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HYPOXIA-INDUCED TRANSCRIPTION OF THE VASCULAR ENDOTHELIAL GROWTH FACTOR GENE IS INDEPENDENT OF FUNCTIONAL AP-1 TRANSCRIPTION FACTOR

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In this study, we investigated the functional role of the transcription factor AP-1 in hypoxia-induced expression of the vascular endothelial growth factor (VEGF) by using dexamethasone as an inhibitor of AP-1 activity. Phorbol ester and platelet-derived growth factor (PDGF) cause an increase in VEGF mRNA expression, which is strongly suppressed in the presence of dexamethasone, whereas hypoxia-induced VEGF expression is not inhibited by dexamethasone. Studies using a VEGF promoter luciferase construct show that the phorbol ester and PDGF-induced VEGF expression is mediated at least in part by transcriptional activation of the VEGF promoter, whereas no transcriptional activation is seen under hypoxic conditions. In contrast, hypoxia leads to an increase in VEGF mRNA stability, as confirmed by experiments using actinomycin D as an inhibitor of transcription. These results indicate that hypoxia-induced VEGF expression is independent of AP-1 mediated transcription.

The development of new blood vessels is required for a variety of fundamental physiological processes including wound healing, corpus luteum formation and embryogenesis (1-3). In addition, angiogenesis is important for tumor growth and metastasis (4, 5), tumor cells being able to produce angiogenic factors which initiate angiogenesis. VEGF is such a factor, being an endothelial cell-specific mitogen and an inducer of angiogenesis *in vivo* (6, 7).

Molecular cloning of the VEGF cDNA revealed that in human cells at least four species of VEGF can occur: a 121, 165, 189 and a 206 amino acid form, which are generated by differential splicing of the VEGF gene (8, 9). Although VEGF contains a signal peptide which enables direct secretion, only the two shorter forms ($VEGF_{121}$ and $VEGF_{165}$) are efficiently secreted, whereas the two high molecular weight forms ($VEGF_{189}$ and $VEGF_{206}$) seem to be cell associated (10).

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Abbreviations: VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; TPA, phorbol 12-myristate 13-acetate; Epo, erythropoietin.

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Several lines of evidence exist, suggesting that VEGF is a very important mediator of tumor angiogenesis: VEGF is highly expressed in areas of active vascular proliferation and in a variety of human and rodent tumor cell lines (10, 11). Moreover, inhibition of VEGF activity by expression of dominant negative VEGF receptor mutants (12) or by using anti-VEGF antibodies (13, 14) inhibits tumor growth *in vivo*.

Analysis of the human VEGF promoter revealed the existence of several potential binding sites for the transcription factors AP-1 and AP-2 (9). Consistant with the presence of these enhancer elements, VEGF expression is inducible in most cell types by phorbol esters, growth factors and cAMP-analogues, indicating that VEGF expression is controlled by protein kinase C and protein kinase A mediated signal transduction pathways (9, 15-17).

VEGF expression has also been shown to be dramatically upregulated under hypoxic conditions (18-20). The mechanism by which oxygen tension regulates VEGF expression is still unclear. Since hypoxia also leads to a rapid and strong increase in the expression of the c-jun and c-fos proto-oncogenes (20, 21), we carried out the following study to investigate the role of functional AP-1 in hypoxia-induced VEGF expression in NIH 3T3 cells.

We show that the increase in VEGF mRNA expression under hypoxic conditions is AP-1 independent and in part due to mRNA stabilization.

MATERIALS AND METHODS

Chemicals. Phorbol 12 -myristate 13-acetate (TPA), dexamethasone and actinomycin D were obtained from Sigma (Deisenhofen, Germany). Platelet derived growth factor (PDGF-BB) was purchased from Saxon Biochemicals (Hannover, Germany). Cell culture media and Geneticin (G 418) were obtained from Gibco (Eggenstein, Germany). (α-^{32}P)-dCTP (3000 Ci/mmol) was purchased from Amersham Buchler (Braunschweig, Germany).

