitutive and TGF α -inducible transcriptional activation. In electrophoretic mobility shift assays, this region binds Sp1-dependent protein complexes constitutively and an additional TGF α -inducible protein complex that is distinct from Sp1 protein. Both AP-2 and Egr-1 transcription factors were detected as components of the TGF α -inducible protein complex in supershift EMSA studies. In co-transfection studies, an AP-2 but not an Egr-1 expression vector activated VPF/VEGF transcription, thus indicating that AP-2 protein is functionally important in TGF α -induced VPF/VEGF gene expression. By clarifying regulatory mechanisms that are critical for angiogenic processes in the skin, these studies may form the basis for new therapeutic strategies to modulate VPF/VEGF expression in cutaneous inflammation and wound healing.

Keywords: gene expression regulation/TGF α /transcription factors/VEGF/VPF

Introduction

Angiogenesis, the formation of new blood vessels from a pre-existing network of capillaries, is a fundamental step in many physiologic and pathophysiologic processes (Folkman and Shing, 1992; Folkman, 1995; Shibuya, 1995). Among several polypeptide growth factors, the vascular permeability factor (VPF) (Senger *et al.*, 1983),

also known as vascular endothelial growth factor (VEGF) (Leung *et al.*, 1989), is viewed as one of the most important inducers of angiogenesis (Plate *et al.*, 1994; Dvorak *et al.*, 1995; Ferrara *et al.*, 1995; Thomas, 1996). VPF/VEGF presents as a strong endothelial cell-specific mitogen in various *in vitro* and *in vivo* systems (Plouet *et al.*, 1989; Pepper *et al.*, 1992; Detmar *et al.*, 1995) and has been shown to increase the permeability of microvessels (Senger *et al.*, 1983; Dvorak *et al.*, 1995; Roberts and Palade, 1995). The homodimeric glycoprotein VPF/VEGF is highly conserved and shares structural homology with placenta growth factor and platelet-derived growth factor (Tischer *et al.*, 1989; Maglione *et al.*, 1991). The effects of VPF/VEGF are mediated through two distinct high-affinity endothelial cell receptors, Flt-1 (De Vries *et al.*, 1992; Seetharam *et al.*, 1995) and KDR/Flk-1 (Terman *et al.*, 1992; Quinn *et al.*, 1993), both of which are receptor protein-tyrosine kinases (Mustonen and Alitalo, 1995). The temporal and spatial correlation of VPF/VEGF overexpression with angiogenesis during tumor growth (Senger *et al.*, 1986, 1994; Potgens *et al.*, 1995; Claffey *et al.*, 1996), inflammation (Aiello *et al.*, 1994; Koch *et al.*, 1994; Brown *et al.*, 1995a,b) and wound healing (Brown *et al.*, 1992; Frank *et al.*, 1995) provides strong evidence for a functional role of VPF/VEGF as a key regulator of angiogenesis. This assumption is further supported by functional studies revealing inhibition of VPF/VEGF-induced angiogenesis by specific antibodies (Kim *et al.*, 1993; Sioussat *et al.*, 1993) or by overexpressing a dominant-negative VPF/VEGF receptor mutant (Millauer *et al.*, 1994).

Most recently, a novel and biologically relevant pathway in the induction of VPF/VEGF gene expression has been identified and elucidated (Detmar, 1996). Transforming growth factor- α (TGF α) was found to function as a potent inducer of VPF/VEGF synthesis (Detmar *et al.*, 1994). Several lines of evidence indicate that the TGF α -induced VPF/VEGF gene expression is fundamental in wound repair and localized tissue inflammation and edema. With respect to angiogenic processes in the skin (Detmar, 1996), recent studies have demonstrated increased VPF/VEGF expression in the hyperplastic epidermis of psoriatic skin (Detmar *et al.*, 1994) and in keratinocytes of healing wounds (Brown *et al.*, 1992; Frank *et al.*, 1995). Additionally, TGF α has been shown markedly to induce the VPF/VEGF synthesis in keratinocytes *in vitro* (Detmar *et al.*, 1994). Since TGF α and its receptor, the epidermal growth factor receptor (EGFR), are overexpressed both in psoriatic epidermis (Elder *et al.*, 1989; Krane *et al.*, 1992; Watts *et al.*, 1994) and in healing wounds (Schultz *et al.*, 1991; Stoscheck *et al.*, 1992; Ono *et al.*, 1994), these findings support a concept of an integral role of TGF α -induced VPF/VEGF expression in cutaneous angiogenesis. Thus, the TGF α -mediated VPF/VEGF induction in keratinocytes

may represent a novel etiopathogenic pathway in cutaneous wound healing and psoriasis.

The objectives of the present studies were to explore molecular mechanisms responsible for the TGF α -mediated transcriptional activation of the VPF/VEGF gene. The 5'-regulatory region of the human VPF/VEGF gene has recently been cloned and characterized (Tischer *et al.*, 1991). Sequence analysis of the VPF/VEGF promoter revealed the lack of a TATA box motif and the presence of dense clusters of GC-rich regions in close proximity to the single major transcription initiation site. In order to determine the sequence requirements for TGF α -mediated VPF/VEGF gene transcription, we utilized 5'-deletional VPF/VEGF gene-based reporter gene constructs in transcriptional activation studies. We further defined involved DNA-protein complexes by employing selected sequences of the 5'-regulatory VPF/VEGF gene region as DNA probes in electrophoretic mobility shift assays (EMSAs). We identified a GC-rich TGF α response element of the VPF/VEGF promoter that binds constitutively expressed and TGF α -inducible protein complexes. These observations are distinctly different from those described in hypoxia-mediated VPF/VEGF gene expression, and the responsive element (RE) identified is also distinct from previously described epidermal growth factor (EGF)-RE. AP-2 transcription factor was detected as a critical component of the TGF α -inducible DNA binding complex and an AP-2 expression vector transcriptionally activated the VPF/VEGF gene. Thus, our observations provide conclusive evidence that AP-2-dependent DNA binding is required for TGF α -induced transcriptional activation of the VPF/VEGF gene.