Cell culture . NIH 3T3 cells were grown in Dulbecco's modified essential medium (DMEM), supplemented with 10 % bovine calf serum, 2 mM fresh glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin, in a 37 ^{o}C, 10 % CO_2 atmosphere. Stably transfected cells (NIH-pLuc 2068) were kept in the same medium in the presence of 200 µg /ml G418. The cells were grown hypoxically using GasPak Pouches (BBL Microbiology systems). In the case of TPA and PDGF-BB induction of VEGF expression, medium was changed to 0.5 % bovine calf serum and the cells were incubated for 24 hours to induce quiescence.

Plasmid construction. The 5.1 kb VEGF-promoter fragment was kindly provided by J.A. Abraham (9) as a M13mp18 derivative. The plasmid pLuc 2068 was constructed by inserting 2068 bp of the human VEGF-promoter (-2018 to + 50) as a *Acc* I -*Nhe* I fragment into pAH 1409 (22).

Construction of stably transfected NIH 3T3 cells. 5 X 10^5 cells were cotransfected with 10 µg pLuc 2068 and 1 µg pWL-neo (Stratagene) by the calcium phosphate precipitation technique (23) using the Stratagene mammalian transfection kit. Forty eight hours later, cells were split 1:10 and put under selection with 800

µg/ml Geneticin (G 418). Colonies of G418 cells were cloned by ring isolation (24) after 14 days of G 418 selection.

Luciferase assay. 6-well-culture plates of confluent cells were washed with 1 ml of phosphate-buffered saline. The cells were removed, sedimented at low speed and washed with 0.2 ml 0,025 M Tris-phosphate pH 7.8, centrifuged again and the pellet resuspended in 100 µl 0.025 M Tris-phosphate pH 7.8. Cells were lysed by three freeze-thaw-cycles and the cell debris sedimented. 10 µl of cell lysate were pipetted into 96-well-micro-titer plate and analysed using a Dynatech Luminometer (Dynatech Laboratories) in the presence of 40 µl assay reagent (20 mM Tricine, 1.07 mM $(MgCO_3)_4$ $Mg(OH)_2$ X 5 H_2O, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 µM coenzyme A, 470 µM luciferin and 530 µM ATP, pH 7.8). The protein concentration was determined using the BCA protein assay kit (Pierce).

Northern blot analysis. Total RNA of confluent cells was prepared using the guanidinium thiocyanate method (25). 10 µg of RNA was electrophoresed in 0,9 % agarose/3 % formaldehyde gels and blotted onto nitrocellulose with 20 X SSC (1x SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). After 2 hours baking at 80 ^{0}C, blots were subjected to prehybridisation at 68 ^{0}C for 30 min in QuickHyb hybridisation solution (Stratagene).

Hybridisation was carried out in the same solution containing 100 µg /ml salmon sperm DNA and 10^6 cpm/ml denatured (^{32}P)dCTP-labeled random primed DNA probes. The probes used for hybridisation were the 1.2 kb *Eco* RI-*Hin* D III chicken ß-actin cDNA fragment of pAcpr2 (26) and the 0.6 kb *Eco* RI-*Bam* HI $VEGF_{165}$ cDNA fragment (11). The last washing step was 0.1 X SSC, 0.1 % SDS at 60 ^{0}C for 30 min. Filters were then autoradiographed using Kodak X-OMAT X-ray films with an intensifying screen.

RESULTS AND DISCUSSION

The synthetic glucocorticoid hormone, dexamethasone, is known to inhibit AP-1 mediated gene transcription by interference of the hormone-activated glucocorticoid receptor with the AP-1 transcription factor (27, 28). We have used dexamethasone as an inhibitor of AP-1 activity in order to investigate the functional role of the AP-1 transcrition factor in hypoxia- and growth factor- induced VEGF expression.