Results

TGF α up-regulates steady-state VPF/VEGF mRNA levels in a time- and concentration-dependent fashion in A431 cells

In order to establish A431 human epidermoid carcinoma cells as an appropriate cell line to study transcriptional regulation of TGF α -mediated VPF/VEGF gene expression, we initially determined constitutive and induced VPF/VEGF mRNA levels in untreated and TGF α -treated cells. Kinetic studies revealed a minimal basilar expression and a successive increase of TGF α -mediated VPF/VEGF mRNA expression for up to 6 h, which was followed by a significant decrease by 16–24 h (Figure 1A). In addition, RNA was isolated from A431 cells which had been exposed to increasing concentrations of TGF α for 4 h (Figure 1B). Basal transcript levels were significantly induced by 10 ng/ml TGF α , with a concentration-dependent further increase by 100 ng/ml. These data demonstrate that the presence of TGF α leads to up-regulation of VPF/VEGF steady-state mRNA levels in a time- and concentration-dependent fashion in A431 cells.

Identification of a TGF α -responsive region between bp –88 and –70 of the VPF/VEGF gene promoter by 5'-deletional analysis in A431 cells

To determine molecular mechanisms responsible for the transcriptional regulation of VPF/VEGF gene expression in response to TGF α , a sequential series of 5'-deletional VPF/VEGF promoter-based reporter gene constructs was

Fig. 1. TGF α induces VPF/VEGF mRNA expression in A431 cells in a (A) time- and (B) concentration-dependent manner. Northern blot analyses of total RNA (20 μ g/lane) extracted from confluent A431 cell cultures. (A) Incubation with 100 ng/ml TGF α for 2, 4, 6, 16 and 24 h or media change 24 h prior to extraction (first lane). (B) Incubation with increasing concentrations of TGF α (0.1, 1, 10 or 100 ng/ml) for 4 h or media change 4 h prior to extraction (first lane). The lower panels display an image of the respective ethidium bromide (EtBr)-stained nylon membrane to demonstrate even loading and transfer.

generated and transiently transfected into A431 cells (Figure 2). Analysis of the respective CAT expressions in untreated and TGF α -stimulated cells revealed notable basal activity of the –1142/+54 VPF CAT construct which was increased after treatment with TGF α by a factor of two to three (Figure 2). While shorter constructs, including the –88/+54 VPF CAT construct, demonstrated comparable constitutive and TGF α -induced expression to the longest reporter gene plasmid, analysis of the –70/+54 VPF CAT construct showed loss of both basal and TGF α -induced transcriptional activity. These findings indicate that critical gene-regulatory elements necessary for both constitutive and TGF α -inducible expression are located between bp –88 and –70 with respect to the transcription initiation site of the VPF/VEGF promoter. The transcriptional activation experiments were also performed on HeLa cells and on the transformed keratinocyte cell line HaCaT, results being comparable with those seen in A431 cells (data not shown). The mechanisms identified for TGF α -mediated transcriptional activation of the VPF/VEGF gene are therefore demonstrable in a variety of epithelial-derived cells.

Constitutive and TGF α -inducible DNA binding activity of nuclear protein extracts to the –88/–65 bp VPF/VEGF promoter sequence

We next explored whether specific protein complexes could be identified that interacted with the VPF/VEGF

Fig. 2. The TGFα-responsive region is localized between bp -88 and -70 of the VPF/VEGF gene promoter. Analysis of a sequential series of 5'-deletional VPF/VEGF promoter-based CAT constructs in A431 cells. Schematic representation of the respective reporter gene constructs on the left, coordinates with respect to the transcription start site in the center, and the normalized CAT activities (expressed as % CAT conversion) in graphic format on the right: white bars, unstimulated controls (Ctrl.); black bars, TGFα-treated A431 cells. Values represent the mean ± SD of duplicate assays; the fold increase in CAT activity after TGFα-treatment is depicted to the right (mean ± SD of three independent duplicate transfections and assays).

promoter region which had been demonstrated to be essential in our transcriptional activation studies. We utilized a double-stranded DNA probe corresponding to the -88/-65 bp VPF/VEGF promoter sequence in electrophoretic mobility shift assays (Figure 3). When incubated with nuclear extracts of untreated A431 cells, constitutive DNA binding activity of several distinct complexes was observed (lane 1). In addition, in lysates of TGFα-stimulated cells, an additional prominent DNA binding complex was detected (lane 2). These data indicate that both constitutively expressed and TGFα-induced protein complexes bind to this sequence. The binding specificity of the complexes formed was determined by exclusive competition through excess unlabeled identical DNA (lane 3) but not irrelevant DNA (lane 4). Sequence analysis for established consensus recognition sites of *trans*-acting factors revealed the presence of two adjacent Sp1 transcription factor binding sites within the -88/-65 bp VPF/VEGF probe (Figure 3). The question thus arose as to whether the detected protein complexes could be attributed to Sp1 protein-dependent binding, either in part or in total, to our identified promoter region of interest. To distinguish between Sp1-dependent and Sp1-independent complex formations, excess unlabeled double-stranded Sp1 consensus oligonucleotides were added in order to neutralize the binding activity of Sp1 or Sp1-like factors. Under these experimental conditions (Figure 3), only the TGFα-inducible DNA binding complex could be detected (lanes 5 and 6). These data thus demonstrate that the TGFα-inducible DNA complex binds to the -88/-65 bp VPF/VEGF probe in a Sp1-independent fashion, whereas the constitutively detected DNA-protein complexes bind in a Sp1-dependent manner. While the TGFα-inducible DNA complex was also detected in lysates of TGFα-treated human keratinocytes and HaCaT cells, this binding complex was absent in nuclear abstracts of human microvascular endothelial cells (HDMEC; data not shown).

Fig. 3. Constitutive and TGFα-inducible DNA binding activity of A431 nuclear protein extracts to the -88/-65 bp VPF/VEGF promoter sequence. Representative EMSAs using nuclear extracts of untreated A431 cells (lanes 1 and 5) and TGFα-treated cells (lanes 2-4 and lane 6) in the absence (lanes 1-4) or presence of unlabeled excess double-stranded Sp1 consensus oligonucleotides (lanes 5 and 6). The DNA sequence of the utilized probe is shown at the top; formation of specific complexes is indicated on the left (the TGFα-inducible complex is marked by a bold arrow). Competitor was used at a concentration of 100 molar excess (specific, unlabeled identical DNA, lane 3; non-specific, unlabeled -77/-63 bp DNA probe of the VCAM-1 promoter, lane 4; unlabeled Sp1 consensus oligonucleotides were consistently used at a final concentration of 0.35 μM, lanes 5 and 6).

Fig. 4. Determination of the minimal sequence requirements for DNA binding of the TGF α -inducible protein complex. Representative EMSAs (at the bottom) using nuclear extracts of TGF α -treated A431 cells and different double-stranded radiolabeled DNA probes corresponding to distinct sequences of the VPF/VEGF promoter (D0 to D4, MT). The TGF α -inducible complex is marked by an arrow on the left. The EMSAs were performed either in the absence (lanes 1, 3, 5, 7, 9 and 11) or presence of unlabeled, double-stranded Sp1 consensus oligonucleotides (lanes 2, 4, 6, 8, 10 and 12). At the top, the respective EMSA probe sequences are depicted. Non-wild-type flanking sequences are expressed in italic letters; the 2 nt mutation in the MT probe is indicated by enlarged letter size; and the TGF α -dependent binding site is underlined.

Determination of the minimal sequence requirements for DNA binding of the TGF α -inducible protein complex

We next determined the 5'- and 3'-minimal sequence requirements for binding of the TGF α -inducible protein complex. Nuclear extracts of TGF α -treated A431 cells were incubated with a series of 5' or 3' single-nucleotide deletional EMSA probes either in the presence or absence of excess unlabeled double-stranded Sp1 consensus oligonucleotides (Figure 4). Within the series of the 5'-single-nucleotide deletional EMSA probes, the D1 probe still demonstrated binding of the TGF α -inducible complex. However, further 5'-deletion and substitution of a single nucleotide (probe D2) resulted in loss of binding of the TGF α -inducible protein complex. Within the series of the 3' single-nucleotide deletional EMSA probes, the D4 probe still permitted binding of the TGF α -inducible complex. By contrast, 3' deletion and substitution of a single additional nucleotide (probe D3) led to loss of binding of TGF α -inducible complex. The pattern of complex formation of the deletional EMSA probes differed from that of the original probe (termed D0). This finding is most likely due to the shorter length of the deletional probes that resulted in loss of either the intact 5'- or 3'-Sp1 consensus binding site. Taken together, these studies define the 3' and 5' nucleotide limits for TGF α -inducible complex formation. The appropriateness of this experimental approach in defining the minimal wild-type sequence

Fig. 5. Functional characterization of the TGF α -responsive region by 5'-deletional and mutational analysis in A431 cells. Analysis of 5'-deletional VPF/VEGF CAT constructs (-88/+54, -80/+54 and -70/+54 bp) in A431 cells. Shown above is the relevant sequence with the minimal region required for TGF α -dependent binding marked by double underline, the 5'-Sp1 binding site marked by underline, and the respective 5'-boundaries of the fragments utilized indicated. In the center are representative duplicate CAT assays, and below are the normalized CAT activities (expressed as % CAT conversion) in graphic format with white bars indicating values for unstimulated controls (Ctrl.) and black bars indicating values for TGF α -treated A431 cells. Fold increase in CAT activity after TGF α treatment: -88/+54 construct, 2.4 ± 0.6 ; -80/+54 construct, 1.9 ± 0.3 (mean $\pm$ SD based upon three independent duplicate transfections and assays).

requirements (bp -78/-70; 5'-CCGGGGGCG-3') for binding of the TGF α -inducible protein complex was confirmed by directed mutations within the identified 9 nt region (termed MT, Figure 4, lanes 11 and 12). Incorporation of a 2 nt mutation resulted in exclusive loss of binding of the TGF α -inducible protein complex. The functional significance of abrogation of the TGF α -inducible complex formation through this mutation has been studied further in transcriptional activation assays (see below and Figure 8).