The effect of dexamethasone on phorbol ester and growth factor-induced VEGF expression in NIH 3T3 cells was analyzed by Northern-blot analysis as shown in Fig.1. In the absence of dexamethasone, TPA as well as PDGF-BB were able to induce the expression of VEGF in serum-starved NIH 3T3 cells, as previously reported (9, 16). In the presence of dexamethasone (0.5 µM), TPA- and PDGF-BB-induced VEGF expression was strongly suppressed, whereas dexamethasone treatment alone had no significant effect on the basal VEGF mRNA levels in uninduced, serum-starved cells. A depression of TPA-induced VEGF expression by dexamethasone was also reported in human mesengial cells (29). Since dexamethasone totally inhibited TPA-induced VEGF expression, it is very likely that TPA may exert its effect mainly via transcriptional activation of the VEGF gene

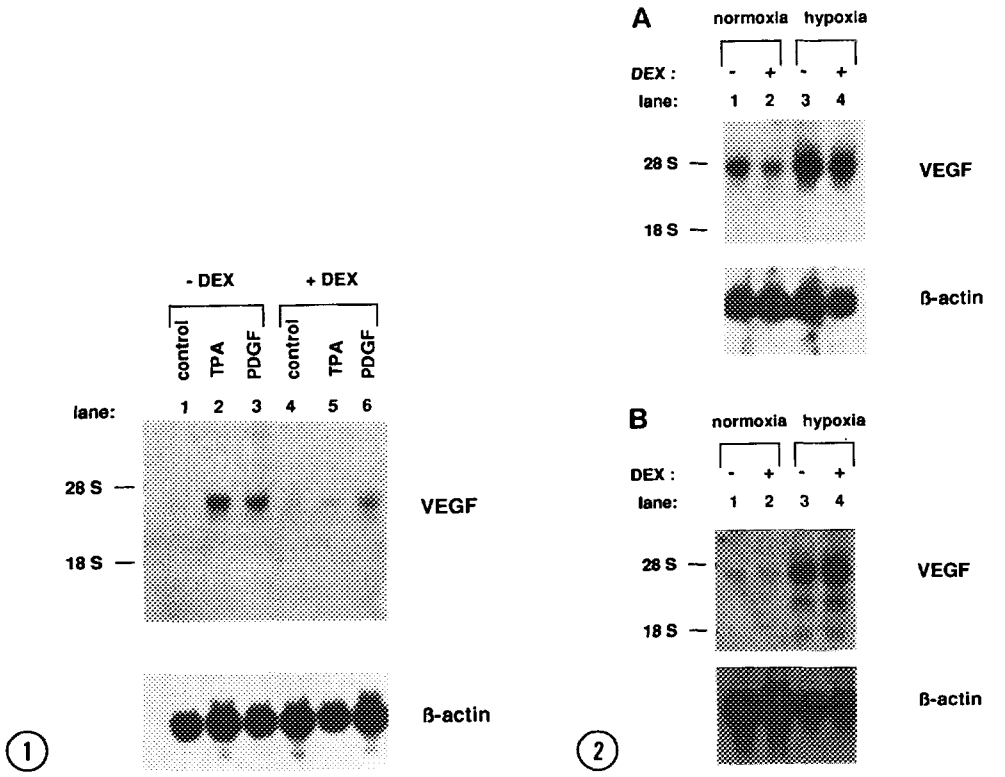

Figure 1. Effect of dexamethasone on TPA- and PDGF-BB-induced VEGF expression. The cells were grown to confluence in medium containing 10 % BCS and were serum-starved for 24 h to induce quiescence. The cells were stimulated for 2 h by addition of 100 ng/ml TPA (lanes 2 and 5) or 50 ng/ml PDGF-BB (lanes 3 and 6). Dexamethasone (0.5 µM) was added to the culture medium together with TPA (lane 5) or PDGF-BB (lane 6). Each lane was loaded with 10 µg total RNA. Transfer membranes were probed with the 0.6-kb *Eco* RI-*Bam* HI human $VEGF_{165}$ cDNA fragment (11) (upper panel) and the 1.2-kb *Eco* RI-*Hind* II chicken ß-actin cDNA fragment (28) (lower panel). The 28 S and 18 S RNA bans are indicated on the left.

Figure 2. Hypoxia -induced expression of VEGF mRNA in NIH 3T3 cells (A) and C6 cells (B). The cells were grown to confluence in medium containing 10 % BCS. Cells were incubated for 24 h under normoxic (lanes 1 and 2) or hypoxic (lanes 3 and 4) conditions. Dexamethasone (0.5 µM) was added to the cultures together with the hypoxic stimulus (lanes 2 and 4). Transfer membranes were probed with the VEGF cDNA fragment (upper panel) and reprobed with the ß-actin cDNA fragment (lower panel) as described for Fig. 1.

involving activation of the AP-1 transcription factor. This hypothesis was confirmed in transfection experiments using a VEGF promoter luciferase construct (Fig.3).