Further functional characterization of the TGF α -responsive region by 5'-deletional promoter analysis in A431 cells

We next constructed and analyzed a -80/+54 VPF/VEGF CAT plasmid which contained the identified binding site of the TGF α -inducible complex, but excluded critical parts of the 5'-Sp1 recognition site (Figure 5). While the -88/+54 VPF CAT construct displayed constitutive baseline activity that was increased by a factor of 2.4 ± 0.6 after TGF α treatment, analysis of the -70/+54 VPF CAT construct showed loss of both the basal and the TGF α -induced transcriptional activation. By comparison, the constitutive expression of the -80/+54 VPF/VEGF CAT plasmid was decreased by ~50%, whereas the TGF α -mediated enhancer qualities were almost completely retained (factor 1.9 ± 0.3). These data indicate that for maximal VPF/VEGF promoter activation, 5' sequences outside the binding site of the TGF α -inducible complex

Fig. 6. Identification of AP-2 and Egr-1 transcription factors in the TGFα-inducible DNA binding complex. Representative EMSAs utilizing nuclear extracts of TGFα-treated A431 cells and the -88/-65 bp VPF/VEGF promoter sequence as a double-stranded probe in the presence of unlabeled double-stranded Sp1 consensus oligonucleotides. Unlabeled AP-2 (lanes 5 and 7) and Egr-1 (lanes 6 and 8) consensus oligonucleotides were added at a final concentration of 40 ng/μl. Specific antibodies (anti-AP-2, lanes 2, 4, 5 and 6; anti-Egr-1, lanes 3, 4, 7 and 8) were added at a final concentration of 100 ng/μl. Specific complex formation is indicated on the left: thin arrows, supershifted complexes; bold arrow, TGFα-inducible complex. The utilized DNA probe and the respective consensus AP-2 (Williams and Tjian, 1991) and Egr-1 (Gashler and Sukhatme, 1995) binding sequences are depicted at the top.

are essential. These findings also suggest that binding of the TGFα-inducible DNA-protein complex may account for the degree of induced transcriptional activation seen with TGFα. To further explore this interpretation, we compared the wild-type -88/+54 VPF CAT plasmid with a mutant construct in which a 2 nt mutation had been generated within the TGFα-dependent minimal binding region. In EMSA analysis, this mutation effectively abolished binding of the TGFα-inducible complex (Figure 4, lanes 11 and 12). Consistent with our interpretation and the EMSA data above, constitutive expression was not substantially affected by the 2 nt mutation (Figure 8), whereas the TGFα-mediated transcriptional activation was virtually abrogated with this mutant construct (data not shown).

AP-2 and Egr-1 transcription factors are detected as components of the TGFα-inducible DNA binding complex

Based upon the minimal sequence required for binding of the TGFα-inducible protein complex, we compared the sequence of this region to additional GC-rich DNA protein recognition sites and found that this 9 nt site displays high homology to both consensus AP-2 (Williams and Tjian, 1991) and Egr-1 (Gashler and Sukhatme, 1995) transcription factor binding sites (Figure 6). We therefore performed supershift assays with antibodies directed against either AP-2 or Egr-1 protein. To eliminate confusion over Sp1-dependent binding and to focus only on the TGFα-inducible complex, these EMSAs were performed with nuclear extracts of TGFα-treated A431 cells in the pres-

ence of unlabeled excess Sp1 consensus oligonucleotides. Consequently, only the TGFα-inducible DNA binding complex was detected (lane 1). Addition of AP-2 Abs led to formation of two more slowly migrating complexes and a significant decrease in the intensity of the original TGFα-inducible complex (lane 2). Additionally, Egr-1 Abs also induced a shift, although intensity of the shifted complexes was significantly less when compared with the AP-2-mediated supershift (lane 3). Increasing concentrations of Abs did not further increase the amount of either supershift (data not shown). However, addition of both Abs resulted in a complete supershift of the TGFα-inducible complex (lane 4). In order to determine the specificity of the shifted complexes, we utilized unrelated Abs in additional EMSAs and also performed supershift-competition assays with excess unlabeled AP-2 and Egr-1 consensus oligonucleotides. Irrelevant Abs directed against the NF-κB transcription factor RelA and c-Rel did not recognize any proteins in the complex (data not shown). The complexes shifted by AP-2 Ab (lane 2) were successfully competed with AP-2 oligonucleotides (lane 5), but not by Egr-1 oligos (lane 6). However, the Egr-1 oligos successfully competed the remainder of the TGFα-inducible complex (lane 6). In contrast, Egr-1 oligos specifically removed the Egr-1 Ab-induced supershift without affecting the remainder of the TGFα-inducible complex (lane 8). While AP-2 oligos did not significantly compete the Egr-1 Ab-induced supershift, they led to competition of the remainder of the TGFα-inducible complex (lane 7). These data thus demonstrate that the TGFα-inducible complex is comprised of distinct proteins which are exclusively recognized by either AP-2 or Egr-1 Abs. In addition, under the given experimental conditions, the majority of the TGFα-inducible complex is detected by AP-2 Ab.

Co-transfection of an expression vector encoding AP-2 transcription factor results in trans-activation of the VPF/VEGF gene promoter in A431 cells

To establish the functional importance of either AP-2 or Egr-1 transcription factor in the transcriptional regulation of VPF/VEGF gene expression, we performed co-transfection experiments. Expression vectors encoding AP-2 or Egr-1 protein were transiently transfected in concert with the -88/+54 VPF/VEGF CAT construct, and subsequently the respective CAT expressions were analyzed (Figure 7). Co-transfection of the AP-2 expression vector markedly increased CAT expression of the reporter gene in a fashion comparable with TGFα. The basal activity was elevated by a factor of 2.9 ± 0.6 . By contrast, co-expression of Egr-1 transcription factor did not significantly induce the expression of the VPF/VEGF-based reporter gene (1.2 ± 0.4), and increasing concentrations of transfected Egr-1 expression vector did not result in any significant increase in CAT expression (data not shown). Additionally, co-transfection of both the AP-2 and Egr-1 expression vectors resulted in no further elevation in induced CAT expression when compared with the AP-2 expression vector alone (2.7 ± 0.5). To ensure that the Egr-1 expression vector was indeed expressed, we further demonstrated its ability to *trans*-activate an Egr-1 responsive reporter gene (EBS1³fosc; Gashler *et al.*, 1993; data not shown) in A431 cells. Our findings imply that while both endo-

Fig. 7. Co-transfection of an expression vector encoding AP-2, but not one encoding Egr-1, results in *trans*-activation of the VPF/VEGF gene promoter in A431 cells. Representative duplicate CAT assays following transfection with the -88/+54 VPF/VEGF CAT (10 μ g) construct in concert with the AP-2 expression vector, the AP-2 and Egr-1 expression vectors, the Egr-1 expression vector, or their respective negative control vectors (each at 1 μ g). Schematic representation of the relevant sequence with the TGF α -dependent binding site underlined is displayed at the top; duplicate CAT assays are shown in the center; the normalized CAT activities (expressed as % CAT conversion) in graphic format are displayed below: white bars indicate cells co-transfected with the respective negative control vectors (Ctrl.) lacking the cDNA sequence (AP-2) or containing a non-functional mutated sequence (Egr-1); black bars indicate A431 cells co-transfected with the indicated expression vectors. Fold increase in CAT activity: AP-2, 2.9 ± 0.6 ; AP-2 + Egr-1, 2.7 ± 0.5 ; Egr-1, 1.2 ± 0.4 (mean $\pm$ SD based upon three independent duplicate transfections and assays).

genously activated AP-2 and Egr-1 are capable of binding to the TGF α -dependent binding site of the VPF/VEGF promoter, AP-2, and not Egr-1, is functionally important in the transcriptional regulation of the VPF/VEGF gene.

Transcriptional activation of the VPF/VEGF gene promoter by AP-2 transcription factor is mediated via the TGF α response element

In order to determine whether the AP-2-induced *trans*-activation of the VPF/VEGF gene was mediated via the putative TGF α -responsive region, a mutation of the -88/+54 CAT plasmid was generated that carried a 2 nt mutation within the TGF α -dependent site (Figure 8). This mutation effectively prevented binding of the TGF α -inducible complex in EMSA analyses (Figure 4, lanes 11 and 12). Whereas co-transfection of the AP-2 expression vector markedly increased CAT expression of the wild-type (wt) reporter gene (2.9 ± 0.6 -fold increase compared with the negative control vector), the mutated (mut) -88/+54 CAT construct significantly hindered AP-2-mediated *trans*-activation of the VPF/VEGF promoter (1.3 ± 0.3 -fold). The constitutive expression with the wild-type construct was essentially retained in this mutant plasmid. Additionally, the indicated mutation abrogated TGF α -inducibility of the -88/+54 CAT construct (data not shown). Taken together, these findings indicate that the

Fig. 8. AP-2-mediated *trans*-activation of the VPF/VEGF gene promoter is prevented by a critical mutation in the AP-2 binding site. Representative duplicate CAT assays following co-transfection of A431 cells with either the -88/+54 wild-type (wt) VPF/VEGF CAT construct or a mutated (mut) -88/+54 VPF/VEGF CAT construct (each at 10 μ g) along with the AP-2 expression vector (black bars) or the AP-2 negative control vector (white bars; each at 1 μ g). Schematic representations of the wild-type and mutated sequence are shown at the top with the TGF α -dependent binding site underlined, the 2 nt mutation indicated by enlarged letter size; duplicate CAT assays are displayed in the center; the normalized CAT activities are shown in graphic format below. Fold increase in CAT activity by co-transfection of the AP-2 expression vector: -88/+54 wt construct, 2.9 ± 0.6 ; -88/+54 mut construct, 1.3 ± 0.3 (mean $\pm$ SD based upon three independent duplicate transfections and assays).

AP-2-mediated transcriptional activation of the VPF/VEGF gene is conferred by the TGF α response element, and thus further substantiate the functional role of AP-2 protein in the transcriptional regulation of the TGF α -induced VPF/VEGF gene expression.

Discussion

The overexpression of VPF/VEGF appears to be an integral part of various angiogenic processes that are associated with vascular hyperpermeability and hyperproliferation. A novel induction pathway has recently been identified. Persuasive evidence indicates that the TGF α -induced expression of VPF/VEGF is critical for the pathogenesis of both malignant and benign settings, in which VPF/VEGF and TGF α as well as their respective receptors are concomitantly overexpressed (Dvorak *et al.*, 1995; Detmar, 1996).

Increased VPF/VEGF expression has recently been demonstrated in the hyperplastic epidermis of psoriatic skin (Detmar *et al.*, 1994) and in keratinocytes of healing wounds (Brown *et al.*, 1992; Frank *et al.*, 1995), both processes in which TGF α and its ligand, the EGFR, are markedly up-regulated (Coffey *et al.*, 1987; Elder *et al.*, 1989; Schultz *et al.*, 1991; Krane *et al.*, 1992; Stoscheck *et al.*, 1992). Since TGF α presents as a potent inducer of VPF/VEGF synthesis in keratinocytes (Detmar *et al.*, 1994), the TGF α -mediated VPF/VEGF expression is likely to play an important role in the initiation and maintenance of increased vascular hyperpermeability and angiogenesis in these conditions.