Hypoxia was previously reported to be a strong inducer of VEGF mRNA in several cell lines and tumor tissues (18-20). However, little is known about the molecular mechanisms responsible for VEGF regulation by hypoxia. Since hypoxia also leads to a strong increase in the expression of the c-jun and c-fos proto-oncogenes (20, 21), we used dexamethasone as an inhibitor of AP-1 transcription factor activity in

order to investigate the role of AP-1 in hypoxia-induced VEGF expression. Incubation of NIH 3T3 cells under hypoxic conditions for 24 hours led to a strong induction of VEGF gene expression (Fig. 2A). In contrast to the inhibitory effect of dexamethasone on TPA-induced VEGF expression (Fig.1), dexamethasone had no effect on hypoxia-induced VEGF expression. This result was also seen in a rat glioma cell line C6 (Fig. 2B), which was previously reported to show hypoxic induction of VEGF mRNA (18).

The finding that dexamethasone is unable to inhibit hypoxia-induced VEGF expression in NIH 3T3- as well as in C6-cells suggests, that hypoxia-enhanced

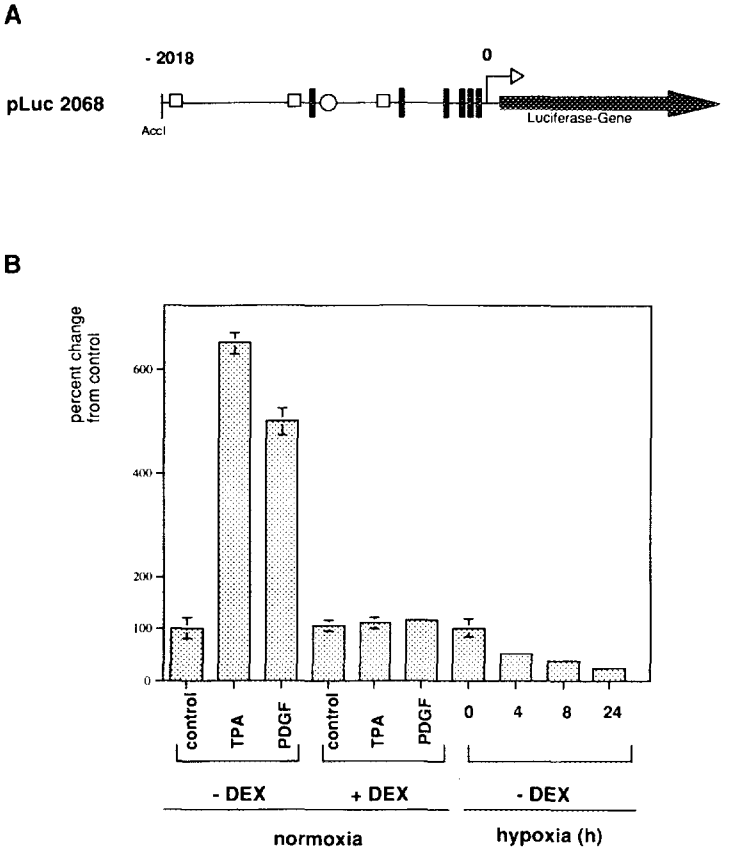

Figure 3. Effect of TPA, PDGF-BB and hypoxia on the VEGF promoter. Transcriptional analysis following stable transfection of NIH 3T3 cells with recombinant pLuc 2068. (A) NIH 3T3 cells were stably transfected with pLuc 2068, containing the human VEGF promoter fragment (-2018 to + 50) fused to the luciferase reporter gene. Open boxes, AP-1 sites; open circle, AP-2 site; closed boxes, SP-1 sites; the arrow indicates the transcriptional start site. (B) Stably transfected cells were serum-starved for 24 h and then treated with 100 ng/ml TPA for 4 h, 50 ng/ml PDGF-BB for 2 h or 0.5 μM dexamethasone as indicated. Cells were exposed to hypoxia for 4, 8 and 24 h as indicated. Means +/- S.D. from three independent experiments, performed in duplicates, were calculated in arbitrary units and expressed as percent change from control.