TGF α expression has also been recognized as a common characteristic of various neoplasms and tumor cells (Derynck, 1992). Endogenous TGF α synthesis, which is particularly prevalent in epithelial and other ectodermally derived tumors (Derynck *et al.*, 1987), has been implicated in facilitating both malignant cell transformation and proliferation (Derynck, 1992; Lee *et al.*, 1995). The ability of TGF α to induce angiogenesis *in vivo* is recognized as an important attribute to promote tumor cell proliferation (Schreiber *et al.*, 1986). There is strong correlative evidence that the angiogenic properties of TGF α may be mediated in part through autocrine induction of VPF/VEGF synthesis (Goldman *et al.*, 1993; Detmar *et al.*, 1994). We here show that the human epidermoid carcinoma cell line A431 utilizes the TGF α /EGFR pathway to markedly up-regulate VPF/VEGF production (Figure 1). Since TGF α is capable of inducing VPF/VEGF synthesis in other malignant cells as well (e.g. HeLa cells; data not shown), it is rational to hypothesize that the overexpression of VPF/VEGF observed in many neoplasms (Senger *et al.*, 1986, 1994) may be additionally mediated in part through the induction by endogenous TGF α .

In the present studies, we examined the transcriptional regulation of the VPF/VEGF gene in response to TGF α within epithelial-derived cells. A431 cells have previously been shown to synthesize the major bioactive VPF/VEGF isoforms (Myoken *et al.*, 1991), and have also been found to express a profile of VPF/VEGF mRNA and protein variants comparable with that seen in human cultured keratinocytes (Ballaun *et al.*, 1995). In addition, our observations show that A431 cells are capable of up-regulating VPF/VEGF mRNA levels by TGF α in a concentration- and time-dependent fashion. In 5'-deletional analysis of reporter gene constructs, we have identified a region which confers TGF α -mediated transactivation of the VPF/VEGF gene. This GC-rich region contains two adjacent Sp1 transcription factor binding sites and was subsequently utilized in EMSAs. These analyses showed constitutive Sp1-dependent complex formation and an additional TGF α -inducible binding complex that was distinct from Sp1. By testing the nuclear binding activity within a series of 3' or 5' single-nucleotide deletional EMSA probes, we subsequently identified a 9 nt region (5'-CCGGGGGCG-3'; bp -78/-70) that was required for binding of the TGF α -inducible protein complex. While this sequence heavily overlaps with the 3'-located Sp1 consensus binding site, there is only a single nucleotide overlap with the 5' Sp1 binding site. Thus, it is likely that Sp1-like factors and Sp1-independent binding complexes may bind simultaneously. This tentative assumption is supported by CAT analysis of a VPF/VEGF construct that contained the binding site of the TGF α -inducible complex but excluded critical parts of the 5'-Sp1 recognition site (Figure 5). In comparison with the 5'-Sp1 site-containing plasmid, analysis of the truncated -80 bp construct showed a decrease in the constitutive expression by ~50%, yet the TGF α -mediated induction of VPF/VEGF transcription was essentially unaffected. These observations indicate that, for maximal VPF/VEGF promoter activation, additional 5' sequences outside the binding site of the TGF α -inducible complex are essential. Most plausibly, binding to the 5'-Sp1 recognition site is needed. This conclusion is also supported by the analysis of CAT constructs that

contain a critical mutation within the binding sequence of the TGF α -dependent site. These studies revealed a particular loss of TGF α -mediated induction, but not of the baseline transcriptional activity (Figure 8). These findings thus support a model in which binding of the TGF α -inducible DNA-protein complex accounts for the degree of TGF α -induced transcriptional activation, whereas binding to the 5'-Sp1 binding site is critical for maximal transcription rates either constitutive or induced. The VPF/VEGF promoter lacks a TATA sequence motif (Tischer *et al.*, 1991). In the absence of a TATA box, mechanisms other than direct recruitment of TATA-binding proteins have been implicated in positioning of the basal transcription complex and initiation of transcription from a definite site. Precisely, Sp1 transcription factors have been shown to function as crucial proteins in accurate transcription initiation from so-called TATA-less promoters. Previous studies have demonstrated that Sp1 binding sites upstream of the transcription start site may act as anchoring factors to position the basal transcription complex and may thereby lead to exact site determination of transcriptional initiation (Azizkhan *et al.*, 1993). These observations appear to apply for the VPF/VEGF promoter as well, since our studies show that deletion of the distal Sp1 binding site significantly decreases basal and induced CAT expression levels.

The minimal sequence required for binding of the TGF α -inducible protein complex shows remarkable homology to known GC-rich DNA consensus recognition sites for AP-2 (Williams and Tjian, 1991) and Egr-1 (Gashler and Sukhatme, 1995) transcription factors (Figure 6). Our combinatorial supershift and competition assays indeed demonstrate that the TGF α -inducible complex contained distinct proteins which are exclusively recognized by either AP-2 or Egr-1 Abs; however, the majority of the TGF α -inducible complex was detected by AP-2 Ab. Nevertheless, the ability of nuclear proteins to bind does not indicate that a given protein factor is actually involved in specific gene transcription induction, only that, within the system of cells, stimuli and genetic elements being studied, it is a viable candidate. Our functional expression data are therefore critical, in that they clearly demonstrate that while Egr-1 is capable of binding in EMSA, co-transfection of Egr-1 does not drive expression, while co-transfection of AP-2 clearly does. While the EMSA data provide guidance and candidates, the reporter assays with co-transfection clearly demonstrate which factor is capable of interacting and driving expression. The co-transfection studies confirm a functional role of AP-2 protein in VPF/VEGF gene transcription, and our studies demonstrate for the first time that AP-2 is critically involved in the regulated expression of the VPF/VEGF gene. AP-2 is an inducible transcriptional regulator controlling gene expression in embryonic development and adult cell differentiation (Williams *et al.*, 1988). The formation of dimers appears to be an essential requirement for DNA interaction. Next to contact with bases of consensus AP-2 binding sites, DNA interaction is also likely to be facilitated and stabilized by contact with the phosphate backbone (Williams and Tjian, 1991). AP-2 activity is subject to distinct regulatory mechanisms. Phorbol esters and signals that elevate cyclic AMP concentrations have been found to induce AP-2 activity independent of increased AP-2

mRNA and protein expression (Imagawa *et al.*, 1987). Conversely, treatment of teratocarcinoma cells with retinoic acid transcriptionally activates the AP-2 gene (Luscher *et al.*, 1989). Additionally, an alternatively spliced AP-2 gene product may negatively regulate AP-2 activity (Buettner *et al.*, 1993; Moser *et al.*, 1995). Our studies clearly indicate that TGF α may function as a novel inducer of AP-2-mediated *trans*-activation. However, the mechanisms by which TGF α affects nuclear AP-2 activity are yet to be elucidated. AP-2 protein has been shown to be expressed in a cell type-specific fashion. Previous studies revealed that AP-2 is absent in human hepatoma HepG2 cells (Williams *et al.*, 1988). Pertinent to our findings, AP-2 protein is present and may be activated in A431 cells. Whereas the AP-2-containing, TGF α -inducible DNA binding complex also forms in human keratinocytes and in transformed HaCaT cells, this complex formation is absent in nuclear extracts of TGF α -treated human dermal microvascular endothelial cells (HDMEC), a finding consistent with our observations that TGF α fails to induce VPF/VEGF mRNA expression in HDMEC (J.Gille, S.W.Caughman and R.A.Swerlick, unpublished observations). Intriguingly, however, the Sp1-dependent complexes still form with HDMEC nucleoproteins in a manner comparable with that seen with A431 lysates. Whether this lack of TGF α -mediated induction of VPF/VEGF expression in HDMEC is due to an absence of AP-2 protein in EC, to different cell type-specific signaling pathways of TGF α that may not lead to AP-2 activation in EC, to the presence of an inhibitory isoform of AP-2 in EC, or to some other mechanism is currently under investigation in our laboratories.

Previous studies on molecular mechanisms responsible for the regulated expression of VPF/VEGF have focused on hypoxia as an additional important and potent inducer of VPF/VEGF gene expression (Minchenko *et al.*, 1994; Ikeda *et al.*, 1995; Liu *et al.*, 1995; Stein *et al.*, 1995). Hypoxia has been demonstrated to result in a rapid and lasting transcriptional activation of the VPF/VEGF gene and also in a gradual increase in VPF/VEGF mRNA stability (Ikeda *et al.*, 1995; Levy *et al.*, 1995, 1996). These observations revealing both transcriptional and post-transcriptional regulatory mechanisms in hypoxia-induced VPF/VEGF gene expression also appear to apply to the induction of VPF/VEGF by hypoglycemia within keratinocytes (M.Detmar *et al.*, unpublished results). Whether this multi-level regulation of expression holds true for the TGF α -mediated VPF/VEGF induction is yet to be explored. Our current studies have concentrated on molecular mechanisms involved in the transcriptional activation of the VPF/VEGF gene in response to TGF α . These observations reveal mechanisms distinct from those described for hypoxia-induced VPF/VEGF expression. While two separate studies have located a hypoxia-responsive element between nucleotides -1180 and -887 (Ikeda *et al.*, 1995) and -985 and -951 (Liu *et al.*, 1995), our observations show that TGF α -responsiveness is conferred by a region much closer to the transcription initiation site between bp -88 and -65 of the 5' regulatory region. Consequently, distinctly different mechanisms appear to mediate hypoxia- and TGF α -mediated transcriptional activation of the VPF/VEGF gene. In addition, the identified TGF α -RE is distinctly different from previously

described EGF-RE in other genes. Though our TGF α -RE exhibits homology to the EGF-RE within the human gastrin promoter (Merchant *et al.*, 1991), this RE is bound by neither Egr-1 nor AP-2 transcription factor. Therefore, clarification and delineation of molecular mechanisms responsible for VPF/VEGF gene expression are important, especially with respect to the development of new therapeutic strategies. Additionally, these presented observations enhance our understanding of the molecular mechanisms by which TGF α up-regulates VPF/VEGF *in vitro* and possibly *in vivo*.

Materials and methods

Reagents

Human recombinant TGF α was purchased from R&D Systems (Minneapolis, MN).

Cell culture

A431 cells, HeLa cells (American Type Culture Collection, Rockville, MD) and HaCaT cells (generously provided by Dr Norbert E.Fusenig; Boukamp *et al.*, 1988) were cultured at 37°C and 5% CO₂ in Dulbecco's modified essential medium (Gibco Life Technologies Inc., Grand Island, NY), supplemented with 5% fetal bovine serum (Hyclone, Logan, UT), 2 mM glutamine (Gibco), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco) and 250 µg/ml amphotericin B (Gibco). Human dermal microvascular endothelial cells (HDMEC) were isolated and cultured as previously described (Gille *et al.*, 1996).