expression of the VEGF gene is independent of functional AP-1 transcription factor activity. In order to prove that this result was not gained simply because dexamethasone was inactivated during the 24 hour incubation period, NIH 3T3 cells were preincubated for 24 hours with 0.5 μM dexamethasone and then stimulated for 2 hours by the addition of TPA or PDGF-BB. Again, a strong suppression of TPA- or PDGF-BB-induced VEGF expression was observed similar to that in Fig. 1 (data not shown).

To further investigate the function of the three AP-1 recognition sites in the VEGF 5′- flanking region (9), we have stably transfected NIH 3T3 cells with a VEGF promoter luciferase construct. Treatment of these cells with TPA or PDGF-BB increased the expression of the luciferase reporter gene 6.5- and 5 -fold over control, respectively (Fig. 3B). In the presence of dexamethasone, TPA- and PDGF-BB-induced transcription was abolished, indicating that the dexamethasone- dependent depression of VEGF mRNA expression is due to a transcriptional control mechanism. When the cells were grown under hypoxic conditions for 4, 8 and 24 hours, no transcriptional activation of the VEGF promoter in relation to normoxic control incubations could be observed; in fact, a slight decrease in the expression of the luciferase reporter gene could be detected.

Two 10-base pair sequences 5′ to the VEGF transcription start site have been identified by computer analysis to have 90 % homology to a region within the human Epo 3′ hypoxia-responsive enhancer (20). Using the numbering employed in

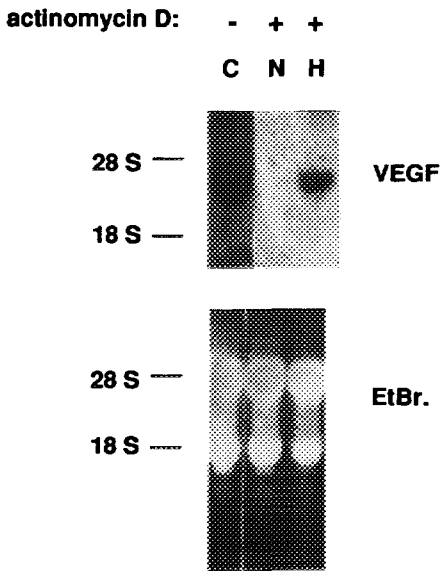

Figure 4. Northern blot analysis of VEGF mRNA after transcriptional blockade with actinomycin D (3 μg/ml) for 24 h. C, VEGF mRNA level before transcriptional blockade and before exposure to hypoxia; N, VEGF mRNA level after 24 h of transcriptional blockade under normoxic conditions; H, VEGF mRNA level after 24 h of transcriptional blockade under hypoxic conditions.

Genbank accession number M 63971, these sequences are located at base pairs 544 through 553 and 1442 through 1451 of human VEGF. These sequences correspond to base pairs -1808 through -1799 and -910 through -901 in the VEGF promoter fragment, that we have used in our studies (Fig. 3A). Our current results suggest that it is unlikely that these elements contribute positively to the hypoxia-induced transcriptional activation of the VEGF gene. However, we have not as yet ruled out the possible existence of further hypoxia-responsive enhancer elements in the native VEGF promoter, that are not present in our promoter fragment. The lack of transcriptional activation of the VEGF promoter luciferase construct under hypoxic conditions suggests that hypoxia might affect VEGF mRNA stability. This hypothesis was confirmed by using actinomycin D as an inhibitor of transcription. In the presence of 3 µg/ml actinomycin D, a 24 h incubation of NIH 3T3 cells under hypoxic conditions was found to lead to stabilization of VEGF mRNA in relation to the normoxic control incubation (Fig. 4).

In conclusion, these results suggest that hypoxia-induced VEGF expression is independent of functional AP-1 transcription factor activity and appears to be regulated at the posttranscriptional level most likely involving modulation of mRNA stability.

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