Northern blot analysis

Total cellular RNA was isolated from confluent cell cultures by a single-step procedure using RNeasyTM B (Biotech Laboratories, Houston, TX). 20 µg of total cellular RNA were fractionated on 1% agarose-6.6% formaldehyde gels, transferred to nylon membranes (Nytan plus, Schleicher & Schuell, Keene, NH) using an electroblotting chamber (Trans Blot Cell; Bio-Rad, Hercules, CA), and were covalently linked by ultraviolet irradiation (UV Stratalinker 1800; Stratagene, La Jolla, CA). Membranes were prehybridized at 65°C for 30 min in Rapid-hyb buffer (Amersham Life Science, Arlington Heights, IL). Hybridization was performed at 65°C for 2 h using the Rapid-hyb buffer containing ~0.5 × 10⁶ c.p.m./ml of α -³²P-labeled DNA probe (specific activity > 10⁹ c.p.m./µg DNA). Membranes were washed twice with 0.1% SDS/0.1 × SSC for 20–30 min at 60°C and exposed to X-ray film with intensifying screens at -70°C for 1 day. α -³²P-labeled probes were prepared by using random primer oligonucleotides (*rediprime* DNA labeling system, Amersham). A 183 bp Exon 3 fragment of the human VPF/VEGF cDNA (generously provided by Dr Judith A.Abraham; Tischer *et al.*, 1989) was used as a specific probe. Autoradiographs and prints were scanned on a LaCie flat bed scanner (LaCie Ltd, Beaverton, OR) utilizing the Adobe Photoshop program (Adobe Systems, Inc., Mountain View, CA). Digitized images were processed by Microsoft software programs (Microsoft Powerpoint and Word; Microsoft Corp., Redmond, WA) and printed on a laser printer. Each image is derived from a representative autoradiograph and depicts characteristic band and background densities.

Reporter gene constructs and expression vectors

The different VPF/VEGF CAT constructs were generated by inserting the corresponding promoter sequences into the pCAT Basic vector from Promega (Madison, WI). The DNA fragments (including flanking 5'-HindIII and 3'-XhoI enzyme restriction sites to facilitate directional cloning into the parent vector) were synthesized by PCR technique. For construction of site-directed and deletion mutant plasmids, primers containing the respective mutations were utilized. Correctness of the generated constructs was confirmed by gel sequencing using the Sequenase Quick-Denature Plasmid Sequencing Kit (Amersham). The expression vectors pCB.Egr-1 (containing the full-length murine Egr-1 cDNA coding sequence), the pCB.Egr-1Δ331–374 (lacking the first and part of the second Egr-1 zinc finger domains) were generously provided by Dr Vikas P.Sukhatme (Gashler *et al.*, 1993). The SPRSV-AP2 (containing the full-length AP-2 cDNA coding region) and SPRSV-NN (containing the vector backbone only) were generously provided by Dr Trevor Williams (Williams and Tjian, 1991).

Transient transfection of reporter gene constructs and analysis of CAT expression

Cells were transfected with 10 µg of appropriate reporter and control plasmids (parent vector) using standard calcium phosphate precipitation techniques (Sambrook *et al.*, 1989). At 16 h after transfection, cells were rinsed with Hank's balanced salt solution and replenished with media; 40 h after transfection, control transfectants were left untreated (media change) and test transfectants received 100 ng/ml TGF α for 16 h. Lysates were prepared by rapid freeze-thaw cycles. Chloramphenicol acetyl transferase (CAT) expression was determined by acetylation of ^{14}C -labeled chloramphenicol (Amersham) and analyzed by thin-layer chromatography followed by scintillation counting. Amounts of lysates used for CAT activity analysis were normalized to the activity of the co-transfected pSV- β -Galactosidase Control vector (Promega). β -Galactosidase activity was determined by ELISA using the Enzyme Assay System from Promega.

Preparation of nuclear extracts and gel mobility shift analysis

Confluent cell cultures were left untreated or were stimulated with TGF α (100 ng/ml) for 1 h. Nuclear proteins were extracted as previously described by Dignam *et al.* (1983). The oligonucleotide D0 was synthesized to span the region between -88 bp and -65 bp of the human VPF/VEGF promoter: 5'-TTTCCGGGGCGGGCCGGGGCGGGGTAT-3'. The underlined sequence served as a template for the synthesis of the second strand (random non-wild-type flanking sequences are shown in italic letters). Radiolabeled double-stranded DNA was synthesized by annealing an oligonucleotide complementary to the underlined sequence listed above (5'-ATACCCCGCCCCCG-3'), and by extension of the second strand with Klenow fragment, 50 µCi of [α - ^{32}P]dCTP, unlabeled dATP, dGTP and dTTP. Unincorporated nucleotides were removed by column chromatography. Unlabeled double-stranded DNA was made identically, except that unlabeled dCTP was substituted for labeled dCTP. The DNA binding reaction was performed for 30 min at room temperature in a volume of 20 µl, containing 5 µg of nuclear protein extract, 2.5 mg/ml bovine serum albumin, 10⁵ c.p.m. α - ^{32}P -labeled probe (-0.5–1.0 ng), 0.1 mg/ml poly[dI:dC] (Sigma), 5 µl of 4 $\times$ binding buffer [1 $\times$ buffer: 10 mM Tris-HCl, pH 7.8, 100 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 10% (v/v) glycerol, 1 mM DTT] with or without 100-fold molar excess of unlabeled competitor. Competitor and antibodies were added 15 min prior to adding the probe. AP-2 and Egr-1 consensus oligonucleotides as well as AP-2 and Egr-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Sp1 consensus oligonucleotides were purchased from Promega. Samples were subjected to electrophoresis on a native 4% polyacrylamide gel for 3–4 h at 120–130 V/10–12 A.

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